

1 **Costs and benefits of mating with fertilized females in a species with first male sperm**  
2 **precedence**

3 Leonor R Rodrigues<sup>1,2</sup>, Alexandre RT Figueiredo<sup>1</sup>, Thomas Van Leeuwen<sup>3</sup>, Isabelle Olivieri<sup>2</sup>, Sara  
4 Magalhães<sup>1</sup>

5 1. cE3c: Centre for Ecology, Evolution, and Environmental Changes, Faculty of Sciences, University of Lisbon,  
6 Edifício C2,3º piso, 1749-016 Lisboa, Portugal

7 2. ISEM: Institut des Sciences de l'Evolution de Montpellier, UMR5554 Université de Montpellier/CNRS/IRD),  
8 Place Eugène Bataillon 34095 Montpellier Cedex 05, France

9 3. Laboratory of Agrozoology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent  
10 University, 9000 Ghent, Belgium

11 Corresponding author: Leonor R Rodrigues; cE3c: Centre for Ecology, Evolution, and Environmental Changes,  
12 Faculty of Sciences, University of Lisbon, Edifício C2,3º piso, 1749-016 Lisboa, Portugal; [alrodrigues@fc.ul.pt](mailto:alrodrigues@fc.ul.pt)

13

14

15

## 16 **Summary**

17 Different patterns of sperm precedence are expected to result in specific mating costs and benefits for  
18 each sex, generating different selection pressures on males and females. However, most studies concern  
19 species with mixed paternity or last male sperm precedence, neglecting species with first male sperm  
20 precedence, in which only the first mating is effective.

21 Here, we measured costs and benefits of multiple mating for both sexes of the spider mite *Tetranychus*  
22 *urticae*. First, we assessed the stability of the sperm precedence pattern, by mating females to one, two  
23 or several males, immediately after the first mating or 24 hours later. We found complete first male  
24 precedence, independently of the mating interval and the number of matings. Females paid a cost of  
25 polyandry, as multiply-mated females laid fewer eggs than once-mated females. However, while first  
26 males had reduced survival when exposed to an intermediate number of virgin females, second males  
27 paid no additional costs by matings with several mated females. Moreover, by mating multiply with  
28 mated females, males decreased the total number of offspring sired by first males, which suggests that  
29 these matings may entail a relative benefit for second males, despite being ineffective.

30 Our results show that complex costs and benefits may arise in males in species with first male  
31 precedence. How these costs and benefits affect the maintenance of selection for polyandry remains an  
32 open question.

33

## 34 **Keywords**

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

## 36 **Introduction**

37 Multiple matings are prevalent in internally fertilizing species (Simmons, 2001). However, this  
38 behaviour is expected to entail negative consequences for both sexes, such as increased risk of predation  
39 and energy expenditure (G Arnqvist & Nilsson, 2000; Göran Arnqvist & Rowe, 2005). Consequently,  
40 for multiple mating to be selected, the reproductive advantage gained by this behaviour should to be  
41 superior to the costs incurred.

42 Polyandry, i.e., multiple mating in females with different males within a breeding season (G Arnqvist  
43 & Nilsson, 2000; Taylor, Price, & Wedell, 2014), is expected to be selected whenever females obtain  
44 direct benefits, such as increased fecundity and survival with each mating via nuptial gifts or nutritious  
45 ejaculates. For instance, in the bruchid beetle *Callosobruchus maculatus*, substances in the seminal  
46 fluids lead to an increase in the number of offspring produced (Eady, Wilson, & Jackson, 2000).  
47 Alternatively, but not exclusively, females can mate multiply to increase the quality or diversity of their  
48 offspring, in which case their benefit is indirect (Kvarnemo & Simmons, 2013; Snook, 2014). This has  
49 been observed, for example, in the bumble bee *Bombus terrestris*, in which polyandry can increase  
50 colony resistance to parasites by maximizing the chances that at least some individuals survive (Schmid-  
51 Hempel & Baer, 1999).

52 From a male's perspective, the benefits of multiple mating are quite straightforward, assuming that  
53 more matings result in more offspring production. Yet, polyandry, an expected outcome of increased  
54 male mating rate, can result in the offspring of a single female being shared by several males. As a  
55 result, males have evolved adaptations to sperm competition, such as harassment, altered male genitalia  
56 and toxic ejaculates, so as to sire a higher share of offspring of each female (Simmons, 2001). This, in  
57 turn, can be costly for females, decreasing their fitness (e.g., Chapman et al. 1995). Consequently, the  
58 balance between costs and benefits obtained with each mating is probably not the same for both sexes  
59 (Bateman, 1948). This imbalance can give rise to sexual conflicts, in which one sex employs  
60 reproductive tactics to enforce matings while the other resists them, depending on which sex will benefit  
61 the most from each mating (Göran Arnqvist & Rowe, 2005).

62 From the interaction between adaptations that have evolved in males and females to increase their  
63 reproductive success, emerges a pattern of sperm precedence. Such patterns may be more beneficial for  
64 one sex than for the other, thereby generating different selection pressures on each sex. In species with  
65 mixed sperm precedence, in which paternity is shared by several males, selection in males should favour  
66 increased mating frequency as a result of offensive and defensive adaptations to sperm competition  
67 (Mark Ridley, 1989) but for females the benefits obtained from each additional mating are not as  
68 straightforward (Göran Arnqvist & Rowe, 2005; Bateman, 1948). Indeed, females may obtain genetic  
69 benefits from a more diverse or fit offspring, but may pay the cost of excessive matings, which can lead  
70 to sexual conflict over mating rate. However, under complete first male precedence, in which only the  
71 first mating of a female is effective, genetic benefits cannot be obtained. Thus, in the absence of direct  
72 benefits, selection in these species should favour monandry, as both sexes are expected to invest all  
73 resources in copulations involving virgin females only (Thomas, 2011), thereby limiting the scope for  
74 sexual conflict (Hosken, Stockley, Tregenza, & Wedell, 2009). However, monandry may be imposed  
75 by males on females, as a result of evolved defensive adaptations against sperm competition. Once  
76 monandry is achieved, selection for this enforcement may be relaxed, potentially leading to polyandry  
77 being restored. In addition, females may be selected to gain the opportunity to choose the sperm they  
78 will use to sire their offspring (Dougherty, Simmons, & Shuker, 2016), and second males may be  
79 selected to obtain a share of the females' offspring (offensive traits). For instance, it has been suggested  
80 that second males may increase their relative reproductive success from matings with mated females  
81 without obtaining any paternity share, by displacing or killing the sperm of the first male inside the  
82 female (Harshman and Prout 1994, Macke et al. 2012). All these patterns suggest that the sperm  
83 precedence observed in a species is not necessarily evolutionarily stable (Dougherty et al., 2016).  
84 Furthermore, in several species, the pattern of sperm precedence has been shown to vary according to  
85 multiple ecological factors, such as the number of matings (Zeh & Zeh, 1994), the interval between  
86 mating events (Bullini, Coluzzi, & Bianchi Bullini, 1976), the effectiveness of the first mating  
87 (Welding, Toft, & Larsen, 2011), or differences in male traits (*e.g.*, size) (Bissoondath & Wiklund,  
88 1997).

89           Because of the lability of this pattern, it is important to know (a) how it translates into cost  
90 and benefits for both sexes and (b) if it is maintained under different ecological conditions.  
91 Nevertheless, most of the research has been done in species with mixed or last male sperm precedence  
92 (Simmons, 2001) and empirical studies addressing costs and benefits for both sexes in species with first  
93 male precedence are remarkably scarce (but see Fisher et al. 2013; Boulton and Shuker 2015, 2016).

94           Here, we study the consequences of mating for both sexes in the two-spotted spider mite,  
95 *Tetranychus urticae*. Earlier studies suggest that in this species only the first copulation of a female is  
96 effective (Helle, 1967). This leads to the expectation that males should only mate with virgin females  
97 to avoid unnecessary costs. Accordingly, males actively guard juvenile quiescent females and mating  
98 occurs as soon as females moult into virgin adults (Potter, Wrensch, & Johnston, 1976), a behaviour  
99 that is consistent across species with first male sperm precedence (M. Ridley, 1989). In addition, when  
100 given the choice between mated and virgin females, males, prefer to mate with virgins, basing their  
101 decision upon volatiles and chemical trails (Oku, 2010; Rodrigues, Figueiredo, Varela, Olivieri, &  
102 Magalhães, 2017). Nevertheless, mated females are often observed mating (Clemente, Rodrigues,  
103 Ponce, Varela, & Magalhães, 2016; Oku, 2010). Here, we provide a comprehensive account of potential  
104 costs and benefits of polyandry for both sexes in spider mites. First, we performed paternity tests, using  
105 a recessive mutation that codes for resistance to a pesticide as a genetic marker, in order to describe the  
106 pattern of sperm precedence. To account for the ecological lability of the pattern sperm precedence, we  
107 varied the number of matings and the interval between mating events. We then measured the fecundity  
108 and survival of females that re-mated at different time points, to assess potential costs or benefits of  
109 polyandry for females. In addition, we analysed differences in male survival in the presence of different  
110 numbers of virgin or mated females to assess potential costs of multiple mating for first and second  
111 males, respectively. Finally, we compared the total number of offspring sired by first males mated to  
112 females with different mating status, to assess the potential benefits for second males.

113

## 114 **Materials and Methods**

### 115 **Spider mite populations, rearing conditions**

116 To study the costs of multiple mating in males, we used a population collected in Carregado, Portugal,  
117 established at the University of Lisbon in 2010 from approximately 300 individuals (TuTOM). To study  
118 the other traits, we used the EtoxR strain, resistant to etoxazole (Van Leeuwen et al., 2012), and the  
119 LondonS strain (Grbić et al., 2011), susceptible to the same pesticide, both established at the University  
120 of Lisbon in 2013 from approximately 2000 individuals. Etoxazole is a pesticide that interferes with  
121 chitin synthesis, affecting spider mite embryos and juvenile stages at the time of hatching or ecdysis  
122 (i.e., at the quiescent stage; Van Leeuwen et al. 2012). In the EtoxR strain, resistance to Etoxazole is  
123 recessive and conferred by a single chitin synthase 1 (CHS1) amino acid change (Van Leeuwen et al.,  
124 2012). We used pesticide resistance as a marker for paternity.

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

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

### 133 **Experimental Setup**

#### 134 Mating protocol

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

139 or the opposite. Thus, these females were allocated to 5 different treatments: O, TI, MI, TL and ML. A  
140 description of these treatments is provided in Figure 1a. Briefly, EtoxR quiescent females were isolated  
141 for 24 hours on leaf discs on water-saturated cotton without males. Once they became adult (one day  
142 later), groups of 5 females were placed with 5/6 susceptible - or resistant - males on 0.8 cm<sup>2</sup> leaf discs.  
143 The patches were observed for 2 hours and once a female had successfully mated, it was transferred to  
144 a new patch, either empty or with males of the other strain, in the same proportion (5 females: 6 males).  
145 Half of the females placed with males on the second patches were observed for two more hours and  
146 isolated when mated (TI). The other half was left unobserved on the patch with males for 24 hours (MI),  
147 which, in this species, is a sufficiently large time interval to ensure the occurrence of multiple matings  
148 (authors Pers. Obs., Krainacker and Carey 1989; Magalhães et al. 2007). The females left alone after  
149 the first mating on the first day were either left alone for one more day (O) or transferred to patches  
150 with males of the alternative strain. Again, half the females were observed for 2 more hours and isolated  
151 if mated (TL) and the other half was left unobserved on the patch with males for 24 hours, thus allowing  
152 for multiple matings (ML). After mating, two- to three-days old females were isolated on a 2.55 cm<sup>2</sup>  
153 leaf disc placed on water-soaked cotton, in order to measure the life-history traits mentioned below. To  
154 maximize the effectiveness of the first mating, we (a) isolated males prior to testing them, (b) limited  
155 their copulations to 5 females, a value below their daily reproductive limit (Krainacker & Carey, 1989)  
156 and (c) discarded first matings interrupted by other individuals on the patch. Due to excessive  
157 experimental effort, this experiment was done on 22 separate days, all treatments being represented at  
158 each day.

## 159 Data Collection

### 160 *Effect of multiple mating on offspring number and paternity*

161 We tested whether the number of matings and the interval between matings affected offspring number,  
162 indicating potential costs or benefits of mating multiple times for females. Moreover, we assessed the  
163 paternity of that offspring, as a measure of potential benefits for first or second males. For this purpose,  
164 females isolated on leaf discs were transferred every three days to a new leaf and the eggs laid on the

165 old leaves were counted. Daily fecundity was measured as the total number of eggs laid per female  
166 divided by the number of days the female was alive (Fig. 1b). To assess paternity, the eggs laid by the  
167 isolated females were allowed to develop until they reached the first juvenile stage (three days after  
168 female transfer to a new disc), then leaf discs were transferred to water-soaked cotton with diluted  
169 etoxazole (500 ppm). Six days later, the number of adult daughters, adult sons and dead juveniles on  
170 each leaf disc was recorded (Fig. 1b). Spider mites are haplodiploid, producing haploid sons, which  
171 result from unfertilized eggs, and diploid daughters, stemming from fertilized eggs (Helle & Sabelis,  
172 1985). Therefore, the number of alive daughters and dead juveniles indicate, respectively, the number  
173 of offspring sired by resistant and by susceptible males. The survival of sons should not be affected by  
174 pesticide application since all females used were resistant to etoxazole and sons only inherit the genetic  
175 material of their mothers. Note however, that natural death in the quiescent stage may be confounded  
176 with death by pesticide exposure. Yet, because this occurs in all treatments, including the once-mated  
177 treatment that serves as control, the differences between treatments are a true measure of paternity share,  
178 excluding natural death at the quiescent stage. In total, we analysed the daily fecundity of 485 females  
179 and assessed the paternity of offspring from 377 females.

#### 180 *Effect of multiple mating on male and female survival*

181 To determine whether mating multiply benefited females by increasing their survival, we tested whether  
182 female survival varied with the number of matings and the interval between matings. To this aim, the  
183 same females used to assess paternity and daily fecundity were used to measure female survival (Fig.  
184 1b). The survival of mated females was followed daily after female isolation on a 2.55 cm<sup>2</sup> leaf disc  
185 placed on water-soaked cotton. In total, we analysed the survival of 485 females.

186 A different experiment was performed to measure the costs of mating in terms of survival in  
187 first and second males. To this aim, males and females were isolated separately at the quiescent stage,  
188 to control their age and ensure virginity prior to the experiment. When these individuals became adults  
189 (*circa* 24 hours later), groups of ten females were either left isolated (virgin – V) or placed with 15  
190 males (mated – M). The latter were left with the males for 24 hours to ensure the occurrence of multiple



191 matings (authors Pers. Obs., Krainacker and Carey 1989; Magalhães et al. 2007). The next day, focal  
192 virgin one-day old males were placed on a leaf circle with the previously isolated females: Males were  
193 allocated to leaves with either 1, 5 or 20 virgin females (V1, V5, V20), which allowed testing costs for  
194 first males, or with 1, 5 or 20 mated females (M1, M5, M20), thereby testing for potential costs in  
195 second males. To normalize densities across treatments, patch size varied according to the number of  
196 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  
197 focal male was then transferred daily to a new patch with the same number of (mated or virgin) females  
198 in every treatment except for the ones with 20 females. In this last treatment, as male mating capacity  
199 decreases with age (Krainacker & Carey, 1989), from the third day onwards, the focal male was placed  
200 with 12, instead of 20 females (size of the patch: 6.25cm<sup>2</sup>). Every day until death, male survival was  
201 recorded. In total, the survival of 180 males was analysed. Due to excessive experimental effort, and  
202 the very high number of females required for each replicate, this experiment was carried out on 66  
203 separate days.

#### 204 Potential benefits of ineffective matings for males

205 Mating with mated females may provide a relative increase in the fitness of second males,  
206 despite first male sperm precedence. For example, by mating with mated females, males may displace  
207 or kill the sperm inside the female and thereby increase their relative reproductive success (Macke et  
208 al. 2012). Here, we tested whether multiple matings could reduce the genetic contribution of first males.  
209 The total number of daughters (i.e. male genetic contribution to the next generation) sired by first males  
210 mated to females with different number of matings and mating intervals, was compared. The same  
211 females used to assess paternity and daily fecundity were used to measure this trait (Fig. 1b). Because  
212 the aim was to study lifetime fecundity, females who died due to artificial causes (drowning in water-  
213 soaked cotton) were excluded from the analysis. In total, we analysed the total number of daughters  
214 produced by 427 females.

#### 215 **Statistical analyses**

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

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

229 To analyse the effects of mating on male survival, the female status (i.e., M: mated; V: virgin) and the  
230 number of females on each patch (1, 5, 20) were fit as fixed explanatory variables, and day was fit as a  
231 random explanatory variable.

### 232 Effect of multiple mating on offspring number and paternity

233 To analyse the proportion of offspring sired by the first male, we redistributed the data of offspring  
234 survival into two variables called contribution of the 1<sup>st</sup> male (1M) and contribution of the second male  
235 (2M) to offspring. 1M corresponds to the number of dead juveniles or the number of alive daughters,  
236 depending on whether the first male was susceptible or resistant, 2M corresponds to the number of alive  
237 daughters or the number of dead juveniles, depending on whether the first male was susceptible or  
238 resistant. These parameters were computed using the function cbind, with 1M, 2M and the number of  
239 sons as arguments. Since the model was greatly over-dispersed, we used a generalized linear mixed  
240 model with a beta-binomial error distribution and added the term  $z_{i,j} \sim 1$  to the model  
241 (glmmTMB, glmmTMB package) (Brooks et al., 2017).

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

#### 244 Effect of multiple mating on male and female survival

245 Male and female survival were analysed using a Cox proportional hazards mixed-effect models (coxme,  
246 coxme package). In the analysis of male survival (MS), because the interaction between the fixed  
247 factors was significant, we analysed separately each level of female status for the effect of female  
248 number.

#### 249 Potential benefits of ineffective matings for males

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

253

## 254 **Results**

#### 255 Effect of multiple mating on offspring number and paternity

256 Overall, there was no significant effect of the mating treatment ( $X^2_4=1.411$ ,  $P=0.842$ ) on the proportion  
257 of offspring sired by the first males (Fig. 2a). Therefore, first male sperm precedence is virtually  
258 complete. This also indicates no differences in sex-ratio across treatments. However, mating treatment  
259 affected daily fecundity significantly ( $F_{4,389.95}=8.633$ ,  $P<0.001$ ). Contrast analyses revealed that females  
260 that mated multiple times 24h after their first mating had significantly lower fecundity compared to  
261 once-mated females, while females from all other treatments laid the same number of eggs (O vs TI:  
262  $Z=-0.025$ ,  $P=1.00$ , O vs TL:  $Z=-0.725$ ,  $P=1.00$ , O vs MI:  $Z=-1.976$ ,  $P=0.193$  and O vs ML:  $Z=-$   
263  $4.151$ ,  $P<0.001$ ; Fig. 2b, Table S3).

#### 264 Effect of multiple mating on male and female survival

265 The mating treatment the female was subjected to affected significantly the survival of females  
266 ( $X^2_4=10.899$ ,  $P=0.0277$ ). However, no significant differences were found when comparing all  
267 treatments to the once-mated control (O vs TI:  $Z=-0.203$ ,  $P=1.00$ , O vs TL:  $Z=1.235$ ,  $P=0.867$ , O vs  
268 MI:  $Z=1.379$ ,  $P=0.671$  and O vs ML:  $Z=-1.719$ ,  $P=0.343$ ; Fig. 3a). As for males, their survival was  
269 significantly affected by the interaction between female status and the number of females on each patch  
270 ( $X^2_2=7.198$ ,  $P=0.027$ ). Indeed, males placed with virgin females survived less in the presence of 5  
271 females than in presence of 1 female per day (V1 vs V5:  $Z=2.349$ ,  $P=0.038$ ; V1 vs V20:  $Z=0.353$ ,  
272  $P=1.00$ ; Fig. 3b, Table S3). However, no significant differences in survival were observed when males  
273 were placed with mated females ( $X^2_2=0.497$ ,  $P=0.78$ ; Fig. 3c).

#### 274 Potential benefits of ineffective matings for males

275 A significant effect of mating treatment was found for the total number of fertilized offspring sired by  
276 the first male ( $X^2_4=15.956$ ,  $P=0.003$ ). Indeed, multiply-mated females with an interval of 24 hours  
277 between first and subsequent matings produced fewer fertilized offspring, compared to once mated  
278 females (O vs ML:  $Z=3.174$ ,  $P=0.006$ ; Fig. 4, Table S3). This suggests that second males benefit by  
279 mating with mated females. However, females belonging to all other treatments produced the same  
280 number of fertilized offspring than once-mated females (O vs TI:  $Z=-0.024$ ,  $P=1.00$ , O vs TL:  $Z=-$   
281  $0.315$ ,  $P=1.00$ ; O vs MI:  $Z=-0.367$ ,  $P=1.00$ ; Fig. 4, Table S3).

282

## 283 **Discussion**

284 Our study revealed that nearly all fertilized offspring was sired by the first male, independently of the  
285 mating interval and the number of matings. In addition, a decrease in fecundity, but not in survival, was  
286 found in females that had multiple mating opportunities after an interval of 24 hours between the first  
287 and subsequent matings. Males, however, suffered increased costs of mating when placed with 5 virgin  
288 females daily, but not when placed with mated females, revealing costs for first, but not for second  
289 males. In addition, first males produced fewer daughters when the females they mated with re-mated  
290 with other males.

291 We cannot disentangle complete, to nearly complete, first male sperm precedence, because natural death  
292 in the quiescent stage may be confounded with death by pesticide exposure. Thus, there is a non-null  
293 threshold of detection for fertilization by second males. Still the contribution of second males to siring  
294 offspring in these conditions, if any, is extremely small, and is not likely to explain the existence of  
295 polyandry in this species. Some species with first male sperm precedence have been shown to change  
296 their pattern of sperm precedence with mating interval and number of matings. For instance, in the silk  
297 worm *Bombix mori*, the paternity share of the first male changes from 0.95 to 0.06 in two hours (Suzuki,  
298 Okuda, & Shinbo, 1996). However, other species, such as the wasp *Diadromus pulchellus*, keep their  
299 pattern of sperm precedence across mating intervals (Agoze, Poirié, & Périquet, 1995).

300 Still other factors, that could influence the pattern of sperm precedence remain to be tested. For instance,  
301 in *Drosophila pseudoobscura*, a mostly monandrous fly, females use the sperm from the second mating  
302 whenever the first mating opportunity failed (Fisher et al., 2013). The authors make a distinction  
303 between true polyandry and pseudopolyandry, that occurs when females remate but no sperm  
304 competition takes place, owing for instance to lack of sperm transfer. This could be the case in spider  
305 mites as well, a hypothesis that remains to be tested. Indeed, sperm depletion, or incomplete sperm  
306 transfer in the first male may allow for some paternity share between the first and second as sperm  
307 depletion is a phenomenon potentially common in spider mites (Krainacker & Carey, 1989). In addition,  
308 an early study, in which the effect of several mating intervals on sperm precedence in spider mites was  
309 tested without controlling for sperm depletion, found that second males can sire some offspring when  
310 the interval between copulations is shorter than 24 hours (Helle 1967). Unfortunately, the frequency of  
311 sperm-depleted matings in spider mite natural populations, which is expected to determine its role in  
312 shaping the evolution of sperm precedence, is unknown.

313 The fact that we could not detect evidence for first male sperm precedence being incomplete  
314 suggests that indirect genetic benefits of polyandry for females are absent in this species. Moreover,  
315 females that mated multiply paid a cost of fecundity. Most of the few studies that explored the costs  
316 and benefits of polyandry in species with first male sperm precedence show that male ejaculates provide  
317 benefits to the females (e.g. Thailayil et al. 2011; Helinski and Harrington 2012). However, in *Nasonia*

318 *vitripennis*, a mostly monandrous wasp, female costs have been observed in patches with intense male  
319 harassment (Rebecca A. Boulton & Shuker, 2015). Since no differences in sex-ratio were observed  
320 between treatments, the mating costs observed here are, most likely, a reflection of the negative effects  
321 of multiple matings coupled with increased number of mating attempts, as observed in *N. vitripennis*.  
322 The fact that we only found a cost when the interval between the first and subsequent matings was of  
323 24 hours, may be explained by differences in female receptivity across different mating intervals.  
324 Indeed, females that mated 24 hours after the first mating, independently of the number of matings,  
325 took longer to mate and interrupted matings more often than females that re-mated immediately after  
326 the first mating (authors personal observations, Clemente et al. 2016). This suggests that females  
327 become more resistant to mating sometime after the first copulation, consequently suffering increased  
328 costs.

329 In the absence of clear benefits of multiple mating for females and of direct benefits for males,  
330 if the costs associated with those matings are low, males may mate with mated females because there  
331 is no selection pressure to eliminate such behaviour. In several species, the cost of mating for males  
332 varies with the mating status of females. Matings with mated females may entail fewer costs, if males  
333 allocate sperm differently according to the reproductive value of females (“strategic ejaculates”;  
334 Simmons 2001; Kelly and Jennions 2011). Accordingly, in species with first male sperm precedence,  
335 we expect males to invest more in matings with virgin females, as those are the ones with the highest  
336 reproductive value. Our results are in line with this prediction, as they show that only first males (those  
337 that mate with virgins) pay a cost of mating. This suggests that males invest more in mating with these  
338 females, either increasing their mating rate or transferring more sperm in each copulation, although it  
339 is intriguing that fewer costs were detected at the highest female density. In contrast, second males,  
340 which mate with mated females, payed the same survival costs in patches with different female  
341 densities. Previous results show that copulations with virgin females occur at a faster rate and last longer  
342 than copulations with mated females (Rodrigues et al., 2017), which is in line with these results.  
343 Therefore, males may engage into matings with virgins, which result in high offspring yield but also a  
344 survival cost, or into matings with mated females, yielding no offspring but also fewer costs.

345 Despite being ineffective, matings with mated females may still yield some benefits to males. A  
346 decrease in the total fecundity of multiply-mated females has been observed in spider mites (Macke et  
347 al., 2012), a result that we recover here. This may translate into fewer offspring being sired by first  
348 males (the Relative Fitness hypothesis, Macke et al. 2012). Here, we validated this hypothesis by  
349 showing that first males produced fewer offspring (i.e., daughters) when mating with females that mated  
350 multiply 24 hours later. Because the proportion of daughters remained unchanged, this decrease in the  
351 number of daughters is probably due to a decrease in fecundity of females owing to costs of mating and  
352 male harassment. Therefore, mating with mated females can increase the relative reproductive success  
353 of subsequent males, by reducing the genetic contribution of the first males to the following generations.  
354 This strategy requires that the harming males (or their brothers) produce some descendance and pay a  
355 low penalty with this behaviour. Apart from the life-history costs of the behaviour, which we showed  
356 here to be low, they could lose mating opportunities with virgin females. Therefore, this behaviour  
357 should be most favoured in expanding populations: males can first mate with virgin females, then later,  
358 when these become scarce, turn to harming mated females, hence not suffering much from lost  
359 opportunities. Moreover, the uncovered benefits should be dependent on population structure. Indeed,  
360 in large populations, benefits should be mitigated as they are shared by all other males of the population,  
361 while In small populations, relatedness can be high, in which case, reducing the fitness of other, related,  
362 males in the population may not be advantageous (e.g., Carazo et al. 2014). Yet, this is contingent on  
363 the scale of competition, because if competition occurs locally, males are not expected to behave  
364 differentially towards related males, as these are their only competitors (Pizzari et al. 2015). Finally,  
365 the effectiveness of this behaviour relies on a collective action as one mating is not enough to reduce  
366 the fitness of the first. Therefore, the precise population structure in which this behaviour will be  
367 beneficial may be very specific. Roughly, it should correspond to a situation of budding dispersal  
368 (Gardner, Arce, & Alpedrinha, 2009), in which related males arrive together in a patch occupied by  
369 unrelated individuals, and collectively reduce the fitness of unrelated males for the next generation.  
370 Although spider mites are expected to face different conditions during their dispersal-colonization  
371 phases, it is not clear that such beneficial conditions occur often enough for this behaviour to be selected.  
372 Hence, the probability of selecting this trait in males will hinge on how often they will encounter the

373 conditions favouring it. It is thus important to design experiments varying population structure to test  
374 these ideas.

375 Altogether, our results show that multiple mating is costly for females but that matings with mated  
376 females are potentially beneficial for males. The latter was not expected, given that we are dealing with  
377 a species with first male sperm precedence. The consequences of polyandry in such species have been  
378 seldom explored, leaving a gap in our knowledge (but see Dougherty et al. 2016). Indeed, if we had  
379 found no benefits of polyandry in both sexes, we could speculate that selection would be favouring  
380 monandry with time. Conversely, if males and females benefited with polyandry, we could expect that  
381 selection would maintain polyandry, which in turn, would open the door for an evolution of the sperm  
382 precedence pattern itself. Because we found that some males may benefit from mating with mated  
383 females, but that females suffer costs with polyandry, conflicts between sexes should be present and the  
384 direction of selection on polyandry will depend on which sex is winning this conflict.

385

### 386 **Acknowledgements**

387 The authors wish to thank Inês Santos for help with the maintenance of mite cultures at cE3c and  
388 Emilie Macke, João Alpedrinha, Alison Duncan, Diogo Godinho and Suzanne Alonzo for insightful  
389 comments on the manuscript. These experiments were funded by Portuguese (FCT, Fundação para a  
390 Ciência e Tecnologia) and French (ANR, Agence Nationale de la Recherche) funds through an FCT-  
391 ANR project (FCT-ANR//BIA-EVF/0013/2012) to SM and IO. LRR had a Ph.D. Grant  
392 (SFRH/BD/87628/2012) funded by FCT. The authors have no conflict of interest to declare.

### 393 **Authors Contributions**

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

397



## 398 **References**

- 399 Agoze, M. El, Poirié, M., & Périquet, G. (1995). Precedence of the first male sperm in successive  
400 matings in the Hymenoptera *Diadromus pulchellus*. *Entomologia Experimentalis et Applicata*,  
401 75, 251–255. doi:10.1111/j.1570-7458.1995.tb01934.x
- 402 Arnqvist, G., & Nilsson, T. (2000). The evolution of polyandry: multiple mating and female fitness in  
403 insects. *Animal Behaviour*, 60, 145–164. doi:10.1006/anbe.2000.1446
- 404 Arnqvist, G., & Rowe, L. (2005). *Sexual Conflict*. Princeton University Press.
- 405 Bateman, A. J. (1948). Intrasexual Selection. *Heredity*, 2, 349–368. doi:10.1038/hdy.1948.21
- 406 Bissoondath, C. J., & Wiklund, C. (1997). Effect of male body size on sperm precedence in the  
407 polyandrous butterfly *Pieris napi* L. (Lepidoptera: Pieridae). *Behavioral Ecology*, 8(5), 518–  
408 523. doi:10.1093/beheco/8.5.518
- 409 Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H., & White,  
410 J. S. S. (2008). Generalized linear mixed models: a practical guide for ecology and evolution.  
411 *Trends in Ecology and Evolution*, 24, 127–135. doi:10.1016/j.tree.2008.10.008
- 412 Boulton, R. A., & Shuker, D. M. (2015). The costs and benefits of multiple mating in a mostly  
413 monandrous wasp. *Evolution*, 69, 939–949. doi:10.1111/evo.12636
- 414 Boulton, R. A., & Shuker, D. M. (2016). Polyandry is context dependent but not convenient in a  
415 mostly monandrous wasp. *Animal Behaviour*, 112, 119–125. doi:10.1016/j.anbehav.2015.12.001
- 416 Breeuwer, J. A. J. (1997). *Wolbachia* and cytoplasmic incompatibility in the spider mites *Tetranychus*  
417 *urticae* and *T. turkestani*. *Heredity*, 79, 41–47. doi:10.1038/sj.hdy.6881810
- 418 Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., ...  
419 Bolker, B. M. (2017). glmmTMB balances speed and flexibility among packages for zero-  
420 inflated generalized linear mixed modeling. *The R Journal*, 9, 378–400. doi:10.3929/ethz-b-  
421 000240890

- 422 Bullini, L., Coluzzi, M., & Bianchi Bullini, A. P. (1976). Biochemical variants in the study of  
423 multiple insemination in *Culex pipiens* L. (Diptera, Culicidae). *Bulletin of Entomological*  
424 *Research*, 65, 683–685. doi:10.1017/S0007485300006374
- 425 Carazo, P., Tan, C. K. W. W., Allen, F., Wigby, S., & Pizzari, T. (2014). Within-group male  
426 relatedness reduces harm to females in *Drosophila*. *Nature*, 505, 672–675.  
427 doi:10.1038/nature12949
- 428 Chapman, T., Liddle, L. F., Kalb, J. M., Wolfner, M. F., & Partridge, L. (1995). Cost of mating in  
429 *Drosophila melanogaster* females is mediated by male accessory-gland products. *Nature*, 373,  
430 241–244. doi:10.1038/373241a0
- 431 Clemente, S. H., Rodrigues, L. R., Ponce, R., Varela, S. A. M., & Magalhães, S. (2016). Incomplete  
432 species recognition entails few costs in spider mites, despite first-male precedence. *Behavioral*  
433 *Ecology and Sociobiology*, 70, 1161–1170. doi:10.1007/s00265-016-2124-0
- 434 Crawley, M. J. (2007). *The R book*. John Wiley & Sons, Ltd.
- 435 Dougherty, L. R., Simmons, L. W., & Shuker, D. M. (2016). Postcopulatory sexual selection when a  
436 female mates once. *Animal Behaviour*, 116, 13–16. doi:10.1016/j.anbehav.2016.03.003
- 437 Eady, P. E., Wilson, N., & Jackson, M. (2000). Copulating with multiple mates enhances female  
438 fecundity but not egg-to-adult survival in the bruchid beetle *Callosobruchus maculatus*.  
439 *Evolution*, 6, 2161–2165.
- 440 Fisher, D. N., Doff, R. J., & Price, T. A. R. (2013). True polyandry and pseudopolyandry: why does a  
441 monandrous fly remate? *BMC Evolutionary Biology*, 13, 157. doi:10.1186/1471-2148-13-157
- 442 Gardner, A., Arce, A., & Alpedrinha, J. (2009). Budding dispersal and the sex ratio. *Journal of*  
443 *Evolutionary Biology*, 22, 1036–1045. doi:10.1111/j.1420-9101.2009.01719.x
- 444 Grbić, M., Van Leeuwen, T., Clark, R. M., Rombauts, S., Rouzé, P., Grbić, V., ... Van de Peer, Y.  
445 (2011). The genome of *Tetranychus urticae* reveals herbivorous pest adaptations. *Nature*, 479,

- 446 487–92. doi:10.1038/nature10640
- 447 Helinski, M. E. H., & Harrington, L. C. (2012). The role of male harassment on female fitness for the  
448 dengue vector mosquito *Aedes aegypti*. *Behavioral Ecology and Sociobiology*, *66*, 1131–1140.  
449 doi:10.1007/s00265-012-1365-9
- 450 Helle, W. (1967). Fertilization in the two-spotted spider mite (*Tetranychus urticae*: Acari).  
451 *Entomologia Experimentalis et Applicata*, *10*, 103–110. doi:10.1007/BF00338618
- 452 Helle, W., & Sabelis, M. W. (1985). *Spider Mites: Their Biology, Natural Enemies and Control*.  
453 Elsevier Science Publishing Company.
- 454 Hosken, D. J., Stockley, P., Tregenza, T., & Wedell, N. (2009). Monogamy and the battle of the  
455 sexes. *Annual Review of Entomology*, *54*, 361–378. doi:10.1146/annurev.ento.54.110807.090608
- 456 Kelly, C. D., & Jennions, M. D. (2011). Sexual selection and sperm quantity: Meta-analyses of  
457 strategic ejaculation. *Biological Reviews*, *86*, 863–884. doi:10.1111/j.1469-185X.2011.00175.x
- 458 Krainacker, D. A., & Carey, J. R. (1989). Reproductive limits and heterogeneity of male twospotted  
459 spider mites. *Entomologia Experimentalis et Applicata*, *50*, 209–214. doi:10.1007/BF00341169
- 460 Kvarnemo, C., & Simmons, L. W. (2013). Polyandry as a mediator of sexual selection before and  
461 after mating. *Philosophical Transactions of the Royal Society of London B*, *368*, 20120042.  
462 doi:10.1098/rstb.2012.0042
- 463 Macke, E., Magalhães, S., Do-Thi Khanh, H., Frantz, A., Facon, B., & Olivieri, I. (2012). Mating  
464 modifies female life history in a haplodiploid spider mite. *The American Naturalist*, *179*, 147–  
465 162. doi:10.1086/665002
- 466 Magalhães, S., Fayard, J., Janssen, A., Carbonell, D., & Olivieri, I. (2007). Adaptation in a spider mite  
467 population after long-term evolution on a single host plant. *Journal of Evolutionary Biology*, *20*,  
468 2016–2027. doi:10.1111/j.1420-9101.2007.01365.x
- 469 Manier, M. K., Belote, J. M., Berben, K. S., Novikov, D., Stuart, W. T., & Pitnick, S. (2010).

- 470 Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. *Science*,  
471 328, 354–357. doi:10.1126/science.1187096
- 472 Oku, K. (2010). Males of the two-spotted spider mite attempt to copulate with mated females: Effects  
473 of double mating on fitness of either sex. *Experimental and Applied Acarology*, 50, 107–113.  
474 doi:10.1007/s10493-009-9306-7
- 475 Pizzari, T., Biernaskie, J. M., & Carazo, P. (2015). Inclusive fitness and sexual conflict: How  
476 population structure can modulate the battle of the sexes. *BioEssays*, 37, 155–166.  
477 doi:10.1002/bies.201400130
- 478 Potter, D. A., Wensch, D. L., & Johnston, D. E. (1976). Aggression and mating success in male  
479 spider mites. *Science*, 193, 160–161. doi:10.1126/science.193.4248.160
- 480 Ridley, M. (1989). The incidence of sperm displacement in insects: four conjectures, one  
481 corroboration. *Biological Journal of the Linnean Society*, 38, 349–367. doi:10.1111/j.1095-  
482 8312.1989.tb01582.x
- 483 Ridley, M. (1989). The timing and frequency of mating in insects. *Animal Behaviour*, 37, 535–545.  
484 doi:10.1016/0003-3472(89)90033-X
- 485 Rodrigues, L. R., Figueiredo, A. R. T., Varela, S. A. M., Olivieri, I., & Magalhães, S. (2017). Male  
486 spider mites use chemical cues, but not the females mating interval to choose between mates.  
487 *Experimental and Applied Acarology*, 70, 1–13. doi:10.1007/s10493-016-0103-9
- 488 Schmid-Hempel, P., & Baer, B. (1999). Experimental variation in polyandry affects parasite loads and  
489 fitness in a bumble-bee. *Nature*, 397, 151–154. doi:10.1038/16451
- 490 Simmons, L. W. (2001). *Sperm competition and its evolutionary consequences in the insects*.  
491 Princeton University Press.
- 492 Snook, R. R. (2014). The evolution of polyandry. In D. M. Shuker & L. W. Simmons (Eds.), *The*  
493 *Evolution of Insect Mating Systems* (pp. 159–180). Oxford University Press.

- 494 Suzuki, N., Okuda, T., & Shinbo, H. (1996). Sperm precedence and sperm movement under different  
495 copulation intervals in the silkworm, *Bombyx mori*. *Journal of Insect Physiology*, *42*, 199–204.  
496 doi:10.1016/0022-1910(95)00104-2
- 497 Taylor, M. L., Price, T. A. R., & Wedell, N. (2014). Polyandry in nature: a global analysis. *Trends in*  
498 *Ecology & Evolution*, *29*, 376–383. doi:10.1016/J.TREE.2014.04.005
- 499 Thailayil, J., Magnusson, K., Godfray, H. C. J., Crisanti, A., & Catteruccia, F. (2011). Spermless  
500 males elicit large-scale female responses to mating in the malaria mosquito *Anopheles gambiae*.  
501 *Proceedings of the National Academy of Sciences*, *108*, 13677–81.  
502 doi:10.1073/pnas.1104738108
- 503 Thomas, M. L. (2011). Detection of female mating status using chemical signals and cues. *Biological*  
504 *Reviews*, *86*, 1–13. doi:10.1111/j.1469-185X.2010.00130.x
- 505 Van Leeuwen, T., Demaeght, P., Osborne, E. J., Dermauw, W., Gohlke, S., Nauen, R., ... Clark, R.  
506 M. (2012). Population bulk segregant mapping uncovers resistance mutations and the mode of  
507 action of a chitin synthesis inhibitor in arthropods. *Proceedings of the National Academy of*  
508 *Sciences*, *109*, 4407–12. doi:10.1073/pnas.1200068109
- 509 Weldingh, D. L., Toft, S., & Larsen, O. N. (2011). Mating duration and sperm precedence in the  
510 spider *Linyphia triangularis*. *Journal of Ethology*, *29*, 143–152. doi:10.1007/s10164-010-0237-x
- 511 Zeh, J. A., & Zeh, D. W. (1994). Last-male sperm precedence breaks down when females mate with  
512 three males. *Proceedings of the Royal Society of London B*, *257*, 287–292.  
513 doi:10.1098/rspb.1994.0127
- 514
- 515

516 **Figure 1. Protocol followed to assess sperm precedence and female fecundity and survival. a)**

517 **Mating Protocol.** Groups of 5 females and 5/6 males were placed together on patches until they mated.

518 Females mated once (O), twice (T) or multiply (M) with a mating interval between the first and

519 subsequent matings of either 0 hours (I, Immediately) or 24 hours (L, later). Females are bigger than

520 males and are always resistant to pesticide (white). Males are smaller and represented in black or white.

521 Different male colours represent different phenotypes after pesticide exposure: black males are

522 susceptible, while white males are resistant to pesticide. Note, that in this scheme only one male type

523 order is represented (resistant males first). However, both orders were performed. Dotted arrows:

524 females were transferred immediately from one patch to the next; full arrows: females were maintained

525 on a patch for 24 hours with males; dashed arrows: females were maintained on a patch for 24 hours

526 without males. **b) Data Collection.** Each female was isolated on a leaf disc placed in water-soaked

527 cotton and its survival was checked daily. Every 3 days, the female was transferred to a new leaf disc

528 where she could continue to lay eggs. The number of eggs laid by the female on each leaf disc was

529 measured after each transfer. On the 6<sup>th</sup> day, the leaf disc was moved into a container with cotton soaked

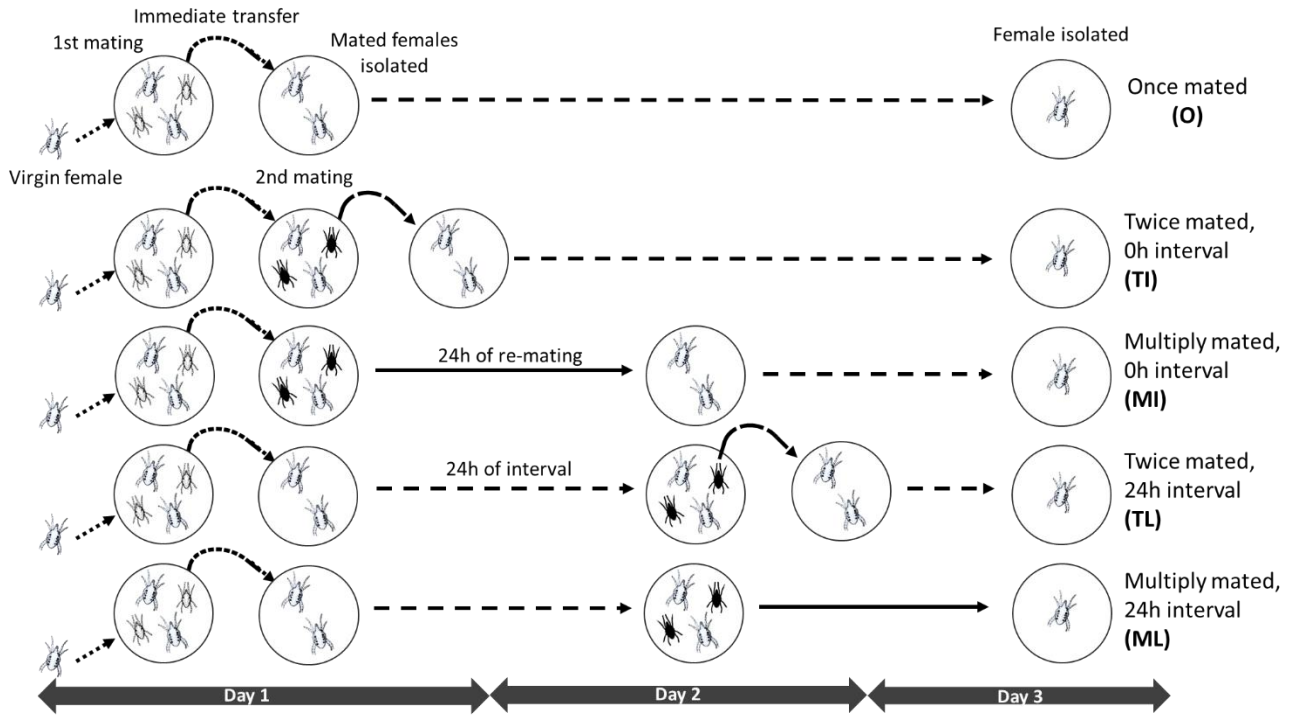
530 in diluted pesticide. On the 12<sup>th</sup> day offspring sex-ratio and survival were measured, to extract offspring

531 paternity and the total number of daughters sired by the first male. X, death owing to pesticide.

532

533

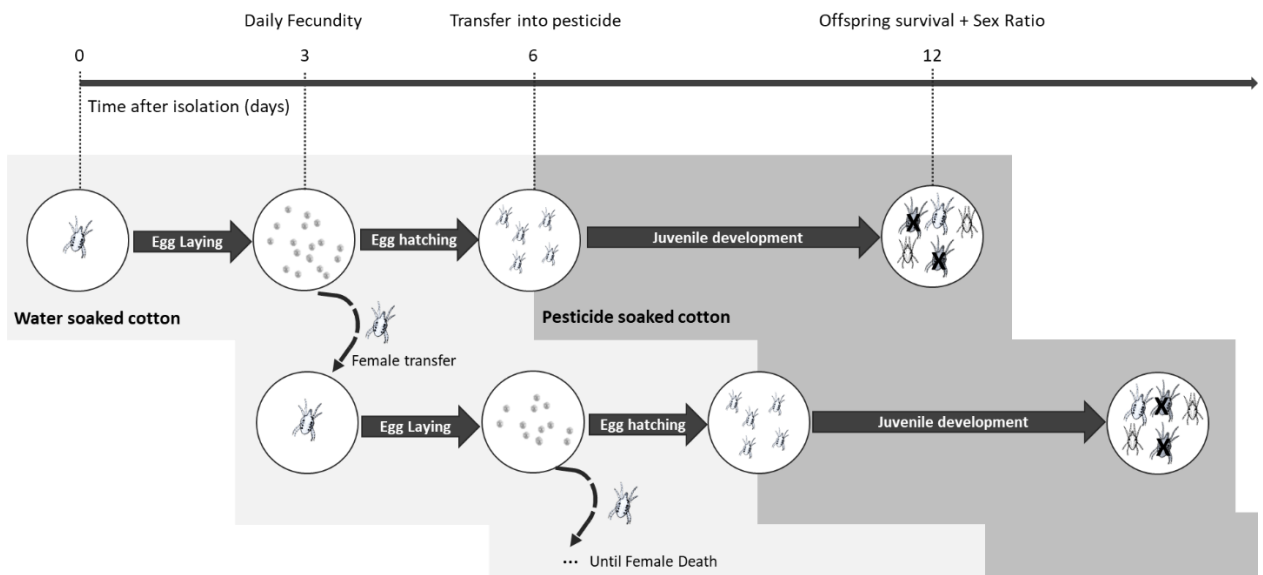
534 a)



535

536

537 b)

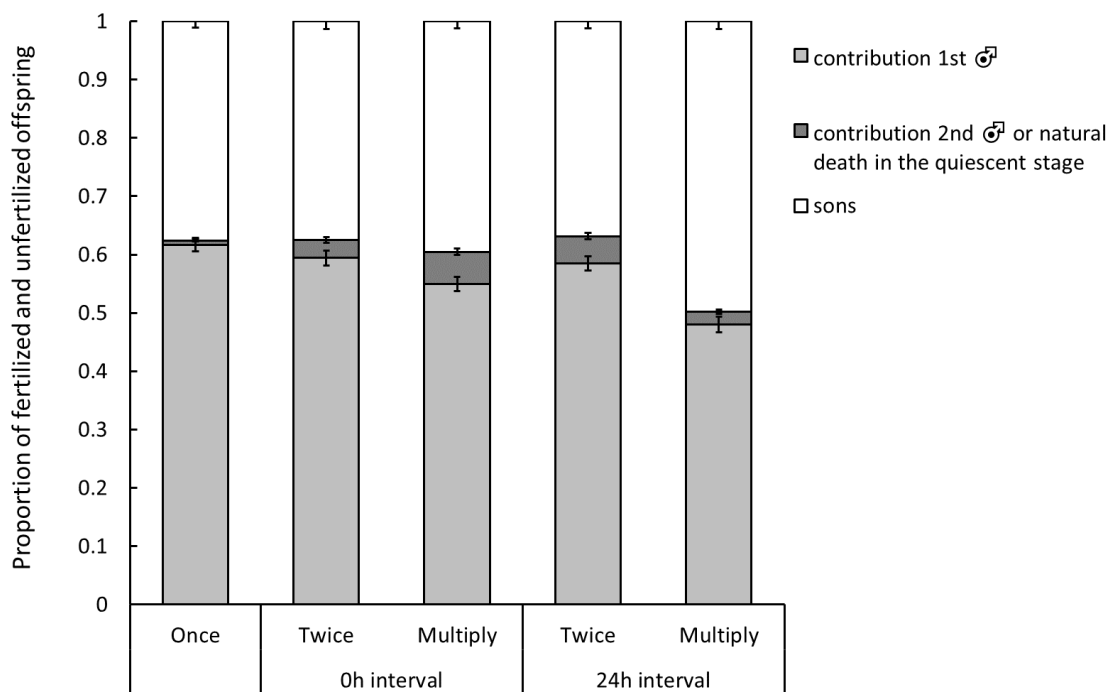


538

539

540 **Figure 2. Effect of multiple mating on offspring number and paternity.** Females mated once (O),  
541 twice (T), or multiply (M). Re-mating was set immediately (0h interval; I) or 24 hours after the first  
542 mating (24h interval; L). **a) Proportion of fertilized and unfertilized offspring across treatments.**  
543 Fertilized offspring (i.e. daughters) is divided into proportion of daughters sired by the first (light grey)  
544 and by the second male (dark grey). Note however, that natural death in the quiescent stage may be  
545 confounded with death by pesticide exposure, in both bars representing fertilized offspring. Unfertilized  
546 offspring (sons) is represented in white. Vertical bars correspond to standard errors of the mean. **b)**  
547 **Mean number of eggs laid daily by females.** Vertical bars correspond to standard errors of the mean.  
548 Asterisk (\*) represent significant level ( $P < 0.05$ ).

549 a)



550

551

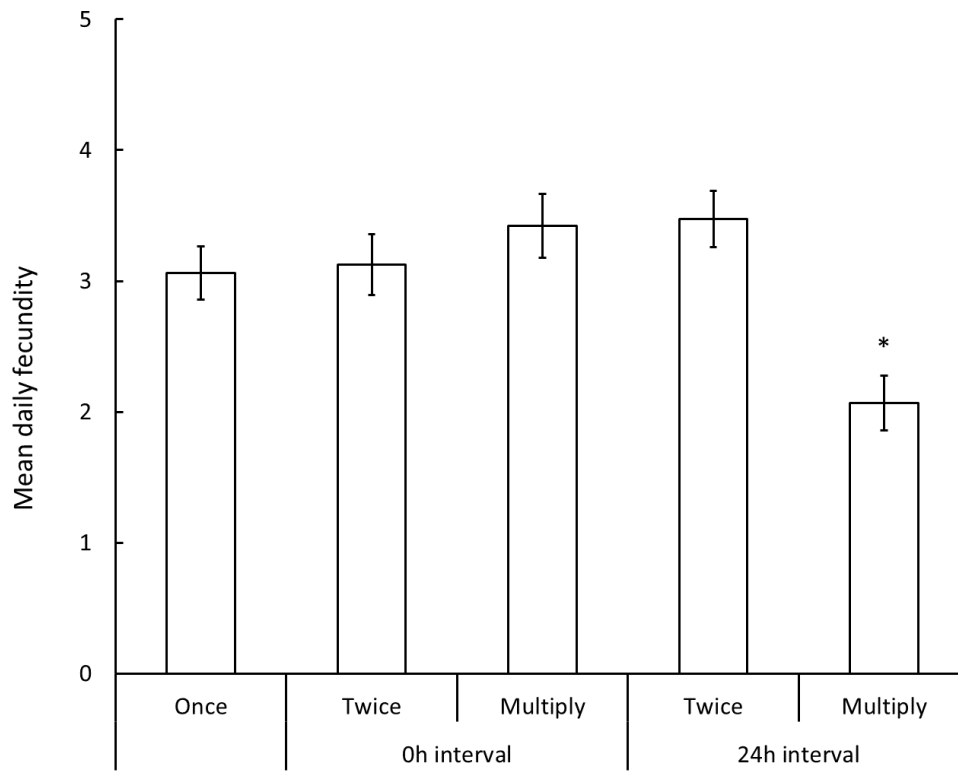
552

553



554

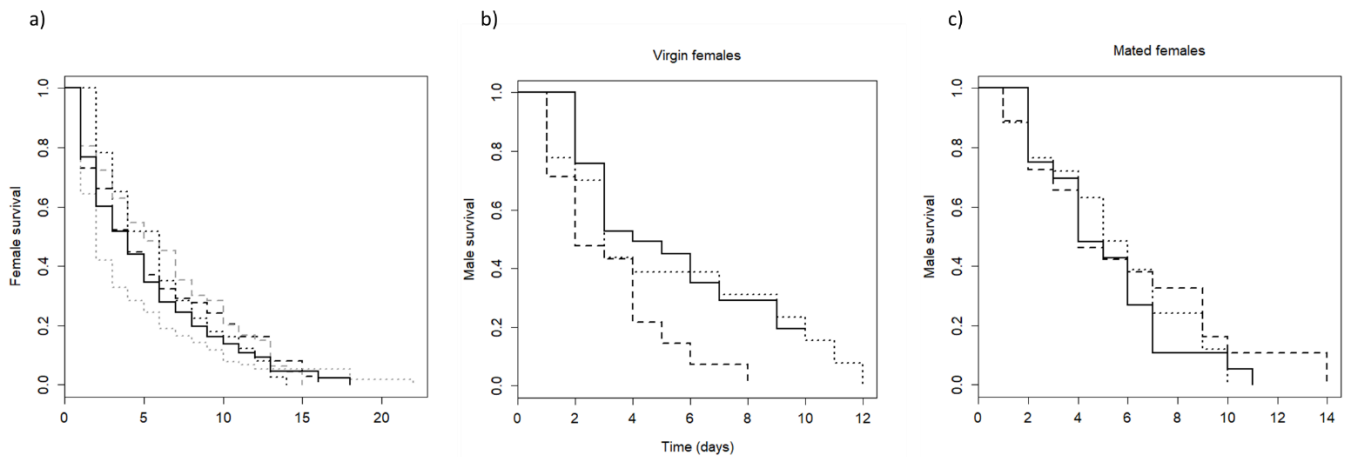
555 **b)**



556

557

558 **Figure 3. Effect of multiple mating on male and female survival. a) Female survival curve.** Females  
559 mated once (O), twice (T), or multiply (M). Re-mating was set immediately (0h interval; I) or 24 hours  
560 after the first mating (24h interval; L). Each line corresponds to different number of matings: continuous  
561 line, one mating; dashed line, two matings; dotted line, multiple matings. Grey lines correspond to L  
562 rematings; black dashed and black dotted lines correspond to I re-matings. **b) and c) Male survival**  
563 **curves.** Males were placed in patches with 1, 5 or 20 virgin (b) or mated (c) females every day and its  
564 survival was followed. Distinct types of lines represent different number of females per patch status:  
565 continuous line, one female; dashed line, 5 females; dotted line, 20 females.

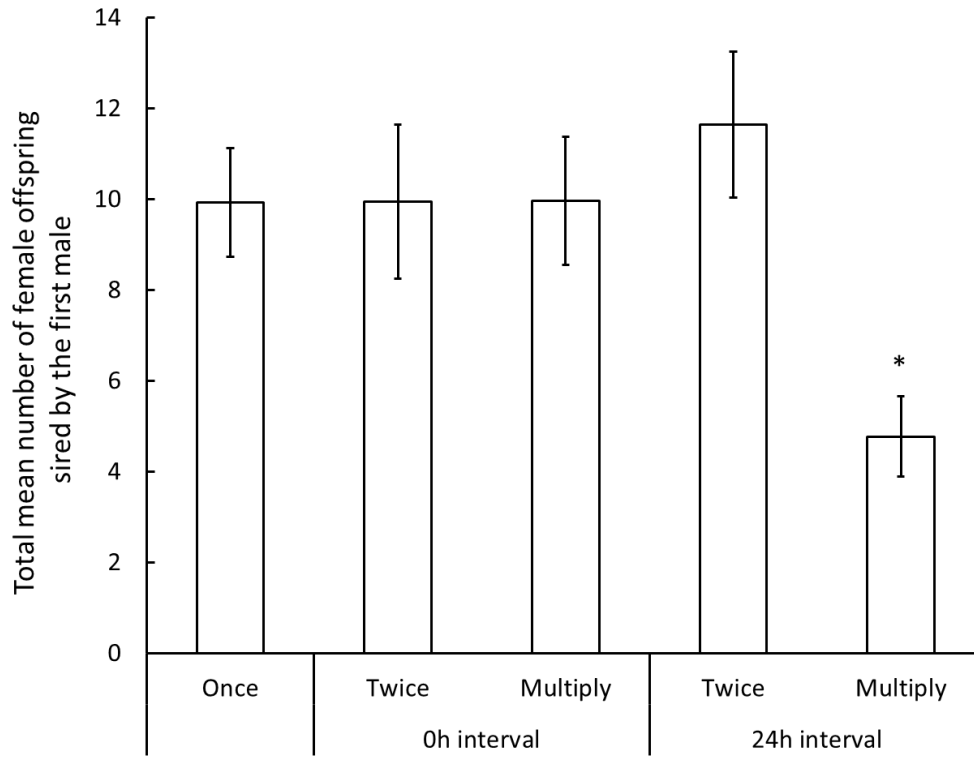


566

567

568

569 **Figure 4. Total mean number of offspring sired by the first male.** Females mated once (O), twice  
570 (T), or multiply (M). Re-mating was set immediately (0h interval; I) or 24 hours after the first mating  
571 (24h interval; L). Vertical bars correspond to standard errors of the mean. Asterisk (\*) represent  
572 significant level ( $P < 0.05$ ).



573

574

575