1 **Despite reproductive interference, the net outcome of reproductive interactions**

2 **among spider mite species is not necessarily costly**

3 Salomé H. Clemente, Inês Santos, Rita Ponce, Leonor R. Rodrigues, Susana A. M.

4 Varela* and Sara Magalhães*[1]

5 salome.hc@gmail.com, inesflsantos@gmail.com, anaritaponce@gmail.com,

6 leonor.rodrigues89@gmail.com, savarela@fc.ul.pt, snmagalhaes@fc.ul.pt

7 *co-last authorship

8 cE3c – Centre for Ecology, Evolution and Environmental Changes

9 Faculdade de Ciências da Universidade de Lisboa

10 Edifício C2, 3º Piso

11 Campo Grande

12 1749-016 Lisbon

13 Portugal

14 [1]: snmagalhaes@fc.ul.pt

15

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18   **Abstract**

19   Reproductive interference is considered a strong ecological force, potentially leading

20   to species exclusion. This supposes that the net effect of reproductive interactions is

21   strongly negative for one of the species involved. Testing this requires a

22   comprehensive analysis of interspecific reproductive interactions, accounting for the

23   order and timing of mating events, and for their effects on either fertility or fecundity.

24   To this aim, we measured reproductive interactions between a focal species,

25   *Tetranychus urticae*, and an invasive (*T.evansi*) and a resident (*T. ludeni*) species,

26   varying the mating sequence and interval, and measuring the effect of such crosses on

27   fecundity and offspring sex ratio (a measure of fertility, as these species are

28   haplodiploid). We found that mating with heterospecifics affected fecundity and sex

29   ratio negatively, but also positively, depending on the species involved, and on the

30   order and timing of mating events. Overall, the net effect of reproductive interactions

31   was weak despite strong effects of particular events. In natural situations the outcome

32   of reproductive interactions will thus hinge upon the frequency of each event.

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38   **Introduction**

39   Reproductive interference, that is, any kind of sexual interaction between two species

40   that diminishes the fitness of at least one of them (Gröning and Hochkirch 2008, Kishi

41   et al. 2009, Burdfield-Steel and Shuker 2011), may have severe effects on the outcome

42   of species interactions. Indeed, theory predicts that reproductive interference may

43   contribute to species exclusion more often than resource competition (Gröning and

44   Hochkirch 2008, Kishi et al. 2009, Kishi and Nakazawa 2013). For example, it has been

45   posited that reproductive interference may underlie the success of some invasive

46   species (e.g. Nishida et al. 2012).

47   Most studies of reproductive interference concern the fitness outcome of

48   interspecific matings of two species that do not produce viable hybrids (Gröning and

49   Hochkirch 2008). In this case, the reproductive effects of the interspecific interaction

50   will be expressed only when organisms mate with both conspecifics and

51   heterospecifics (as mating with conspecifics alone will yield no offspring). Moreover,

52   clearly evaluating the effects of reproductive interference on exclusion in polyandrous

53   species necessitates measuring all possible combinations of mating order (*i.e.*, whether

54   heterospecific matings occur before or after conspecific ones) and timing (*i.e.*, the

55   interval between mating events) between pairs of species. It is also important to test

56   whether reproductive interactions affect fecundity (egg production) or fertility (egg

57   fertilization). This information can then be integrated to predict the net outcome of

58   reproductive interactions between species. Despite the many studies on reproductive

59   interference, none has yet applied this approach. Indeed, some studies attempt to

60   predict how reproductive interference affects species exclusion, but do so by focussing

61   on some sequence events only. For example, Takafuji (1997) used a Lotka-Volterra

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62   modified model to predict the effect of reproductive interference between two mite

63   species (*Panonychus citri* and *P. mori*) on species exclusion, but they used only one

64   possible combination of mating interactions between species. In contrast, other

65   studies consider different orders of mating events (eg, Kyogoku and Nishida 2013), but

66   do not integrate this information to generate a prediction concerning the net effect of

67   reproductive interactions on species distributions.

68   Here, we aimed at testing how the outcome of different mating events among

69   species may affect their life-history traits, using spider mites, a group where

70   reproductive interference has been frequently observed (Collins and Margolies 1994;

71   Takafuji et al. 1997; Ben-David et al. 2009, Sato et al. 2014). Spider mites are

72   haplodiploid, hence the distinction between fecundity and fertilization effects can be

73   made given that fertilized eggs result in female offspring and unfertilized eggs in male

74   offspring. Thus, fertilization failures can be detected by a reduction in the proportion

75   of female offspring, whereas impairment of egg production is detected by a reduction

76   in the total number of offspring.

77   We used a system composed of one focal species, *Tetranychus urticae*, in

78   sexual heterospecific interactions with another resident species, *T. ludeni*, and an

79   invasive species, *T. evansi*. These three herbivorous species co-occur in the

80   Mediterranean region and are often found on the same host plant (Escudero and

81   Ferragut 2005, Boubou et al. 2012, Godinho et al. 2016). Whereas *T. urticae* and *T.*

82   *ludeni* are resident species, *T. evansi* has only recently invaded the European continent

83   (Boubou et al. 2012). Whereas information on the interaction between *T. urticae* and

84   *T. ludeni* is as yet lacking, heterospecific matings have been observed between *T.*

85   *urticae* and *T. evansi* (Sato et al. 2014, 2016, Clemente et al. 2016). Moreover, *T. evansi*

86   can exclude *T. urticae* on tomato plants (Sarmento et al. 2011a), a result that

87   correlates with field observations (Ferragut et al. 2013). Finally, a recent study has

88   shown that, in competition with *T. evansi*, the population growth of *T. urticae* is more

89   severely affected when plants are colonized by virgin females than when plants are

90   colonized by mated females, suggesting that reproductive interference may be

91   responsible for the species distribution patterns observed (Sato et al. 2014).

92   To postulate hypotheses concerning the consequences of heterospecific

93   matings, it is crucial to understand within-species reproductive behaviour. *T. urticae*,

94   the focal species, exhibits first male sperm precedence, with second matings being

95   sometimes effective if they occur within the 24 hours following the first (Helle 1967).

96   However, females that mate multiple times with conspecific males, after a 24h interval

97   between matings, produce fewer fertilized offspring (*i.e.*, females) (Macke et al. 2012),

98   suggesting that sperm displacement after 24h is possible. Here, we hypothesize that

99   mating order and the mating interval will affect the outcome of reproductive

100 interference in *T. urticae*. Also, given that *T. evansi*, the invasive species, displaces *T.*

101 *urticae*, unlike *T. ludeni*, we expect the former to exert stronger effects than the latter.

102 To this aim, we performed crosses between *T. urticae* and the two other species at

103 different time intervals and with different mating orders, and measured the

104 consequences for the two species involved in the cross.

105

106 **Material and Methods**

107 **Stock Cultures**

108 The mite species used in this study were collected in Carregado (39.022260, -

109 8.966566), Portugal, and all laboratory populations were established from an initial

110   pool of 300 mated females. The laboratory population of *T. urticae* was collected on

111   tomato plants (*Solanum lycopersicum)* in May 2010, that of *T. evansi* on *Physalis*

112   *angulata* in May 2012 and that of *T. ludeni* on tomato in September 2012. The

113   populations of *T. evansi* and *T. ludeni* became extinct in August 2012 and May 2013,

114   respectively, being subsequently replaced with populations from the same location,

115   both collected in *Datura stramonium* plants. Both populations of *T. evansi* and *T.*

116   *ludeni* were used in the experiments.

117   Species identity was confirmed through polymerase chain reaction–restriction

118   fragment length polymorphism (PCR–RFLP) of the ITS2 region (Hurtado et al. 2008), on

119   approximately 50 females of each population. Total genomic DNA was extracted from

120   each individual spider mite using the Sigma-Aldrich GenEluteTM Mammalian Genomic

121   DNA Miniprep Kit, following manufacturer´s instructions, except for the elution

122   volume, which we set to 20µL of RNase free water (Qiagen NV, Venlo, The

123   Netherlands) to increase the concentration of DNA obtained from this very small

124   animal (c.a. 300µm long).

125   Adult females from populations used in this experiment were screened for

126   *Wolbachia* using the primers *wsp* (*Wolbachia*-specific primers) 81F and 691R (Braig et

127   al. 1998). We did this to avoid potential cytoplasmic incompatibility as a confounding

128   factor in our measurements. PCR assay procedures were as described in Breeuwer

129   (1997). Results were positive for *Wolbachia* infection and all spider mite populations

130   were thus treated by placing adult females in detached bean leaves with tetracycline

131   (0.025% w/v) for three consecutive generations, then absence of *Wolbachia* was

132   confirmed using the same protocol as above. Other endosymbionts tested

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133   (Arsenophorous, Rickettsia, Spiroplasma and Cardinium) were absent from these

134   populations.

135   Bean (*Phaseolus vulgaris*) and tomato (*Solanum lycopersicum*) plants were planted

136   every week and grown in an herbivore-free greenhouse, being watered two to three

137   times a week. *T. urticae* populations were maintained on trays with 6-10 bean plants

138   whereas those of *T. evansi* and *T. ludeni* were kept on tomato plants at 25°C, both with

139   a 16 L: 8D photoperiod. Plant trays were changed every two weeks, placing old leaves

140   on top of uninfested plants. Cultures were kept inside plastic boxes (28x39x28 cm),

141   with an opening of 25x15 cm polyamide fabric (80 μm mesh width).

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144   **Experimental procedure**

145   Experiments were done on the plant species from which the female tested had been

146   cultured. As in the literature there was no information on whether hybridization is

147   possible between *T. urticae* and *T. ludeni*, we studied the outcome of a single

148   heterospecific mating between these two species (the same analysis for *T. urticae* and

149   *T. evansi* was performed in a previous experiment (Clemente et al. 2016)).

150   Subsequently, we set out to study the heterospecific interactions between *T. urticae*

151   and the invasive *T. evansi* and the resident *T. ludeni* species for which we analysed the

152   outcome of mating with a heterospecific male before or after a conspecific male. Since

153   we focused on interactions with *T. urticae* (the focal species of our study), we

154   performed crosses between *T. urticae* males or females and *T. evansi* or *T. ludeni*

155   males or females, but not between the two latter species. All experiments were

156   performed in an acclimatized room at approximately 25°C.

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157

a) The outcome of a single heterospecific mating between *T. urticae* and *T. ludeni*

159   To determine whether hybridization occurred between *T. urticae* and *T. ludeni*, we

160   measured the offspring sex-ratio resulting from single heterospecific matings. Given

161   that only females develop from fertilized eggs, a whole-male offspring would mean

162   unsuccessful hybridization. However, even in the absence of viable hybrids,

163   heterospecific matings could result in aborted development of heterospecifically-

164   fertilized eggs, meaning that females would produce fewer eggs. To test this, we

165   compared the fecundity of *T. urticae* and *T. ludeni* females that mated with a

166   heterospecific male to that of virgin females and of females mated with a conspecific

167   male.

168   Females were collected from the stock populations, isolated at the quiescent

169   deutonymph stage (which precedes their last moult before reaching adulthood), and

170   kept in groups of approximately 15 females on bean (*Phaseolus vulgaris*) leaf discs

171   (2 cm^2) until emergence, to ensure their virginity. Adult males were collected from the

172   same stock populations and kept isolated in leaf discs (2 cm^2) for at least 24 hours

173   before the assay, to ensure sperm replenishment. Females were placed individually in

174   leaf discs (1 cm^2) with either a conspecific or a heterospecific male and observed

175   continuously until copulation occurred. Only matings that lasted at least 1 minute were

176   considered effective (Boudreaux 1963). These experiments had the maximum duration

177   of 2 hours. If no mating occurred within this time, individuals were discarded.

178   Subsequently, females were isolated in a leaf disc (2 cm^2), then transferred to a new

179   disc every three days until the female's death. The number of eggs laid was registered

180   after female transfer to a new leaf disc. Eggs were left to develop until adulthood

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181   when offspring sex-ratio could be determined. With this data, we tested whether

182   heterospecific matings affected (a) the mean daily fecundity and (b) offspring sex ratio

183   (hence the proportion of fertilized offspring).

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185   **b) The outcome of heterospecific matings that precede or follow conspecific ones**

186   To determine the outcome of mating with a heterospecific male before or after a

187   conspecific male between *T. urticae* and the other two species, we compared the

188   fecundity and offspring sex ratio of those crosses to that of females that mated with

189   two conspecific males. The experimental procedure was as described above, except

190   that we let females mate with a conspecific or a heterospecific male, then placed the

191   focal females with another male. We created the following mating sequences:

192   conspecific-conspecific, conspecific-heterospecific and heterospecific-conspecific. The

193   second mating occurred either immediately after the first mating (0 hours treatment)

194   or 24 hours later. If no mating was observed within 2 hours, the females were

195   discarded. We used the 0h and 24h mating intervals because the time interval was

196   shown to affect the degree of sperm precedence in spider mites (Helle 1967).

197

198   **Statistical analysis**

199   All analyses were carried out using R (version 3.3.2, R Development Core Team 2016).

200   To analyse female fecundity within each species (*T. urticae*, *T. evansi* and *T. ludeni*), we

201   used linear models (LM procedure), considering the mean number of eggs per day as

202   the response variable (oviposition rate). To analyse offspring sex ratio within each

203   species, we used generalized linear models (GLM procedure) with a quasi-binomial

204   distribution – due to overdispersion of the data –, considering the number of female

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205 and male offspring produced by each focal female as the response variables (analysed

206 together with the function cbind).

207   For both types of analyses, we used as fixed factors the mating order (with

208 three levels: the control treatment, where a female mated twice with conspecific

209 males; an experimental treatment where the heterospecific male was the first to mate

210 with the female; and another experimental treatment where the heterospecific male

211 was the second to mate with the female) and the mating interval (with two levels:

212 either 0h or 24h interval between matings). We also tested the interaction among

213 these fixed factors. If the interaction was non-significant, a backward stepwise

214 procedure was used to find the best simplified fitted model. We performed

215 independent analyses for each species within each species pair (i.e. for *T. urticae* and

216 *T. evansi* females in *T. urticae versus T. evansi* crosses; and for *T. urticae* and *T. ludeni*

217 females in *T. urticae versus T. ludeni* crosses), as shown in Table 1.

218   We did a first block of experiments with the populations of *T. evansi* and *T.*

219 *ludeni* collected in 2012 (block 1). For question b) we also did a second block of

220 experiments with populations of those species from 2013 (block 2). In block 2 we did

221 not repeat all treatments, but only the crosses that were not complete before the

222 extinction of block 1 populations, as well as their respective controls – hence, there

223 were no treatments that were only performed in block 2. Because of that, instead of

224 including the factor block in the statistical models as a covariate, we did all the

225 statistical analyses with block 1 only and with block 1 and block 2 together. Since the

226 results were qualitatively similar (Table S1), here we present the results from the

227 analysis with block 1 and block 2 together.

228

229   **Results**

230   **a) The outcome of a single heterospecific mating between _T. urticae_ and _T._**

231   **_ludeni_**

232   Crosses between _T. ludeni_ and _T. urticae_ resulted in 100% male offspring, indicating

233   that hybrid production between these species is inexistent. The fecundity of _T. urticae_

234   females that mated heterospecifically was not significantly different from that of virgin

235   females or from that of females mated with a conspecific male ($F_{2,78}$= 1.886, P= 0.1585;

236   Figure 1). On the other hand, the fecundity of _T. ludeni_ females that mated with

237   conspecifics or heterospecifics was significantly higher than that of virgin females

238   ($F_{2,66}$= 1.886, P= 0.1585; Figure 1 and Table 1). Therefore, mating with heterospecific

239   males does not result in the aborted fertilization of oocytes for _T. urticae_ and _T. ludeni_

240   females.

241

242   **b) The outcome of heterospecific matings that precede or follow conspecific**

243   **ones**

244   _(i)   T. urticae vs T. evansi_

245   The oviposition rate of _T. urticae_ females that mated with either a conspecific and a

246   heterospecific or with two conspecific mates varied significantly according to mating

247   order in interaction with mating interval ($F_{2,136}$ = 6.026, P = 0.0031). Specifically, it was

248   higher for _T. urticae_ females that mated with _T. evansi_ males just before mating with a

249   conspecific male than for any other cross at 0h mating interval (|t| = 4.964, P < 0.0001

250   and |t| = 3.288, P = 0.0009, in comparison with double conspecific matings and with

251   matings with a conspecific followed by a mating with an heterospecific, respectively;

252   Fig. 2a). At the 24h interval, however, mating combinations did not affect this trait. The

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253 proportion of fertilized offspring (*i.e.*, daughters) of females *T. urticae* also varied

254 significantly according to mating order in interaction with mating interval ($F_{2,106}$=

255 4.963, P= 0.0087). But in contrast to the oviposition rate, this trait was affected at the

256 24h interval only, in which mating with a *T. evansi* male after mating with a conspecific

257 male resulted in a decrease in the proportion of fertilized offspring of *T. urticae*

258 females, relative to other mating sequences ($|t| = 5.362$, $P < 0.0001$ and $|t| = 5.103$, P

259 < 0.0001, in comparison with double conspecific matings and with matings with an

260 heterospecific followed by a mating with a conspecific male, respectively; Fig. 2b).

261   The mating order also affected differentially the oviposition rate of *T. evansi*

262 females, depending on the interval between matings ($F_{2,187}$= 4.977, P= 0.0078). *T.*

263 *evansi* females that mated with *T. urticae* males immediately after conspecific mates

264 had reduced oviposition rate relative to other mating sequences at this time interval

265 ($|t| = 2.841$, $P = 0.0050$ and $|t| = 2.692$, $P = 0.0078$ in comparison with double

266 conspecific matings and with matings with a heterospecific followed by a mating with a

267 conspecific male, respectively; Fig. 2c); however, if the heterospecific cross occurred

268 24 hours before the conspecific cross, the oviposition rate of *T. evansi* females

269 increased relative to double conspecific matings at this time interval ($|t| = 2.948$, $P =$

270 0.0036; Fig. 2c). These crosses did not significantly affect sex ratio ($F_{2,111}$= 0.368, P=

271 0.6931; Fig. 2d).

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273   *(ii)*   *T. urticae vs T. ludeni*

274 In crosses with the resident species (*T. ludeni),* the oviposition rate of *T. urticae*

275 females varied significantly according to mating order in interaction with mating

276 interval ($F_{2,144} = 3.694$, $P = 0.0273$). Specifically, we found that, at 0h interval, females

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277   that mated first with a conspecific then with a heterospecific male had lower

278   oviposition rate than females that mated first with a heterospecific then with a

279   conspecific male ($|t|$ = 2.736, P = 0.0070; Fig. 3a) At the 24h interval, the oviposition

280   rate of females that mated first with a conspecific then with a heterospecific male was

281   lower than that of double conspecific crosses. ($|t|$ = 2.505, P = 0.0134; Fig. 3a). *T.*

282   *urticae* females suffered no significant changes in offspring sex ratio from matings with

283   *T. ludeni* males ($F_{2,99}$ = 1.141, P = 0.3237; Figure 3b).

284   In *T. ludeni* females, the oviposition rate and the proportion of fertilized

285   offspring varied significantly according to mating order in interaction with the mating

286   interval (($F_{2,248}$ = 14.098, P < 0.0001 and $F_{2,152}$ = 10.1064, P < 0.0001, for oviposition rate

287   and proportion of fertilized offspring respectively). Compared to the control

288   treatment, *T. ludeni* females had lower oviposition rate when mating with *T. urticae*

289   males immediately before conspecifics males ($|t|$ = 2.605, P = 0.0097; Fig. 3c). At the

290   24 hour interval, the conspecific crosses yielded higher oviposition rate than all other

291   crosses in this time interval (($|t|$ = 4.646, P < 0.0001 and $|t|$ = 3.805, P = 0.0002, in

292   comparison with females mating with a conspecific before an heterospecific male and

293   females mating with an heterospecific before mating with a conspecific, respectively;

294   Fig. 3c). Additionally, when *T. ludeni* females mated with *T. urticae* males 24h after

295   conspecific matings, the proportion of fertilized offspring was significantly lower than

296   that of other crosses at this time interval (($|t|$ = 4.084, P < 0.0001 and $|t|$ = 3.586, P =

297   0.0005, in comparison with double conspecific matings and with females mating with a

298   heterospecific before mating with a conspecific, respectively; Figure 3d). The mating

299   sequence had no effect on the sex ratio at the 0h interval.

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13

301 **Discussion**

302 In this study, we investigated the consequences of mating with heterospecifics for the

303 fertilization success and offspring viability in a system composed of three spider-mite

304 species. We found that heterospecific matings between *T. urticae* and *T. ludeni* did not

305 result in fertilized offspring (i.e., females), nor did it have any negative effects on egg

306 viability, as shown for matings between *T. urticae* and *T. evansi* (Sato et al. 2014,

307 Clemente et al. 2016). In fact, *T. ludeni* females that mate with *T. urticae* males

308 produce more (male) offspring than virgin *T. ludeni* females. Second, the effects of

309 heterospecific matings on the outcome of previous or subsequent matings with

310 conspecifics were highly dependent on the species pair involved, on the trait measured

311 and on the timing and order of mating events. Despite strong effects of particular

312 mating sequences, the results taken as a whole suggest that the net effect of

313 reproductive interactions between species are relatively weak.

314 Positive effects of interspecific reproductive interactions were found for

315 fecundity. This can be due to a stimulation of oogenesis by the sperm of heterospecific

316 males, increasing the availability of oocytes to subsequent matings with conspecifics.

317 Indeed, oogenesis is stimulated by conspecific sperm in several species (Qazi et al.

318 2003, Xu and Wang 2011). This could also be the case with heterospecific sperm. If so,

319 it could explain the higher fecundity found in crosses between *T. urticae* and *T. evansi*.

320 In fact, earlier studies have documented that interactions with heterospecific males

321 are not always negative. In some gynogenetic species, heterospecific mating is a

322 prerequisite for embryogenesis (Gumm and Gabor 2005, Schlupp 2010). Moreover, in

323 some invertebrate species, females receive nuptial gifts from heterospecific males

324 (Vahed 1998, Costa-Schmidt and Machado 2012). However, to our knowledge, this is

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325   the first time that an increase in fecundity following a heterospecific mating is

326   described in the literature. Such effects may thus be rare. Still, earlier studies may have

327   overlooked them because they have not examined the roles of the order of mating in

328   the outcome of heterospecific mating interactions.

329   Nonetheless, we also detected several negative effects of mating with

330   heterospecifics, as found in most studies of reproductive interference (Gröning and

331   Hochkirch 2008, Kishi 2015). We found both a reduction in the number of eggs laid and

332   a decrease in fertilization success (i.e., offspring sex ratio). However, the incidence of

333   these two effects varied according to the species involved, the order of matings and

334   the time interval. Whereas effects on fecundity were found in several mating

335   sequences, an effect on fertilization success was found only when the heterospecific

336   male mated with the female 24 hours after the conspecific male. This is at odds with

337   expectations stemming from findings on conspecific matings, which show (a) first-male

338   precedence and (b) exceptions to this rule only if the second male mates immediately

339   after the first (Helle 1967). Therefore, the mechanisms underlying sperm displacement

340   by heterospecific males in spider mites should be investigated.

341   This also suggests that first male precedence found in conspecific matings

342   cannot be extrapolated to matings involving heterospecific sperm. This contrasts with

343   the recent finding that effects of heterospecific matings in *Drosophila* could be

344   predicted from the harmful effects of conspecific mates (Yassin and David 2015), and

345   that genes involved in conspecific male precedence also affect sperm precedence in

346   multiple matings involving heterospecifics (Civetta and Finn 2014). This indicates that

347   the equivalence of effects of conspecific and heterospecific sperm on the outcome of

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348   conspecific matings is dependent on the type of effect and/or the species involved in

349   the interaction.

350   Since effects of heterospecific matings depend on the order and timing of

351   occurrence, the outcome of interspecific reproductive interactions will depend on the

352   frequency with which those different types of matings occur in nature. This, in turn,

353   will depend on the discrimination abilities between species. First, these interactions

354   will occur only if species discrimination is weak. This, indeed, has been explicitly

355   demonstrated for the *T. evansi/T. urticae* interaction (Clemente et al. 2016), but not

356   for *T. ludeni/T. urticae*. Still, these species do mate with heterospecifics under no

357   choice scenarios, as shown here, hence the scope for the occurrence of reproductive

358   interference does exist.

359   What then, would be the relative frequency of the mating sequences tested

360   here? In spider mites, conspecific males often guard quiescent females (*i.e.*, the last

361   larval stage before becoming adult female), to ensure mating immediately after

362   emergence. If males guard preferentially conspecific females, as shown in other spider

363   mite species pairs (Collins et al. 1993, Takafuji et al. 1997), heterospecific matings will

364   occur more often after rather than before conspecific ones. Moreover, we have shown

365   that *T. urticae* females become less receptive to both conspecific and heterospecific

366   matings if the first mating has occurred 24h before the second (Clemente et al. 2016).

367   Hence, this leads to the prediction that the most common mating sequence among

368   these species will be a heterospecific mating immediately following a conspecific one.

369   Under those circumstances the only effect of heterospecific matings is a fecundity

370   reduction in *T. evansi* upon mating with *T. urticae*. This would mean that the invasive

371   species suffers more from reproductive interference than the resident.

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372   Even assuming that all mating combinations do occur, reproductive interactions

373   between *T. urticae* and *T. evansi* can be positive or negative for the two species,

374   depending on the mating sequence. Therefore, reproductive interference cannot be

375   invoked to explain the exclusion of *T. urticae* in habitats with *T. evansi* (Ferragut et al.

376   2013, Sarmento et al. 2011b). Other factors may contribute to this exclusion, as the

377   production of a dense web by *T. evansi*, which prevents heterospecifics from accessing

378   the surface of the leaves to feed and oviposit (Sarmento et al. 2011b). Importantly,

379   however, we show that the occurrence and strength of reproductive interference

380   cannot be assessed with the unique evaluation of the outcome of a specific type of

381   reproductive interaction. The different types of mating combinations – the order and

382   interval between matings – have great influence on the overall outcome of

383   heterospecific interactions and on the relative frequency of such events. This confirms

384   the importance of using complete experimental designs on the detection and

385   characterization of reproductive interference.

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395

396   **FIGURE LEGENDS**

397

398   **Figure 1** | Average daily fecundity of virgin females, and of females that have mated

399   with a conspecific or a heterospecific male. Tu: *T. urticae* males or females; Tl: *T. ludeni*

400   males or females. Grey bars: matings involving *T. urticae* females; white bars: matings

401   involving *T. ludeni* females. Error bars represent the standard errors of the mean.

402   Numbers on the bottom of bars represent the sample size for each type of mating.

403

404   **Figure 2** | Average daily fecundity and estimated offspring sex ratio resulting from

405   interactions between *T. urticae* (a, b; grey solid bars) and *T. evansi* (c,d; striped bars)

406   females with conspecific and heterospecific males. In each plot, bars on the left side of

407   the dotted straight line correspond to treatments where second matings occurred

408   immediately (0h) after the first one; bars on the right side correspond to treatments

409   where second matings occurred 24h after the first one. "1st M": first male that mated

410   with the female; "2nd M": second male. The interval indicates the time of occurrence

411   of the second mating, i.e., if immediately after the first mating (0h) or 24h later. "Tu":

412   *T. urticae* males; "Te": *T. evansi* males. Letters above the bars indicate significant

413   differences among treatments (small letters: among crosses occurring with a 0h

414   interval; capital letters: among crosses occurring with a 24h interval). Error bars

415   represent the standard errors of the mean. For offspring sex ratio, we obtained the

416   estimates of the probability of being female and correspondent standard errors of the

417   mean from the statistical GLM models. This takes into account sex ratio variation

418   among females, as well as the quasi-binomial correction for overdispersion of the data.

419   Numbers on the bottom of bars represent the sample size for each type of mating.

18

420

421   **Figure 3** | Average daily fecundity and estimated offspring sex ratio resulting from

422   interactions between *T. urticae* (plots a, b; grey bars) and *T. ludeni* (plots c, d; white

423   bars) females with conspecific and heterospecific males. In each plot, bars on the left

424   side of the dotted line correspond to treatments where second matings occurred

425   immediately (0h) after the first one; bars on the right side correspond to treatments

426   where second matings occurred 24h after the first one. "1st M": first male that mated

427   with the female; "2nd M": second male. The interval indicates the time of occurrence

428   of the second mating, i.e., if immediately after the first mating (0h) or 24h later. "Tu":

429   *T. urticae* males; "Tl": *T. ludeni* males. Letters above the bars indicate the significant

430   differences between treatments (small letters: among crosses occurring with a 0h

431   interval; capital letters: among crosses occurring with a 24h interval. Error bars

432   represent the standard errors of the mean. For offspring sex ratio, we obtained the

433   estimates of the probability of being female and correspondent standard errors of the

434   mean from the statistical GLM models. This takes into account sex ratio variation

435   among females, as well as the quasi-binomial correction for overdispersion of the data.

436   Numbers on the bottom of bars represent the sample size for each type of mating.

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579

580 **Supporting Information**

581 The following Supporting Information has been made available in the online version of

582 this article.

583 Table S1 |Statistical tests and contrasts for the comparisons of fecundity and offspring

584 sex ratio in crosses between conspecific and heterospecific males and females, using

585 data from Bloc 1 or Blocs 1+2.

586