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

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

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15

16   **Abstract**

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18   in Evolutionary Biology (http://dx.doi.org/10.24072/pci.evolbiol.100025).

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.

33

34   **Keywords**:

37

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 heterospecifics alone will yield no offspring).

52   Moreover, clearly evaluating the effects of reproductive interference on exclusion in

53   polyandrous species necessitates measuring all possible combinations of mating order

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

55   (*i.e.*, the interval between mating events) between pairs of species. It is also important

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

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

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

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

60   attempt to predict how reproductive interference affects species exclusion, but do so

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

62   Volterra modified model to predict the effect of reproductive interference between

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

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

65   other studies consider different orders of mating events (eg, Kyogoku and Nishida

66   2013), but do not integrate this information to generate a prediction concerning the

67   net effect of 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). *T. urticae* and *T. ludeni* are

82   resident species, and *T. evansi* has recently invaded the European continent, via

83   Portugal (Boubou et al. 2012). Heterospecific matings among these species have been

84 observed, although they mostly do not result in viable offspring (Sato et al. 2014, 2016,

85 Clemente et al. 2016, S. Magalhães pers. obs.). Moreover, *T. evansi* can exclude *T.*

86 *urticae* on tomato plants (Sarmento et al. 2011a), a result that correlates with field

87 observations (Ferragut et al. 2013). Finally, a recent study has shown that, in

88 competition with *T. evansi*, the population growth of *T. urticae* is more severely

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

90 by mated females, suggesting that reproductive interference may be responsible for

91 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**

Stock Cultures

The mite species used in this study were collected in Carregado (39.022260, -8.966566), Portugal, and all laboratory populations were established from an initial pool of 300 mated females. The laboratory population of *T. urticae* was collected on tomato plants (*Solanum lycopersicum)* in May 2010, that of *T. evansi* on *Physalis angulata* in May 2012 and that of *T. ludeni* on tomato in September 2012. The populations of *T. evansi* and *T. ludeni* became extinct in August 2012 and May 2013, respectively, being subsequently replaced with populations from the same location, both collected in *Datura stramonium* plants. Both populations of *T. evansi* and *T. ludeni* were used in the experiments.

Species identity was confirmed through polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) of the ITS2 region (Hurtado et al. 2008), on approximately 50 females of each population. Total genomic DNA was extracted from each individual spider mite using the Sigma-Aldrich GenEluteTM Mammalian Genomic DNA Miniprep Kit, following manufacturer´s instructions, except for the elution volume, which we set to 20µL of RNase free water (Qiagen NV, Venlo, The Netherlands) to increase the concentration of DNA obtained from this very small animal (c.a. 300µm long).

Adult females from populations used in this experiment were screened for *Wolbachia* using the primers *wsp* (*Wolbachia*-specific primers) 81F and 691R (Braig et al. 1998). We did this to avoid potential cytoplasmic incompatibility as a confounding factor in our measurements. PCR assay procedures were as described in Breeuwer (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

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).

142

143

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.

157

158 **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

9

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

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).

184

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**

10

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

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).

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

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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   ($F_{2,66}$= 5.636, P= 0.0055; Figure 1). Therefore, mating with heterospecific males does

239   not result in the aborted fertilization of oocytes for *T. urticae* and *T. ludeni* females.

240

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

242   **ones**

243   *(i)   T. urticae vs T. evansi*

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244   The oviposition rate of *T. urticae* females that mated with either a conspecific and a

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

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

247   higher for *T. urticae* females that mated with *T. evansi* males just before mating with a

248   conspecific male than for any other cross at 0h mating interval ($|t|(1)$ = 4.964,P <

249   0.0001 and $|t|(1)$ = 3.288, P = 0.0009, in comparison with double conspecific matings

250   and with matings with a conspecific followed by a mating with an heterospecific,

251   respectively; Fig. 2a). At the 24h interval, however, mating combinations did not affect

252   this trait. The proportion of fertilized offspring (*i.e*., daughters) of females *T. urticae*

253   also varied significantly according to mating order in interaction with mating interval

254   ($F_{2,106}$= 57.219, P= 0.007). But in contrast to the oviposition rate, this trait was affected

255   at the 24h interval only, in which mating with a *T. evansi* male after mating with a

256   conspecific male resulted in a decrease in the proportion of fertilized offspring of *T.*

257   *urticae* females, relative to other mating sequences ($|t|(1)$ = 5.362, P < 0.0001 and

258      $|t|(1)$ = 5.103, P < 0.0001, in comparison with double conspecific matings and with

259   matings with an heterospecific followed by a mating with a conspecific male,

260   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   ($|t|(1)$ = 2.841, P = 0.0050 and $|t|(1)$ = 2.692, P = 0.0078 in comparison with double

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

13

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 ($|t|(1) = 2.948$, P

270     $= 0.0036$; Fig. 2c). These crosses did not significantly affect sex ratio ($F_{2,111}=3.786$, P=

271   0.6923; Fig. 2d).

272

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

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

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

279   conspecific male ($|t|(1) = 2.736$, P = 0.0070; Fig. 3a). At the 24h interval, the

280   oviposition rate of females that mated first with a conspecific then with a

281   heterospecific male was lower than that of double conspecific crosses. ($|t|(1) = 2.505$,

282   P = 0.0134; Fig. 3a). *T. urticae* females suffered no significant changes in offspring sex

283   ratio from matings with *T. ludeni* males ($F_{2,99} = 10.769$, P = 0.3195; 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 $F_{2,152} = 158.690$, 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 ($|t|(1) = 2.605$, P = 0.0097; Fig. 3c). At

14

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

291   other crosses in this time interval (($|t|(1) = 4.646$, P < 0.0001 and $|t|(1) = 3.805$, P =

292   0.0002, in comparison with females mating with a conspecific before an heterospecific

293   male and females mating with an heterospecific before mating with a conspecific,

294   respectively; Fig. 3c). Additionally, when *T. ludeni* females mated with *T. urticae* males

295   24h after conspecific matings, the proportion of fertilized offspring was significantly

296   lower than that of other crosses at this time interval ($|t|(1) = 4.084$, P < 0.0001 and

297    $|t|(1) = 3.586$, P = 0.0005, in comparison with double conspecific matings and with

298   females mating with a heterospecific before mating with a conspecific, respectively;

299   Figure 3d). The mating sequence had no effect on the sex ratio at the 0h interval.

300

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

15

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

313   reproductive interactions between species is 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

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). Whereas lower fecundity

333   will most likely be costly in all ecological scenarios (assuming no trade-off with other

334   traits), a decrease in fertilization success, leading to an excess of males in the

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335   population, will have an impact on fitness that is contingent upon the structure of

336   spider-mite populations. Indeed, a higher frequency of males is likely to be more

337   detrimental in recently-established populations, generally founded by a single female.

338   In those populations, the optimal sex ratio is highly female-biased, hence a higher

339   proportion of males is probably very costly. This is not the case in more panmitic

340   populations, usually found when mites are established on a plant for a longer period

341   (Roeder 1992, Macke et al. 2011). One also can speculate that a higher proportion of

342   males may impose a strong cost in the other, competing species (assuming detrimental

343   effects of heterospecific matings are stronger than positive effects). However, this

344   behaviour is expected to be selected in males only if the cost they pay in terms of

345   sperm loss is compensated by the benefit they would provide to their sisters. Hence,

346   this behaviour is more likely to be selected in more structured populations.

347   Reproductive interference occurred independently of the order of matings and

348   the time interval. This is at odds with expectations stemming from findings on

349   conspecific matings, which show (a) first-male precedence and (b) exceptions to this

350   rule only if the second male mates immediately after the first (Helle 1967). Therefore,

351   the first male precedence found in conspecific matings cannot be extrapolated to

352   matings involving heterospecific sperm. This contrasts with the recent finding that

353   effects of heterospecific matings in *Drosophila* could be predicted from the harmful

354   effects of conspecific mates (Yassin and David 2015), and that genes involved in

355   conspecific male precedence also affect sperm precedence in multiple matings

356   involving heterospecifics (Civetta and Finn 2014). Thus, the equivalence of effects of

357   conspecific and heterospecific sperm on the outcome of conspecific matings is

17

358   dependent on the type of effect and/or the species involved in the interaction. The

359   mechanisms of sperm displacement in heterospecific matings in spider mites should

360   therefore be investigated.

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

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

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

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

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

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

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

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

369   interference does exist.

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

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

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

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

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

375   occur more often after rather than before conspecific ones. Hence, this leads to the

376   prediction that the most common mating sequence among these species will be a

377   heterospecific mating following a conspecific one. In this case, the detrimental effects

378   of heterospecific matings for *T. urticae* females, are only visible when the two matings

379   happen in close succession, while for *T. evansi* females, these effects are only apparent

380   when the two matings occurred 24 hours apart. Since latency to copulation is not

18

381 statistically different for these time intervals (Clemente et al. 2016), one expects these

382 events to occur at similar frequency. However, since the effect of these heterospecific

383 matings for *T. evansi* females is a reduction in fecundity, while for *T. urticae* it is an

384 increase in the proportion of male offspring, the cost may be higher for *T. evansi*

385 females (cf. above). Moreover, the excess of males produced by *T. urticae* can lead to

386 more detrimental matings with *T. evansi* females, while reducing the probability of *T.*

387 *urticae* females mating with a heterospecific male. This would mean that the invasive

388 species would suffer more from reproductive interference than the resident.

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

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

391 depending on the mating sequence. Moreover, reproductive interference between *T.*

392 *urticae* and *T. ludeni* is stronger than that between *T. urticae* and *T. evansi*. Therefore,

393 reproductive interference cannot be invoked to explain the exclusion of *T. urticae* in

394 habitats with *T. evansi* (Ferragut et al. 2013, Sarmento et al. 2011b). Other factors may

395 contribute to this exclusion, as the production of a dense web by *T. evansi*, which

396 prevents heterospecifics from accessing the surface of the leaves to feed and oviposit

397 (Sarmento et al. 2011b). Importantly, however, we show that the occurrence and

398 strength of reproductive interference cannot be assessed with the unique evaluation

399 of the outcome of a specific type of reproductive interaction. The different types of

400 mating combinations – the order and interval between matings – have great influence

401 on the overall outcome of heterospecific interactions and on the relative frequency of

402 such events. This confirms the importance of using complete experimental designs on

403 the detection and characterization of reproductive interference.

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413

414   **FIGURE LEGENDS**

415

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

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

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

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

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

421

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

438

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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553   production and sperm trigger oviposition in a moth. J Insect Physiol. 57: 385-390.

554

555   **Supporting Information**

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

557   this article.

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

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

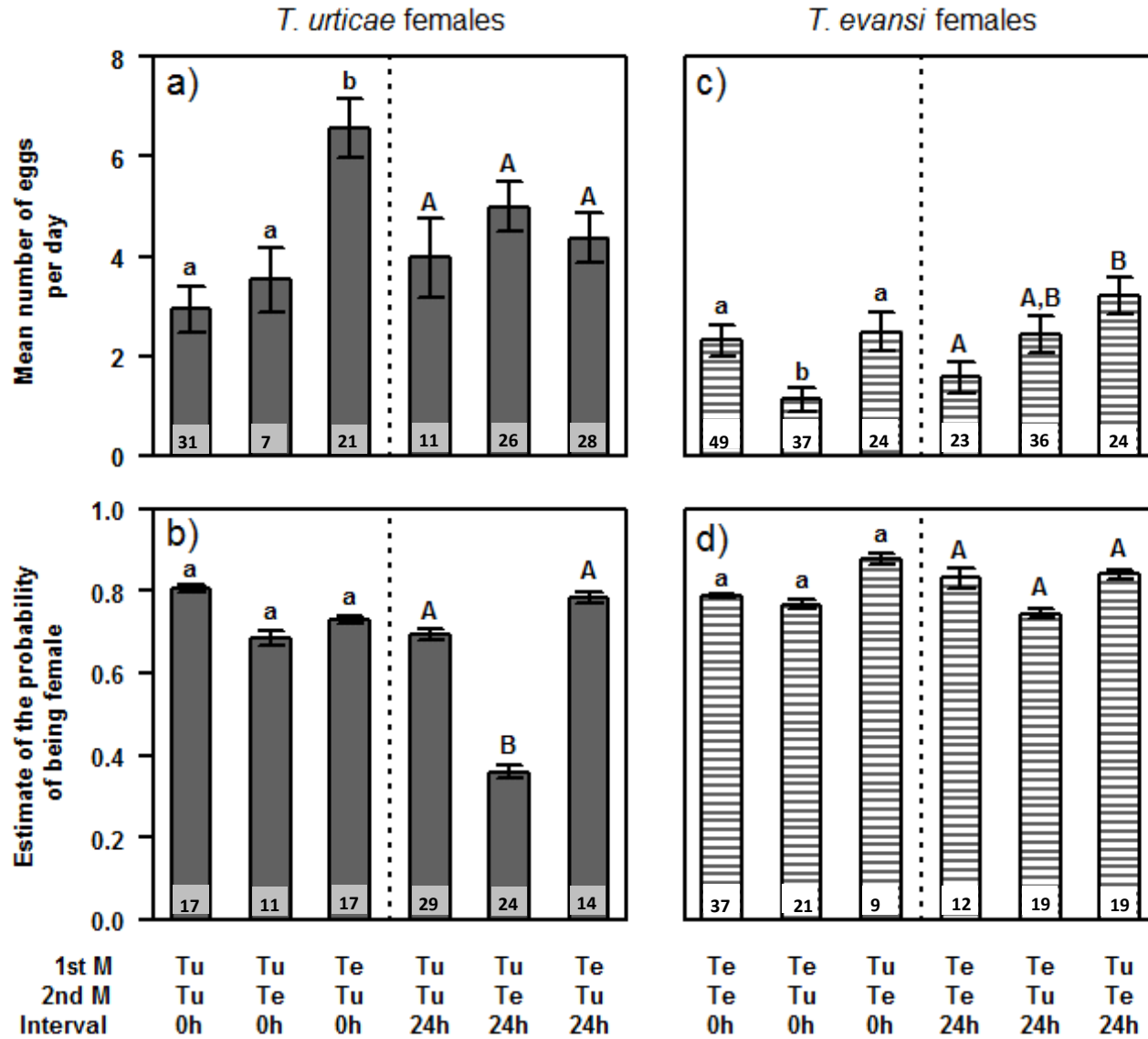560   data from Block 1 or Blocks 1+2.

561

Fig.1
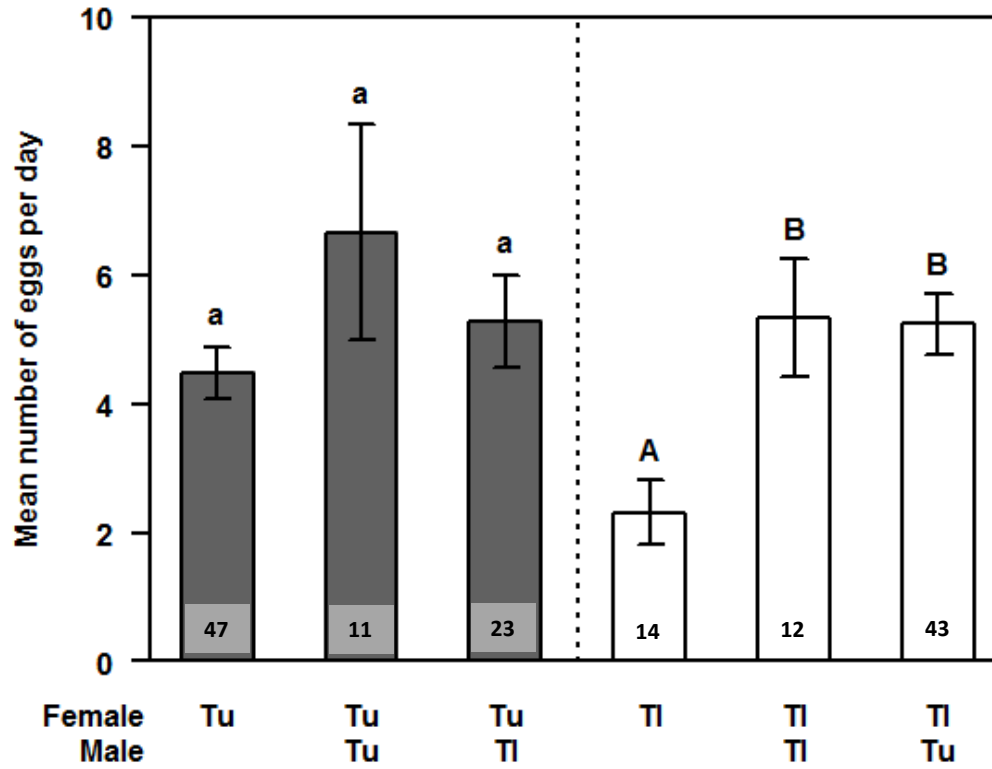
Fig.2

T. urticae versus *T. evansi*

Fig.3

T. urticae versus T. ludeni