Despite reproductive interference, the net outcome of reproductive interactions among spider mite species is not necessarily costly

Salomé H. Clemente, Inês Santos, Rita Ponce, Leonor R. Rodrigues, Susana A. M. Varela* and Sara Magalhães*[1]

salome.hc@gmail.com, inesflsantos@gmail.com, anaritaponce@gmail.com, leonor.rodrigues89@gmail.com, savarela@fc.ul.pt, snmagalhaes@fc.ul.pt

*co-last authorship

cE3c – Centre for Ecology, Evolution and Environmental Changes

Faculdade de Ciências da Universidade de Lisboa

Edifício C2, 3º Piso

Campo Grande

1749-016 Lisbon

Portugal

[1]: snmagalhaes@fc.ul.pt

Abstract

Reproductive interference is considered a strong ecological force, potentially leading to species exclusion. This supposes that the net effect of reproductive interactions is strongly negative for one of the species involved. Testing this requires a comprehensive analysis of interspecific reproductive interactions, accounting for the order and timing of mating events, and for their effects on either fertility or fecundity. To this aim, we measured reproductive interactions between a focal species, *Tetranychus urticae*, and an invasive (*T.evansi*) and a resident (*T. ludeni*) species, varying the mating sequence and interval, and measuring the effect of such crosses on fecundity and offspring sex ratio (a measure of fertility, as these species are haplodiploid). We found that mating with heterospecifics affected fecundity and sex ratio negatively, but also positively, depending on the species involved, and on the order and timing of mating events. Overall, the net effect of reproductive interactions was weak despite strong effects of particular events. In natural situations the outcome of reproductive interactions will thus hinge upon the frequency of each event.

Keywords:

Biological invasions, sperm precedence, *Tetranychus*, reproductive interactions, mating.

Introduction

Reproductive interference, that is, any kind of sexual interaction between two species that diminishes the fitness of at least one of them (Gröning and Hochkirch 2008, Kishi et al. 2009, Burdfield-Steel and Shuker 2011), may have severe effects on the outcome of species interactions. Indeed, theory predicts that reproductive interference may contribute to species exclusion more often than resource competition (Gröning and Hochkirch 2008, Kishi et al. 2009, Kishi and Nakazawa 2013). For example, it has been posited that reproductive interference may underlie the success of some invasive species (e.g. Nishida et al. 2012).

Most studies of reproductive interference concern the fitness outcome of interspecific matings of two species that do not produce viable hybrids (Gröning and Hochkirch 2008). In this case, the reproductive effects of the interspecific interaction will be expressed only when organisms mate with both conspecifics and heterospecifics (as mating with conspecifics alone will yield no offspring). Moreover, clearly evaluating the effects of reproductive interference on exclusion in polyandrous species necessitates measuring all possible combinations of mating order (*i.e.*, whether heterospecific matings occur before or after conspecific ones) and timing (*i.e.*, the interval between mating events) between pairs of species. It is also important to test whether reproductive interactions affect fecundity (egg production) or fertility (egg fertilization). This information can then be integrated to predict the net outcome of reproductive interactions between species. Despite the many studies on reproductive interference, none has yet applied this approach. Indeed, some studies attempt to predict how reproductive interference affects species exclusion, but do so by focussing on some sequence events only. For example, Takafuji (1997) used a Lotka-Volterra

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*

can exclude *T. urticae* on tomato plants (Sarmento et al. 2011a), a result that correlates with field observations (Ferragut et al. 2013). Finally, a recent study has shown that, in competition with *T. evansi*, the population growth of *T. urticae* is more severely affected when plants are colonized by virgin females than when plants are colonized by mated females, suggesting that reproductive interference may be responsible for the species distribution patterns observed (Sato et al. 2014).

To postulate hypotheses concerning the consequences of heterospecific matings, it is crucial to understand within-species reproductive behaviour. *T. urticae*, the focal species, exhibits first male sperm precedence, with second matings being sometimes effective if they occur within the 24 hours following the first (Helle 1967). However, females that mate multiple times with conspecific males, after a 24h interval between matings, produce fewer fertilized offspring (*i.e.*, females) (Macke et al. 2012), suggesting that sperm displacement after 24h is possible. Here, we hypothesize that mating order and the mating interval will affect the outcome of reproductive interference in *T. urticae*. Also, given that *T. evansi*, the invasive species, displaces *T. urticae*, unlike *T. ludeni*, we expect the former to exert stronger effects than the latter. To this aim, we performed crosses between *T. urticae* and the two other species at different time intervals and with different mating orders, and measured the consequences for the two species involved in the cross.

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 were thus treated by placing adult females in detached bean leaves with tetracycline (0.025% w/v) for three consecutive generations, then absence of *Wolbachia* was confirmed using the same protocol as above. Other endosymbionts tested

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

Bean (*Phaseolus vulgaris*) and tomato (*Solanum lycopersicum*) plants were planted every week and grown in an herbivore-free greenhouse, being watered two to three times a week. *T. urticae* populations were maintained on trays with 6-10 bean plants whereas those of *T. evansi* and *T. ludeni* were kept on tomato plants at 25°C, both with a 16 L: 8D photoperiod. Plant trays were changed every two weeks, placing old leaves on top of uninfested plants. Cultures were kept inside plastic boxes (28x39x28 cm), with an opening of 25x15 cm polyamide fabric (80 µm mesh width).

Experimental procedure

Experiments were done on the plant species from which the female tested had been cultured. As in the literature there was no information on whether hybridization is possible between *T. urticae* and *T. ludeni*, we studied the outcome of a single heterospecific mating between these two species (the same analysis for *T. urticae* and *T. evansi* was performed in a previous experiment (Clemente et al. 2016)). Subsequently, we set out to study the heterospecific interactions between *T. urticae* and the invasive *T. evansi* and the resident *T. ludeni* species for which we analysed the outcome of mating with a heterospecific male before or after a conspecific male. Since we focused on interactions with *T. urticae* (the focal species of our study), we performed crosses between *T. urticae* males or females and *T. evansi* or *T. ludeni* males or females, but not between the two latter species. All experiments were 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
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

when offspring sex-ratio could be determined. With this data, we tested whether heterospecific matings affected (a) the mean daily fecundity and (b) offspring sex ratio (hence the proportion of fertilized offspring).

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

To determine the outcome of mating with a heterospecific male before or after a conspecific male between *T. urticae* and the other two species, we compared the fecundity and offspring sex ratio of those crosses to that of females that mated with two conspecific males. The experimental procedure was as described above, except that we let females mate with a conspecific or a heterospecific male, then placed the focal females with another male. We created the following mating sequences: conspecific-conspecific, conspecific-heterospecific and heterospecific-conspecific. The second mating occurred either immediately after the first mating (0 hours treatment) or 24 hours later. If no mating was observed within 2 hours, the females were discarded. We used the 0h and 24h mating intervals because the time interval was shown to affect the degree of sperm precedence in spider mites (Helle 1967).

Statistical analysis

All analyses were carried out using R (version 3.3.2, R Development Core Team 2016). To analyse female fecundity within each species (*T. urticae*, *T. evansi* and *T. ludeni*), we used linear models (LM procedure), considering the mean number of eggs per day as the response variable (oviposition rate). To analyse offspring sex ratio within each species, we used generalized linear models (GLM procedure) with a quasi-binomial distribution – due to overdispersion of the data –, considering the number of female

and male offspring produced by each focal female as the response variables (analysed together with the function cbind).

For both types of analyses, we used as fixed factors the mating order (with three levels: the control treatment, where a female mated twice with conspecific males; an experimental treatment where the heterospecific male was the first to mate with the female; and another experimental treatment where the heterospecific male was the second to mate with the female) and the mating interval (with two levels: either 0h or 24h interval between matings). We also tested the interaction among these fixed factors. If the interaction was non-significant, a backward stepwise procedure was used to find the best simplified fitted model. We performed independent analyses for each species within each species pair (i.e. for *T. urticae* and *T. evansi* females in *T. urticae versus T. evansi* crosses; and for *T. urticae* and *T. ludeni* females in *T. urticae versus T. ludeni* crosses), as shown in Table 1.

We did a first block of experiments with the populations of *T. evansi* and *T. ludeni* collected in 2012 (block 1). For question b) we also did a second block of experiments with populations of those species from 2013 (block 2). In block 2 we did not repeat all treatments, but only the crosses that were not complete before the extinction of block 1 populations, as well as their respective controls – hence, there were no treatments that were only performed in block 2. Because of that, instead of including the factor block in the statistical models as a covariate, we did all the statistical analyses with block 1 only and with block 1 and block 2 together. Since the results were qualitatively similar (Table S1), here we present the results from the analysis with block 1 and block 2 together.

Results

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

Crosses between *T. ludeni* and *T. urticae* resulted in 100% male offspring, indicating that hybrid production between these species is inexistent. The fecundity of *T. urticae* females that mated heterospecifically was not significantly different from that of virgin females or from that of females mated with a conspecific male ($F_{2,78}$= 1.886, P= 0.1585; Figure 1). On the other hand, the fecundity of *T. ludeni* females that mated with conspecifics or heterospecifics was significantly higher than that of virgin females ($F_{2,66}$= 1.886, P= 0.1585; Figure 1 and Table 1). Therefore, mating with heterospecific males does not result in the aborted fertilization of oocytes for *T. urticae* and *T. ludeni* females.

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

(i) T. urticae vs T. evansi

The oviposition rate of *T. urticae* females that mated with either a conspecific and a heterospecific or with two conspecific mates varied significantly according to mating order in interaction with mating interval ($F_{2,136}$ = 6.026, P = 0.0031). Specifically, it was higher for *T. urticae* females that mated with *T. evansi* males just before mating with a conspecific male than for any other cross at 0h mating interval (|t| = 4.964, P < 0.0001 and |t| = 3.288, P = 0.0009, in comparison with double conspecific matings and with matings with a conspecific followed by a mating with an heterospecific, respectively; Fig. 2a). At the 24h interval, however, mating combinations did not affect this trait. The

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).
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 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.
300

Discussion

In this study, we investigated the consequences of mating with heterospecifics for the fertilization success and offspring viability in a system composed of three spider-mite species. We found that heterospecific matings between *T. urticae* and *T. ludeni* did not result in fertilized offspring (i.e., females), nor did it have any negative effects on egg viability, as shown for matings between *T. urticae* and *T. evansi* (Sato et al. 2014, Clemente et al. 2016). In fact, *T. ludeni* females that mate with *T. urticae* males produce more (male) offspring than virgin *T. ludeni* females. Second, the effects of heterospecific matings on the outcome of previous or subsequent matings with conspecifics were highly dependent on the species pair involved, on the trait measured and on the timing and order of mating events. Despite strong effects of particular mating sequences, the results taken as a whole suggest that the net effect of reproductive interactions between species are relatively weak.

Positive effects of interspecific reproductive interactions were found for fecundity. This can be due to a stimulation of oogenesis by the sperm of heterospecific males, increasing the availability of oocytes to subsequent matings with conspecifics. Indeed, oogenesis is stimulated by conspecific sperm in several species (Qazi et al. 2003, Xu and Wang 2011). This could also be the case with heterospecific sperm. If so, it could explain the higher fecundity found in crosses between *T. urticae* and *T. evansi*. In fact, earlier studies have documented that interactions with heterospecific males are not always negative. In some gynogenetic species, heterospecific mating is a prerequisite for embryogenesis (Gumm and Gabor 2005, Schlupp 2010). Moreover, in some invertebrate species, females receive nuptial gifts from heterospecific males (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). 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

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.

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.
386

387 **Acknowledgements**

388 The authors wish to thank Yukie Sato and Maurice W. Sabelis for helpful discussions
389 during the early stages of this work. These experiments and Inês Santos were funded
390 by Portuguese National Funds through an FCT-ANR project (FCT-ANR//BIA-
391 EVF/0013/2012) to SM and Isabelle Olivieri. SHC and LRR had PhD fellowships funded
392 by FCT (SFRH/ BD/ 90156/2012 and SFRH/BD/87628/2012, respectively). SAMV and RP
393 had Post-doctoral fellowships from FCT, (SFRH/BPD/66042/2009) and (SFRH / BPD /
394 42801 /2008) respectively. The authors declare no conflict of interest.
395

FIGURE LEGENDS

Figure 1 | Average daily fecundity of virgin females, and of females that have mated with a conspecific or a heterospecific male. Tu: *T. urticae* males or females; Tl: *T. ludeni* males or females. Grey bars: matings involving *T. urticae* females; white bars: matings involving *T. ludeni* females. Error bars represent the standard errors of the mean. Numbers on the bottom of bars represent the sample size for each type of mating.

Figure 2 | Average daily fecundity and estimated offspring sex ratio resulting from interactions between *T. urticae* (a, b; grey solid bars) and *T. evansi* (c,d; striped bars) females with conspecific and heterospecific males. In each plot, bars on the left side of the dotted straight line correspond to treatments where second matings occurred immediately (0h) after the first one; bars on the right side correspond to treatments where second matings occurred 24h after the first one. "1st M": first male that mated with the female; "2nd M": second male. The interval indicates the time of occurrence of the second mating, i.e., if immediately after the first mating (0h) or 24h later. "Tu": *T. urticae* males; "Te": *T. evansi* males. Letters above the bars indicate significant differences among treatments (small letters: among crosses occurring with a 0h interval; capital letters: among crosses occurring with a 24h interval). Error bars represent the standard errors of the mean. For offspring sex ratio, we obtained the estimates of the probability of being female and correspondent standard errors of the mean from the statistical GLM models. This takes into account sex ratio variation among females, as well as the quasi-binomial correction for overdispersion of the data. Numbers on the bottom of bars represent the sample size for each type of mating.

Figure 3 | Average daily fecundity and estimated offspring sex ratio resulting from interactions between *T. urticae* (plots a, b; grey bars) and *T. ludeni* (plots c, d; white bars) females with conspecific and heterospecific males. In each plot, bars on the left side of the dotted line correspond to treatments where second matings occurred immediately (0h) after the first one; bars on the right side correspond to treatments where second matings occurred 24h after the first one. "1st M": first male that mated with the female; "2nd M": second male. The interval indicates the time of occurrence of the second mating, i.e., if immediately after the first mating (0h) or 24h later. "Tu": *T. urticae* males; "Tl": *T. ludeni* males. Letters above the bars indicate the significant differences between treatments (small letters: among crosses occurring with a 0h interval; capital letters: among crosses occurring with a 24h interval. Error bars represent the standard errors of the mean. For offspring sex ratio, we obtained the estimates of the probability of being female and correspondent standard errors of the mean from the statistical GLM models. This takes into account sex ratio variation among females, as well as the quasi-binomial correction for overdispersion of the data. Numbers on the bottom of bars represent the sample size for each type of mating.

441

References

Alpedrinha J. and Magalhães S. 2016. Sex Allocation: l'enfer c'est les autres? – Curr. Biol. 26: R476-R478.

Arnqvist, G. and Rowe, L. 2005. Sexual Conflict. – Princeton University Press, Princeton.

Ben-David, T. et al. 2009. Asymmetric reproductive interference between two closely related spider mites: Tetranychus urticae and T. turkestani (Acari: Tetranychidae). – Exp. Appl. Acarol. 48: 213-227.

Braig, H. R. et al.1998. Cloning and Characterization of a Gene Encoding the Major Surface Protein of the Bacterial Endosymbiont *Wolbachia pipientis*. – J. Bacteriol. 180: 2373–2378.

Breeuwer, J. A. 1997. *Wolbachia* and cytoplasmic incompatibility in the spider mites *Tetranychus urticae* and *T.* turkestani. – Heredity79: 41–47.

Boubou, A. et al.2012. Test of colonisation scenarios reveals complex invasion history of the red tomato spider mite *Tetranychus evansi*. – PLoS One 7: 35601.

Boudreaux, H. B. 1963. Biological aspects of some phytophagous mites. – Annu. Rev. Entomol. 8: 137–154.

Burdfield-Steel, E. R. and Shuker, D. M. 2011. Reproductive interference. – Curr. Biol. 21: 450–451.

461 Civetta, A. and Finn, S. 2014. Do candidate genes mediating conspecific sperm
462 precedence affect sperm competitive ability within species? A test case in Drosophila.
463 – G3 (Bethesda) 4: 1701-1707.

464 Clemente, S. H. et al. 2016. Incomplete species recognition entails few costs in spider
465 mites, despite first-male precedence. – Behav. Ecol.Sociobiol. in press. DOI
466 10.1007/s00265-016-2124-0

467 Collins, R. D. et al. 1993. Guarding behavior and reproductive isolation in two
468 tetranychid mite species (Acari: Tetranychidae). – Ann. Entomol. Soc. Am. 86: 111-116.

469 Collins, R. and Margolies, D. 1994. The effect of interspecific mating on sex ratios in the
470 twospotted spider mite and the Banks grass mite (Acarina: Tetranychidae). –
471 J. Insect Behav., 8: 189–206.

472 Costa-Schmidt, L. E. and Machado, G. 2012. Reproductive interference between two
473 sibling species of gift-giving spiders. – Anim. Behav. 84: 1201-1211.

474 Crampton, W. G. et al. 2011. Reproductive character displacement and signal ontogeny
475 in a sympatric assemblage of electric fish. – Evolution 65: 1650-1666.

476 Crowder, D. W. et al. 2010. Plasticity in mating behaviour drives asymmetric
477 reproductive interference in whiteflies. – Anim. Behav. 79: 579-587.

478 Del Re, A. C. 2013. compute.es: Compute Effect Sizes. R package version 0.2-2.
479 http://cran.r-project.org/web/packages/compute.es.

480 delBarco-Trillo, J. and Johnston R. 2010. Adult female hamsters require long and
481 sustained exposures to heterospecific males to avoid interspecific mating. – Evol. Ecol.
482 25: 391–401.

483 Escudero, L. and Ferragut, F. 2005. Life-history of predatory mites Neoseiulus
484 californicus and Phytoseiulus persimilis (Acari: Phytoseiidae) on four spider mite
485 species as prey, with special reference to Tetranychus evansi (Acari: Tetranychidae). –
486 Biol. Control 32: 378-384.

487 Ferragut, F. et al. 2013. The invasive spider mite Tetranychus evansi (Acari:
488 Tetranychidae) alters community composition and host-plant use of native relatives. –
489 Exp. Appl. Acarol. 60: 321-341.

490 Godinho, D. P. et al. 2016. Down-regulation of plant defence in a resident spider mite
491 species and its effect upon con-and heterospecifics. – Oecologia 180: 161-167.

492 Gotoh, T. et al. 2015. Development and reproduction of five Tetranychus species
493 (Acari: Tetranychidae): Do they all have the potential to become major pests? – Exp.
494 Appl. Acarol. 66: 453-479.

495 Grbić, M. et al. 2011. The genome of Tetranychus urticae reveals herbivorous pest
496 adaptations. – Nature 479: 487-492.

497 Gröning, J. and Hochkirch, A. 2008. Reproductive interference between animal species.
498 – Q. Rev. Biol. 83: 257-282.

499 Gumm, J. M. and Gabor, C. R. 2005. Asexuals looking for sex: conflict between species
500 and mate-quality recognition in sailfin mollies (Poecilia latipinna). – Behav. Ecol.
501 Sociobiol. 58: 558–565.

502 Helle, W. 1967. Fertilization in the two-spotted spider mite (Tetranychus urticae:
503 Acari). – Entomol. Exp. Appl. 10: 103–110.

504 Hochkirch A., Gröning J. & Bücker A. 2007. Sympatry with the devil: reproductive
505 interference could hamper species coexistence. Journal of Animal Ecology 76(4): 633–
506 642.

507 Hurtado M. A. et al. 2008. Sequence analysis of the ribosomal internal transcribed
508 spacers region in spider mites (Prostigmata: Tetranychidae) occurring in citrus orchards
509 in Eastern Spain: use for species discrimination. – Ann. Appl. Biol. 153: 167-174.

510 Kishi, S. 2015. Reproductive interference in laboratory experiments of interspecific
511 competition. – Popul. Ecol. 57: 283-292.

512 Kishi, S. et al. 2009. Reproductive interference determines persistence and exclusion in
513 species interactions. – J. Anim. Ecol. 78: 1043-1049.

514 Kishi, S. and Nakazawa, T. 2013. Analysis of species coexistence co-mediated by
515 resource competition and reproductive interference. – Popul. Ecol. 55: 305-313.

516 Kyogoku, D. and Nishida, T. 2013. The mechanism of the fecundity reduction in
517 Callosobruchus maculatus caused by Callosobruchus chinensis males. – Popul. Ecol. 55:
518 87-93.

519 Kyogoku, D. 2015. Reproductive interference: ecological and evolutionary
520 consequences of interspecific promiscuity. – Popul. Ecol. 57: 253-260.

521 Lievens, E. J. et al. 2016. Maladaptive Sex Ratio Adjustment in the invasive brine
522 shrimp Artemia franciscana. – Curr. Biol. 26: 1463- 1467.

523 Macke, E. et al. 2012. Mating modifies female life history in a haplodiploid spider mite.
524 – Am. Nat. 179: E147-E162.

525 Matsuda, T. et al. 2013. DNA-based identification of spider mites: molecular evidence
526 for cryptic species of the genus Tetranychus (Acari: Tetranychidae). – J. Econ. Entomol.
527 106: 463-472.

528 McPeek, M.A., and Gavrilets, S. 2006. The evolution of female mating preferences:
529 differentiation from species with promiscuous males can promote speciation. –
530 Evolution 60: 1967-1980.

531 Nakagawa, S. and Poulin, R. 2012. Meta-analytic insights into evolutionary ecology: an
532 introduction and synthesis. – Evol. Ecol. 26: 1085–1099.

533 Navajas, M. 1998. Host plant associations in the spider mite *Tetranychus urticae* (Acari:
534 Tetranychidae): insights from molecular phylogeography. – Exp. Appl. Acarol. 22: 201-
535 214.

536 Navajas, M. et al. 2013. Review of the invasion of *Tetranychus evansi*: biology,
537 colonization pathways, potential expansion and prospects for biological control. – Exp.
538 Appl. Acarol. 59: 43-65.

539 Noriyuki, S. et al. 2012. Asymmetric reproductive interference between specialist and
540 generalist predatory ladybirds. – J. Anim. Ecol. 81: 1077-1085.

541 Qazi M. C. B. et al. 2003. The developments between gametogenesis and fertilization:
542 ovulation and female sperm storage in Drosophila melanogaster. – Dev. Biol. 256: 195–
543 211.

544 Sarmento, R. A. et al. 2011a. A herbivore that manipulates plant defence. – Ecol. Lett.
545 14: 229-236.

546 Sarmento, R. A. et al. 2011b. A herbivorous mite down-regulates plant defence and
547 produces web to exclude competitors. – PLoS One 6: 23757.

548 Sato, Y. et al. 2014. Testing for reproductive interference in the population dynamics of
549 two congeneric species of herbivorous mites. – Heredity 113: 495–502.

550 Sato, Y. et al. 2016. Why do males choose heterospecific females in the red spider
551 mite? – Exp. Appl. Acarol. 68: 21-31.

552 Satoh, Y. et al. 2001. Mating strategy of spider mite, *Tetranychus urticae* (Acari:
553 Tetranychidae) males: Postcopulatory guarding to assure paternity. – Appl. Entomol.
554 Zool. 36: 41– 45.

555 Schlupp, I. 2010. Mate Choice and the Amazon Molly: How Sexuality and Unisexuality
556 Can Coexist. – J. Hered. 101: S55–S61.

557 Servedio, M. R. and Kirkpatrick, M. 1997. The effects of gene flow on reinforcement. –
558 Evolution 51: 1764-1772.

559 Takafuji, A. 1986. Effectiveness of second mating for two incompatible types of the
560 citrus red mite, Panonychus citri (McGregor). – Res. Popul. Ecol. 28: 91-101.

561 Takafuji, A. et al. 1997. Reproductive interference and its consequences for the
562 competitive interactions between two closely related Panonychus spider mites. – Exp.
563 Appl. Acarol. 21: 379-391.

564 Takakura, K. I. et al. 2015. Conflicting intersexual mate choices maintain interspecific
565 sexual interactions. – Popul. Ecol. 57: 261-271.

566 Vahed, K. 1998. The function of nuptial feeding in insects: review of empirical studies.
567 – Biol. Rev. Camb. Philos. 73: 43–78.

568 Vala, F. et al. 2000. *Wolbachia*-induced hybrid breakdown in the two--spotted spider
569 mite *Tetranychus urticae* Koch. – P. Roy. Soc. Lond. B Bio. 267: 1931-1937.

570 Viechtbauer, W. 2010. Conducting meta-analyses in R with the metafor package. – J.
571 Stat. Softw. 36: 1-48.

572 Wang, P. et al. 2010. Crossing experiments and behavioral observations reveal
573 reproductive incompatibility among three putative species of the whitefly *Bemisia*
574 *tabaci*. – Insect Sci. 17: 508-516.

575 Yassin, A. and David, J. R. 2016. Within-species reproductive costs affect the
576 asymmetry of satyrization in Drosophila. – J. Evol. Biol. 29: 455-60.

577 Xu, J. and Wang, Q. 2011. Seminal fluid reduces female longevity and stimulates egg
578 production and sperm trigger oviposition in a moth. – J. Insect Physiol. 57: 385-390.

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

Fig.1

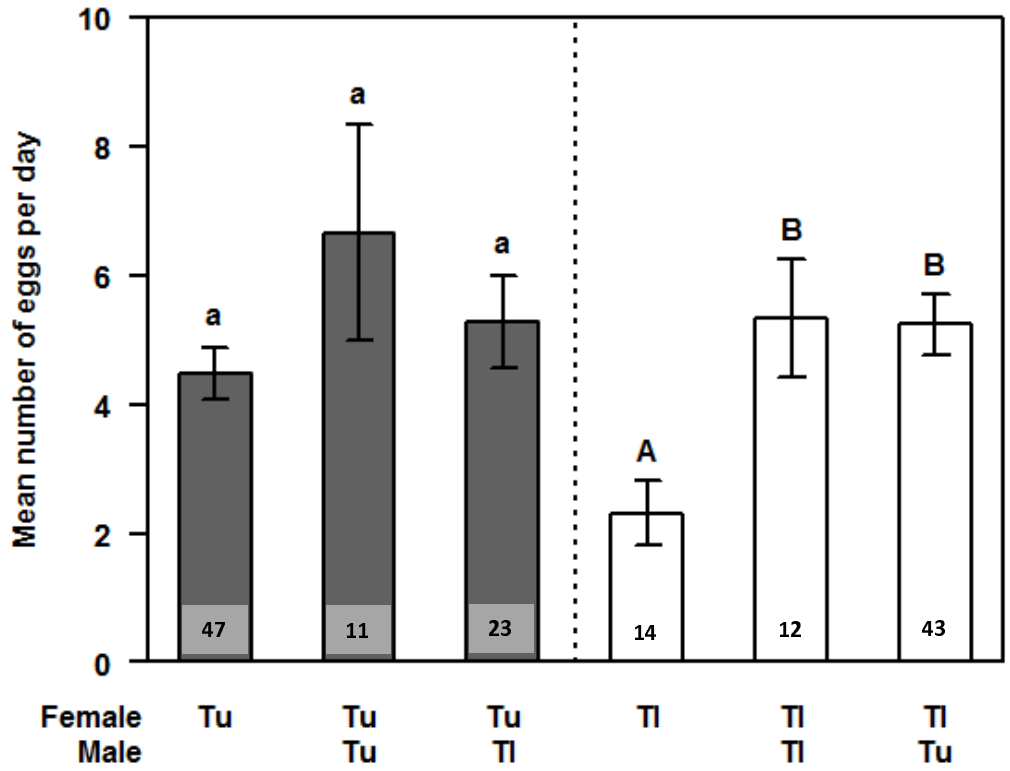

Fig.2

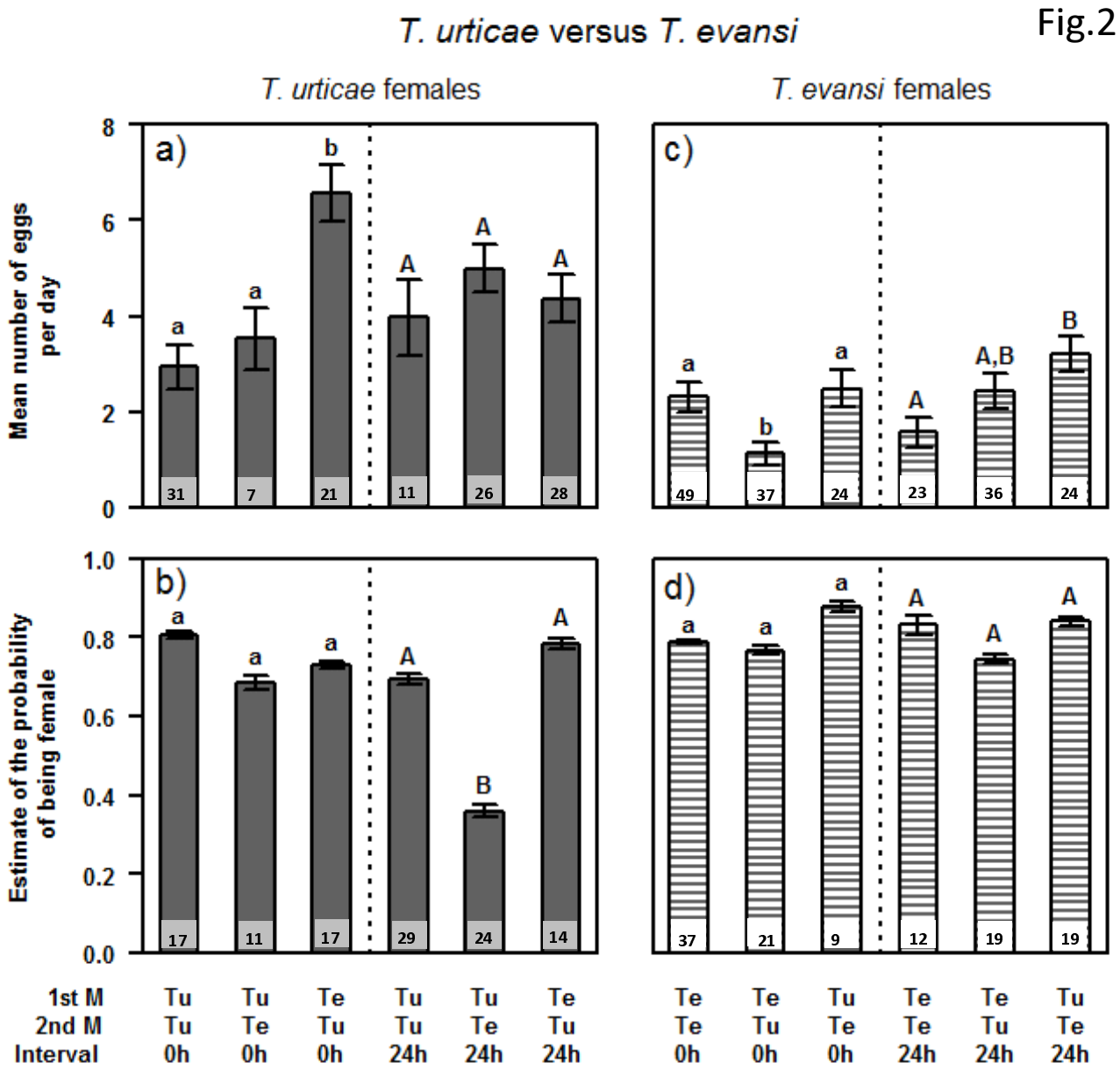

Fig.3