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

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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. To assess the net effect of these interactions on *T. urticae*, we performed a meta-analysis on the data obtained. This revealed that the net effect of the interaction *T. urticae – T. evansi* was neutral, whereas that between *T. urticae* and *T. ludeni* was slightly positive for *T. urticae*. Therefore, the net effect of such interactions may be 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, meta-analysis.

Introduction

Reproductive interference refers to 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). It can occur at different levels: overlapping or masking conspecific sexual signals (signal jamming), interrupting conspecific sexual interactions, or promoting heterospecific matings, thereby reducing the frequency or outcome of conspecific matings, or inducing hybridization, leading to a lower offspring fitness (Gröning and Hochkirch 2008). Given these negative effects, reproductive interference may lead to the exclusion of one of the species involved (Gröning and Hochkirch 2008; Kishi et al. 2009). Indeed, theory predicts that reproductive interference may contribute to species exclusion more often than resource competition (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), if it is stronger between invasive and residents than among residents.

The bulk of studies of reproductive interference concerns 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 species exclusion 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. Moreover, it is 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 while not measuring all possible effects of this interaction. For example, Takafuji (1997) used a Lotka-Volterra modified model to predict the effect of reproductive interference between two *Panonychus* mite species from Japan (*Panonychus citri* and *P. mori*) on species exclusion. However, only one possible combination of mating interactions between these two species (a female mating first with a heterospecific then with a conspecific) was tested. In contrast, other studies consider different orders of mating events (eg, Kyogoku and Nishida 2013), but do not integrate this information to generate a prediction concerning the net effect of reproductive interactions on species distributions.

Here, we aimed at testing how the outcome of different mating events among spider mite species can be integrated into a net measure of the effect of reproductive interactions on a focal species. Spider mites are haplodiploid, hence the distinction between fecundity and fertilization effects can be made given that fertilized eggs result in female offspring and unfertilized eggs in male offspring. Thus, fertilization failures can be detected by a reduction in the proportion of female offspring, whereas impairment of egg production is detected by a reduction in the total number of offspring. Moreover, reproductive interference has been frequently observed in this group (Collins and Margolies 1994; Takafuji et al. 1997; Ben-David et al. 2009, Sato et al. 2014).

We studied the outcome of reproductive interactions in a system composed of one focal species – the spider mite *Tetranychus urticae* – in sexual heterospecific interactions with another resident species, *T. ludeni*, and an invasive species, *T. evansi*. These three herbivorous species co-occur in the Mediterranean region and are often found on the same host plants (Escudero and Ferragut 2005, Boubou et al. 2012, Godinho et al. 2016). Whereas T. urticae and T. ludeni are resident species, T. evansi has only recently invaded the European continent (Boubou et al. 2012). We used *T. urticae* as the focal species because it is the spider-mite species for which most information is available. Indeed, it has been shown that this 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 the 24h interval, produce fewer fertilized offspring (i.e., females) (Macke et al. 2012), suggesting that sperm displacement after 24h is possible. Hence, we hypothesize that mating order and the mating interval will affect the outcome of reproductive interference in *T. urticae*. Whereas information on the interaction between *T. urticae* and *T. ludeni* is as yet lacking, heterospecific matings have been observed between *T. urticae* and *T. evansi* (Sato et al. 2014, 2016, Clemente et al. 2016). Moreover, *T. evansi* has been shown to 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).

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 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.

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

To determine whether hybridization occurred between *T. urticae* and *T. ludeni*, we measured the offspring sex-ratio resulting from single heterospecific matings. Given that only females develop from fertilized eggs, a whole-male offspring would mean unsuccessful hybridization. However, even in the absence of viable hybrids, heterospecific matings could result in aborted development of heterospecifically-fertilized eggs, meaning that females would produce fewer eggs. To test this, we compared the fecundity of *T. urticae* and *T. ludeni* females that mated with a heterospecific male to that of virgin females and of females mated with a conspecific male.

Females were collected from the stock populations, isolated at the quiescent deutonymph stage (which precedes their last moult before reaching adulthood), and kept in groups of approximately 15 females on bean (*Phaseolus vulgaris*) leaf discs (2 cm^2) until emergence, to ensure their virginity. Adult males were collected from the same stock populations and kept isolated in leaf discs (2 cm^2) for at least 24 hours before the assay, to ensure sperm replenishment. Females were placed individually in leaf discs (1 cm^2) with either a conspecific or a heterospecific male and observed continuously until copulation occurred. Only matings that lasted at least 1 minute were considered effective (Boudreaux 1963). These experiments had the maximum duration

of 2 hours. If no mating occurred within this time, individuals were discarded. Subsequently, females were isolated in a leaf disc (2 cm^2), then transferred to a new disc every three days until the female's death. The number of eggs laid was registered 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

233 statistical analyses with block 1 only and with block 1 and block 2 together. Since the
234 results were qualitatively similar, here we present the results from the analysis with
235 block 1 and block 2 together.

236 With the outputs from these analyses, we further compared the general net
237 effects of reproductive interference from the invasive and resident species on *T.*
238 *urticae* with a meta-analysis procedure (Borenstein et al. 2009; Nakagawa and Poulin
239 2012). This procedure allowed us to test which species, within each species pair, exerts
240 the strongest effect on the other; and whether, between species pairs, invasive-
241 resident heterospecific sexual interactions are more severe than resident-resident
242 interactions. For that we calculated the effect sizes of the statistical results obtained
243 from the LM and GLM analyses described above and shown in Table 1, converting p-
244 values and sampling sizes into the Fishers' *z* transformation of the correlation
245 coefficient (Z_r) and its corresponding variance (Var_{Zr}). The correlation coefficient varies
246 between -1 and 1 and can be interpreted as the strength of female response with
247 respect to oviposition rate and offspring sex ratio: the more significant the p-values
248 obtained from the LM and GLM models the greater the departure from a random
249 response, and so the "stronger" the effect of reproductive interference of *T. evansi*
250 and *T. ludeni* on *T. urticae* and vice versa.

251 We used the p-values from the contrasts between the control and the two
252 experimental treatments. However, to avoid duplicating the contribution of the
253 control to the effect sizes (Borenstein et al. 2009), we did two independent analysis,
254 one for when a female's first mating was with a heterospecific male and a second
255 analysis for when a female's second mating was with a heterospecific male. The effect
256 sizes are shown in Table S1 from the Supplementary Material. Additionally, because

each female contributed with two data outputs (oviposition rate and offspring sex ratio), and to avoid redundancy in our data again, we calculated a synthetic effect size that was defined as the mean between oviposition rate and offspring sex ratio and their variance (Borenstein et al. 2009). To calculate the variance of the mean, we had to calculate a correlation between outcomes (Borenstein et al. 2009). We did this using a Pearson correlation, and obtained 0.18 (shown in Table S2 from the Supplementary Material).

The effect sizes could be either positive or negative, depending on whether the interactions of *T. urticae* with the other species were beneficial or costly to *T. urticae*: positive effects occurred when oviposition rate and offspring sex ratio increased in *T. urticae* females or decreased in *T. evansi* and *T. ludeni* females; negative effects occurred in the opposite way.

We used the Compute.es package (Del Re 2013) to convert p-values and sample sizes into Z_r and Var_{Zr} (see Tables S1 and S2 in Supplementary Material) and the Metafor package v1.9-8 (Viechtbauer 2010) for the meta-analysis (Table S3 in Supplementary Material). We used a meta-analytic fixed-effects linear model (using the rma.uni function in Metafor) with the interfering species (Invasive versus Resident) as the explanatory variable.

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*

281 females that mated heterospecifically was not significantly different from that of virgin
282 females or from that of females mated with a conspecific male (Figure 1 and Table 1).
283 On the other hand, the fecundity of *T. ludeni* females that mated with conspecifics or
284 heterospecifics was significantly higher than that of virgin females (Figure 1 and Table
285 1). Therefore, mating with heterospecific males does not result in the aborted
286 fertilization of oocytes for *T. urticae* and *T. ludeni* females.
287
288 **b) The outcome of heterospecific matings that precede or follow conspecific**
289 **ones**
290 *(i) T. urticae vs T. evansi*
291 The oviposition rate of *T. urticae* females that mated with either a conspecific and a
292 heterospecific or with two conspecific mates varied significantly according to mating
293 order in interaction with mating interval (Table 1). Specifically, it was higher for *T.*
294 *urticae* females that mated with *T. evansi* males just before mating than for any other
295 cross at 0h mating interval (Fig. 2a, Table 1). At the 24h interval, however, mating
296 combinations did not affect this trait. The proportion of fertilized offspring (*i.e.*,
297 daughters) of females *T. urticae* also varied significantly according to mating order in
298 interaction with mating interval (Table 1). But in contrast to the oviposition rate, this
299 trait was affected at the 24h interval only, in which mating with a *T. evansi* male after
300 mating with a conspecific male resulted in a decrease in the proportion of fertilized
301 offspring of *T. urticae* females, relative to other mating sequences (Fig. 2b, Table 1).
302 The mating order also affected diferentially the oviposition rate of *T. evansi*
303 females, depending on the interval between matings. *T. evansi* females that mated
304 with *T. urticae* males immediately after conspecific mates had reduced oviposition rate

305 relative to other mating sequences at this time interval (Fig. 2c; Table 1); however, if
306 the heterospecific cross occurred 24 hours before the conspecific cross, the oviposition
307 rate of *T. evansi* females increased relative to other mating sequences at this time
308 interval (Fig. 2c; Table 1). These crosses did not significantly affect sex ratio (Fig. 2d;
309 Table 1).
310
311 *(ii) T. urticae vs T. ludeni*
312 In crosses with the resident species (*T. ludeni*), the oviposition rate of *T. urticae*
313 females varied significantly according to mating order in interaction with mating
314 interval (Table 1). Specifically, we found no effect of the mating order at 0h interval,
315 but at 24h interval the oviposition rate of females that mated first with a conspecific
316 then with a heterospecific male was lower than that of other crosses at this time
317 interval. (Fig. 3a; Table 1). *T. urticae* females suffered no significant changes in
318 offspring sex ratio from matings with *T. ludeni* males (Figure 3b; Table 1).
319 In *T. ludeni* females, the oviposition rate and the proportion of fertilized
320 offspring varied significantly according to mating order in interaction with the mating
321 interval (Table 1). Compared to the control treatment, *T. ludeni* females had lower
322 oviposition rate when mating with *T. urticae* males immediately before conspecifics
323 males, or when hetero- and conspecific matings had 24h interval, irrespective of the
324 mating order (Fig. 3c, table 1). Additionally, when *T. ludeni* females mated with *T.*
325 *urticae* males 24h after conspecific matings, the proportion of fertilized offspring was
326 significantly lower than that of other crosses at this time interval (Figure 3d; Table 1).
327 The mating sequence had no effect on the sex ratio at the 0h interval.
328

329 **c) Meta-analysis on the effects of mating with heterospecifics**

330 Because the effects of mating with heterospecifics were contingent upon the species
331 involved, but also the order and timing of mating events, we performed a meta-
332 analysis on these results to obtain the net effect of each interaction (Figure 4 and
333 Tables S1, S2 and S3).

334 The overall effect of mating with heterospecifics was neutral, though slightly
335 positive, for *T. urticae*, both when the female's first and second matings were with a
336 heterospecific male (first male heterospecific: $Z_r = 1.088$, $P = 0.277$; second male
337 heterospecific: $Z_r = 1.439$, $P = 0.150$). Matings involving the invasive species did not
338 result in overall net costs or benefits for *T. urticae* (first male heterospecific: $Z_r = -$
339 0.460, $P = 0.646$; second male heterospecific: $Z_r = 0.087$, $P = 0.931$). Matings with the
340 resident species, on the other hand, were mainly beneficial, both for first ($Z_r = 1.878$,
341 $P = 0.060$, marginally significant) and second matings with a heterospecific male
342 ($Z_r = 1.989$, $P = 0.047$). The difference between matings with the invasive and the
343 resident species was, however, non-significant (first male heterospecific: $Z_r = 1.598$,
344 $P = 0.110$; second male heterospecific: $Z_r = 1.376$, $P = 0.169$).

345 Concerning the effect of the mating interval, when matings occurred at the 0h
346 interval, the net effect for *T. urticae* from both invasive (first male heterospecific:
347 $Z_r = 0.080$, $P = 0.936$; second male heterospecific: $Z_r = 1.234$, $P = 0.217$) and resident
348 (first male heterospecific: $Z_r = 0.497$, $P = 0.619$; second male heterospecific: $Z_r = -0.671$,
349 $P = 0.502$) species was mainly neutral, with no significant differences between the net
350 effect from the invasive and the resident species (first male heterospecific: $Z_r = -0.279$,
351 $P = 0.781$; second male heterospecific: $Z_r = 1.310$, $P = 0.190$). When matings occurred
352 at the 24h interval, the net effect for *T. urticae* from matings with the invasive species

353 was again neutral (first male heterospecific: Z_r = -0.787, P = 0.431; second male
354 heterospecific: Z_r = -1.237, P = 0.216). Contrastingly, however, the net effect for *T.*
355 *urticae* from matings with the resident species was significantly positive for both first
356 (Z_r = 2.219, P = 0.027) and second matings (Z_r = 3.223, P = 0.001) with heterospecifics.
357 Additionally, there were significant differences between the invasive and resident
358 species (first male heterospecific: Z_r = -2.051, P = 0.040; second male heterospecific:
359 Z_r = -3.099, P = 0.002).
360
361 **Discussion**
362 In this study, we investigated the consequences of mating with heterospecifics for the
363 fertilization success and offspring viability in a system composed of three spider-mite
364 species. We found that heterospecific matings between *T. urticae* and *T. ludeni* did not
365 result in fertilized offspring (i.e., females), nor did it have any negative effects on egg
366 viability, as shown for matings between *T. urticae* and *T. evansi* (Sato et al. 2014,
367 Clemente et al. 2016). In fact, a *T. ludeni* female that mates with a *T. urticae* male will
368 produce more male offspring than a virgin *T. ludeni* female. Second, the effects of
369 heterospecific matings on the outcome of previous or subsequent matings with
370 conspecifics were highly dependent on the species pair involved, on the trait measured
371 and on the timing and order of mating events. Despite strong effects of particular
372 mating sequences, our meta-analysis for the net effect of reproductive interactions on
373 *T. urticae* revealed a neutral net effect of the interaction with *T. evansi,* and a positive
374 net effect of the interaction with *T. ludeni*.
375 Positive effects of interspecific reproductive interactions were found for
376 fecundity. This can be due to a stimulation of oogenesis by the sperm of heterospecific

377 males, increasing the availability of oocytes to subsequent matings with conspecifics.
378 Indeed, oogenesis is stimulated by conspecific sperm in several species (Qazi et al.
379 2003, Xu and Wang 2011). This could also be the case with heterospecific sperm. If so,
380 it could explain the higher fecundity found in crosses between *T. urticae* and *T. evansi*.
381 In fact, earlier studies have documented that interactions with heterospecific males
382 are not always negative. In some gynogenetic species, heterospecific mating is a
383 prerequisite for embryogenesis (Gumm and Gabor 2005, Schlupp 2010). Moreover, in
384 some invertebrate species, females receive nuptial gifts from heterospecific males
385 (Vahed 1998, Costa-Schmidt and Machado 2012). However, to our knowledge, this is
386 the first time that an increase in fecundity following a heterospecific mating is
387 described in the literature. Such effects may thus be rare. Still, earlier studies may have
388 overlooked them because they have not examined the roles of the order of mating in
389 the outcome of heterospecific mating interactions.

390 Nonetheless, we also detected several negative effects of mating with
391 heterospecifics, as found in most studies of reproductive interference (Gröning and
392 Hochkirch 2008, Kishi 2015). We found both a reduction in the number of eggs laid and
393 a decrease in fertilization success (i.e., offspring sex ratio). However, the incidence of
394 these two effects varied according to the species involved, the order of matings and
395 the time interval. Whereas effects on fecundity were found in several mating
396 sequences, an effect on fertilization success was found only when the heterospecific
397 male mated with the female 24 hours after the conspecific male. This is at odds with
398 expectations stemming from findings on conspecific matings, which show (a) first-male
399 precedence and (b) exceptions to this rule only if the second male mates immediately

after the first. Therefore, the mechanisms underlying sperm displacement by heterospecific males in spider mites should be investigated.

The meta-analysis confirmed this finding, showing that effects were stronger at the 24h interval. Also, it showed that effects were similar irrespective of the order of the mating events. In fact, in some cases, effects of mating with heterospecifics are stronger if such matings follow conspecific ones. This suggests that first male precedence found in conspecific matings cannot be extrapolated to matings involving heterospecific sperm. This contrasts with the recent finding that effects of heterospecific matings in *Drosophila* could be predicted from the harmful effects of conspecific mates (Yassin and David 2015), and that genes involved in conspecific male precedence also affect sperm precedence in multiple matings involving heterospecifics (Civetta and Finn 2014). This indicates that the equivalence of effects of conspecific and heterospecific sperm on the outcome of conspecific matings is dependent on the type of effect and/or the species involved in the interaction.

Despite the fact that many interactions have a negative outcome, the meta-analysis also revealed that the overall effect of mating with heterospecifics is neutral for *T. urticae*. This is because the negative impact of mating with heterospecifics is compensated by the negative impact that *T. urticae* males have on fertility and fecundity of the other species. This leads to the prediction that selection for species discrimination should be low in *T. urticae*, as the net outcome of interspecific reproductive interactions is not costly. Indeed, it has been shown that both males and females of *T. urticae* show weak, if not absent, discrimination between conspecifics and *T. evansi* mates (Sato et al. 2014, 2016, Clemente et al. 2016). However, it may be possible that costs are found if matings with heterospecifics become very frequent.

424 Since effects of heterospecific matings depend on the order and timing of
425 occurrence, the outcome of these interactions will depend on the frequency with
426 which those different types of matings occur in nature. In the species studied here,
427 conspecific males tend to guard quiescent females (i.e, last larval stage before
428 becoming adult female), to ensure mating immediately after emergence. If males
429 guard preferentially conspecific females, as has been shown in other spider mite
430 species pairs (Collins et al. 1993, Takafuji et al. 1997), heterospecific matings will occur
431 more often after rather than before conspecific ones. If this is the case, the effects of
432 *T. evansi* and of *T. ludeni* on the offspring of *T. urticae* females will not be the same.
433 Indeed, whereas mating with *T. evansi* males after a conspecific male leads to a
434 reduction in offspring fertilization in *T. urticae*, *T. ludeni* matings that follow conspecific
435 ones have no effect on the offspring of *T. urticae* females. Moreover, we have shown
436 that females become less receptive to both conspecific and heterospecific matings if
437 the first mating has occurred 24h before the second (Clemente et al. 2016). This leads
438 to the prediction that the most common mating sequence among these species will be
439 a heterospecific mating immediately following a conspecific one. If this is the case,
440 then we predict that the effect of heterospecific matings in *T. urticae* will be relatively
441 mild.

442 The meta-analysis also showed that the net effect of mating with *T. ludeni*, the
443 resident species, was positive, whereas that of mating with *T. evansi*, the invasive
444 species, was neutral. Therefore, our hypothesis that reproductive interference could
445 be more costly (or less beneficial) between resident and invasive species than between
446 residents is confirmed by our results. However, as the net outcome of the resident-
447 invasive interaction was neutral, reproductive interference cannot be invoked to

448 explain the exclusion of *T. urticae* in habitats with *T. evansi* (Ferragut et al. 2013,
449 Sarmento et al. 2011b). Other factors may contribute to this exclusion, as the
450 production of a dense web by *T. evansi*, which prevents heterospecifics from accessing
451 the surface of the leaves to feed and oviposit (Sarmento et al. 2011b). Importantly,
452 however, we show that the occurrence and strength of reproductive interference
453 cannot be assessed with a partial evaluation of the outcome of reproductive
454 interactions. Indeed, the order and interval between matings have great influence on
455 the outcome of heterospecific interactions. Therefore, the net outcome will hinge on
456 the frequency of such events. This confirms the importance of using complete
457 experimental designs on the detection and characterization of reproductive
458 interference.
459
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469

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.

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.

493 **Figure 3** | Average daily fecundity and estimated offspring sex ratio resulting from
494 interactions between *T. urticae* (plots a, b; grey bars) and *T. ludeni* (plots c, d; white
495 bars) females with conspecific and heterospecific males. In each plot, bars on the left
496 side of the dotted line correspond to treatments where second matings occurred
497 immediately (0h) after the first one; bars on the right side correspond to treatments
498 where second matings occurred 24h after the first one. "1st M": first male that mated
499 with the female; "2nd M": second male. The interval indicates the time of occurrence
500 of the second mating, i.e., if immediately after the first mating (0h) or 24h later. "Tu":
501 *T. urticae* males; "Tl": *T. ludeni* males. Letters above the bars indicate the significant
502 differences between treatments (small letters: among crosses occurring with a 0h
503 interval; capital letters: among crosses occurring with a 24h interval. Error bars
504 represent the standard errors of the mean. For offspring sex ratio, we obtained the
505 estimates of the probability of being female and correspondent standard errors of the
506 mean from the statistical GLM models. This takes into account sex ratio variation
507 among females, as well as the quasi-binomial correction for overdispersion of the data.
508
509 **Figure 4 |** Mean strength of reproductive interference by the invasive (*T. evansi*) and
510 resident (*T. ludeni*) species on *T. urticae*, when a female's first (A) or second (B) mating
511 is heterospecific. Squares show the mean effect size estimates derived from the meta-
512 analytic models; the squares' size represent the weights given to the observed effects
513 during the model fitting; and the bars show the 95% confidence intervals (CI) around
514 the mean effect size estimates. Negative or positive effects towards *T. urticae* are
515 significant when the effect size and both anchors of the CI fall below or above zero.
516 The results of the meta-analytic models testing the effect of the interfering species

517 (invasive versus resident) on all effect sizes and for each subgroup of explanatory
518 variables (Mating intervcal with a first male at 0h or 24h) are shown with the "NS",
519 "S*" and "S**" symbols: "NS" for non-significant differences ($p>0.05$); "S*" for
520 significant differences ($p<0.05$); and "S**" for significant differences ($p<0.01$). At the
521 bottom is a summary effect size representing pooled effect sizes. The effect sizes were
522 defined as the mean between female fecundity and offspring sex ratio and their
523 variance. To obtain the variance of the mean, we calculated a correlation between
524 outcomes, which was 0.18.
525
526
527

528 **Table 1 |** Statistical tests and contrasts for the comparisons of fecundity and offspring

529 sex ratio in crosses between con- and heterospecific males and females.

	Matings			Fecundity (F-test)	Sex-ratio (F-test)
a) Single mated females					
T. urticae vs T. ludeni					
With *T. urticae* females					
Mating order				$F_{2,78} = 1.886$, P = 0.1585	
Contrasts	No mating	*vs*	Tu	|t| = 0.922; P = 0.3595	
		vs	Tl	|t| = 1.885; P = 0.0631	
	Tu	*vs*	Tl	|t| = 1.083; P = 0.2822	
With *T. ludeni* females					
Mating order				$F_{2,66} = 5.636$, P = 0.0055	
Contrasts	No mating	*vs*	Tl	|t| = 2.621; P = 0.0109	
		vs	Tu	|t| = 3.240; P = 0.0019	
	Tl	*vs*	Tu	|t| = 0.105; P = 0.9170	
b) Matings with an invasive species					
T. urticae* vs *T. evansi					
With *T. urticae* females					
Mating order				$F_{2,136} = 7.919$, P = 0.0006	$F_{2,109} = 16.371$, P < 0.0001
Mating interval				$F_{1,136} = 0.039$, P = 0.8440	$F_{1,108} = 6.878$, P = 0.0100
Mating order x Mating interval				$F_{2,136} = 6.026$, P = 0.0031	$F_{2,106} = 4.963$, P = 0.0087
Planned contrasts					
Mating interval 0h:	TuTu	*vs*	TuTe	|t| = 0.712; P = 0.4719	|t| = 1.430; P = 0.1556
		vs	TeTu	|t| = 4.964; P < 0.0001	|t| = 1.116; P = 0.2670
	TuTe	*vs*	TeTu	|t| = 3.288; P = 0.0009	|t| = 0.552; P = 0.5819
Mating interval 24h:	TuTu	*vs*	TuTe	|t| = 1.044; P = 0.2984	|t| = 5.362; P < 0.0001
		vs	TeTu	|t| = 0.406; P = 0.6852	|t| = 1.419; P = 0.1587
	TuTe	*vs*	TeTu	|t| = 0.848; P = 0.3980	|t| = 5.103; P < 0.0001
With *T. evansi* females					
Mating order				$F_{2,187} = 4.680$, P = 0.0104	$F_{2,114} = 2.462$, P = 0.0898
Mating interval				$F_{1,187} = 2.555$, P = 0.1116	$F_{1,113} = 0.045$, P = 0.8320
Mating order x Mating interval				$F_{2,187} = 4.977$, P = 0.0078	$F_{2,111} = 0.368$, P = 0.6931
Planned contrasts					
Mating interval 0h:	TeTe	*vs*	TeTu	|t| = 2.841; P = 0.0050	|t| = 0.295; P = 0.7680
		vs	TuTe	|t| = 0.348; P = 0.7281	|t| = 1.327; P = 0.1870
	TeTu	*vs*	TuTe	|t| = 2.692; P = 0.0078	|t| = 1.377; P = 0.1714

Mating interval 24h:	TeTe	*vs*	TeTu	$\|t\|$ = 1.682; P = 0.0943	$\|t\|$ = 1.016; P = 0.3118
		vs	TuTe	$\|t\|$ = 2.948; P = 0.0036	$\|t\|$ = 0.101; P = 0.9199
	TeTu	*vs*	TuTe	$\|t\|$ = 1.561; P = 0.1203	$\|t\|$ = 1.689; P = 0.0940

c) Matings with a resident species

T. urticae* vs *T. ludeni

With *T. urticae* females

Mating order				$F_{2,144}$ = 6.997, P = 0.0013	$F_{2,102}$ = 2.516, P = 0.0858
Mating interval	-			$F_{1,144}$ = 2.598, P = 0.1092	$F_{1,101}$ = 0.654, P = 0.4206
Mating order x Mating interval				$F_{2,144}$ = 3.694, P = 0.0273	$F_{2,99}$ = 1.141, P = 0.3237
Planned contrasts					
Mating interval 0h:	TuTu	*vs*	TuTl	$\|t\|$ = 0.859; P = 0.3915	$\|t\|$ = 0.005; P = 0.9957
		vs	TlTu	$\|t\|$ = 0.857; P = 0.3931	$\|t\|$ = 1.016; P = 0.3119
	TuTl	*vs*	TlTu	$\|t\|$ = 2.736; P = 0.0070	$\|t\|$ = 1.895; P = 0.0610
Mating interval 24h:	TuTu	*vs*	TuTl	$\|t\|$ = 2.505; P = 0.0134	$\|t\|$ = 0.164; P = 0.8700
		vs	TlTu	$\|t\|$ = 1.115; P = 0.2501	$\|t\|$ = 0.964; P = 0.3370
	TuTl	*vs*	TlTu	$\|t\|$ = 1.382; P = 0.1692	$\|t\|$ = 0.640; P = 0.5230

With *T. ludeni* females

Mating order				$F_{2,248}$ = 10.534, P < 0.0001	$F_{2,155}$ = 2.147, P = 0.1204
Mating interval	-			$F_{1,248}$ = 5.180, P = 0.0237	$F_{1,154}$ = 2.567, P = 0.1112
Mating order x Mating interval				$F_{2,248}$ = 14.098, P < 0.0001	$F_{2,152}$ = 10.1064, P < 0.0001
Planned contrasts					
Mating interval 0h:	TlTl	*vs*	TlTu	$\|t\|$ = 1.297; P = 0.1957	$\|t\|$ = 0.853; P = 0.3952
		vs	TuTl	$\|t\|$ = 2.605; P = 0.0097	$\|t\|$ = 0.631; P = 0.5292
	TlTu	*vs*	TuTl	$\|t\|$ = 5.141; P < 0.0001	$\|t\|$ = 1.619; P = 0.1075
Mating interval 24h:	TlTl	*vs*	TlTu	$\|t\|$ = 4.646; P < 0.0001	$\|t\|$ = 4.084; P < 0.0001
		vs	TuTl	$\|t\|$ = 3.805; P = 0.0002	$\|t\|$ = 0.841; P = 0.4018
	TlTu	*vs*	TuTl	$\|t\|$ = 0.401; P = 0.2020	$\|t\|$ = 3.586; P = 0.0005

Legend: "Tu": matings involving *T. urticae* males. "Te": matings with *T. evansi* males. "Tl": matings with *T. ludeni* males. "0h" and "24h" indicate the time of occurrence of the second mating, i.e., if immediately after the first mating (0h) or 24h later. TuTu means that both mating events were with a *T. urticae* male. TuTe means that the first mating was with a *T. urticae* male and the second with a *T. evansi* male. The same logic applies to TeTe, TeTu, TlTl, TlTu and TuTl.

536

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674

675 **Supporting Information**

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

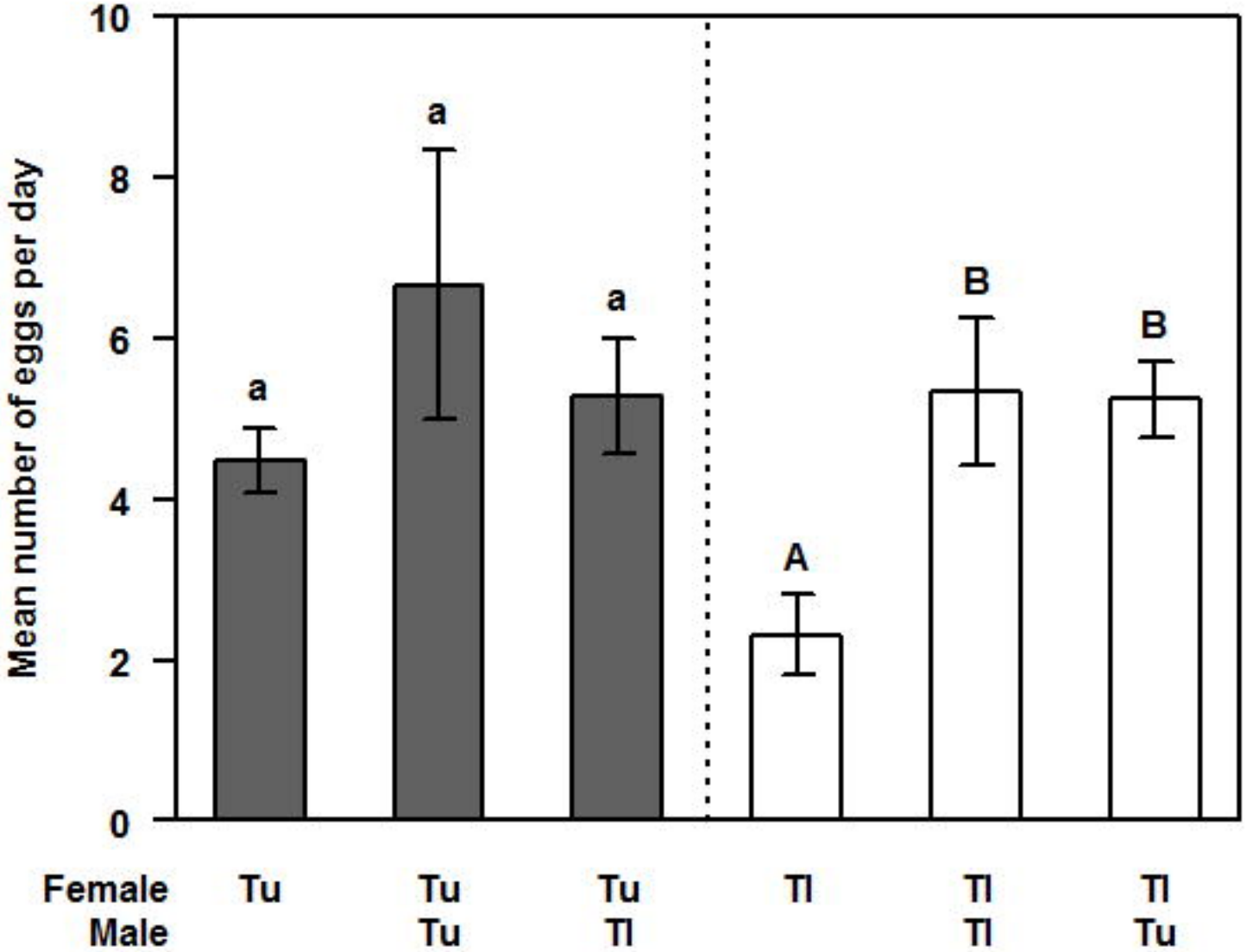
677 this article.

678 Table S1 | All effect sizes extracted for the meta-analyses.

679 Table S2 | Effect sizes used in the meta-analyses.

680 Table S3 | Meta-analysis output.

681


10
8
6
4
2
0
Mean number of eggs per day
a
a
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A
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Female
Male
Tu
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Tl
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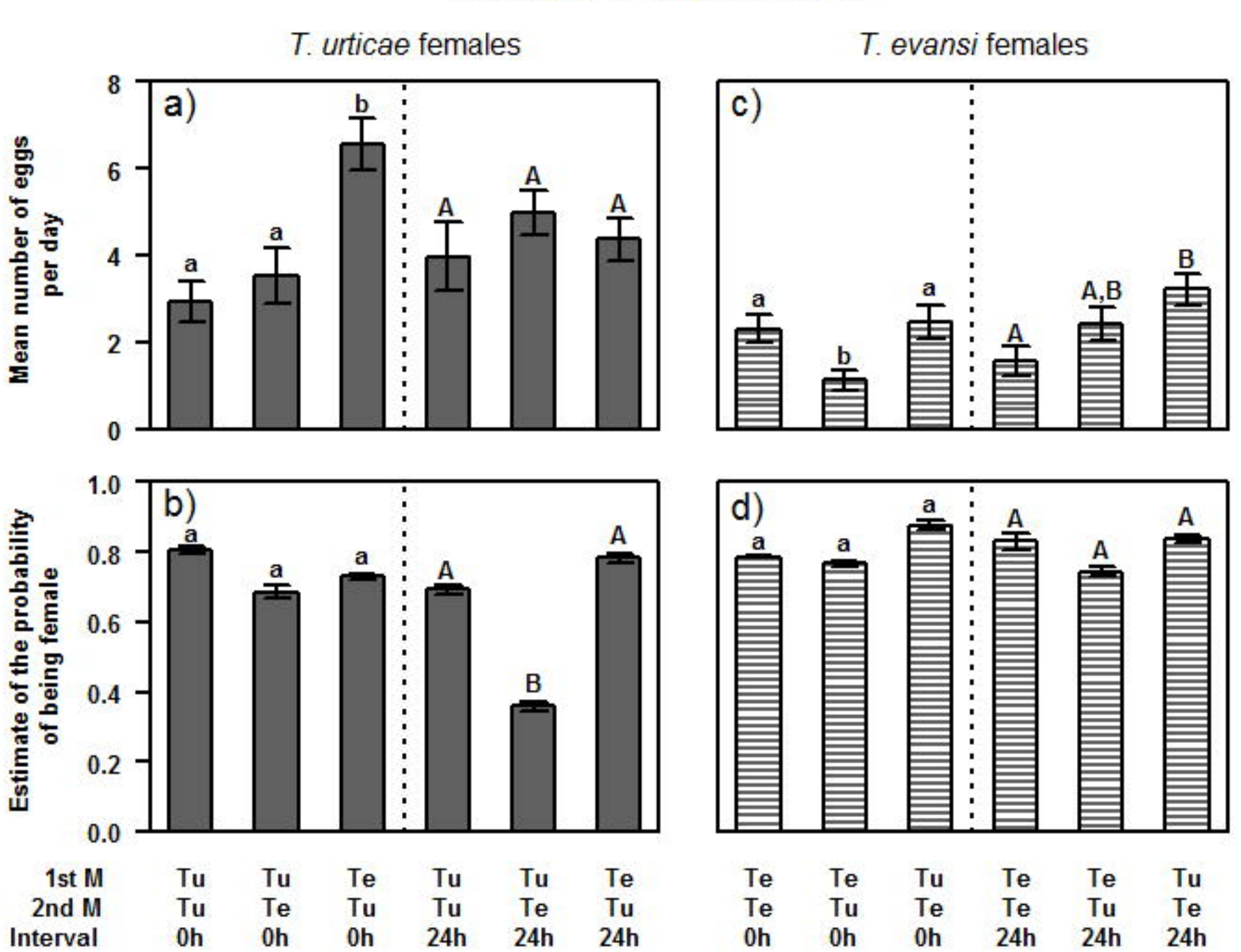
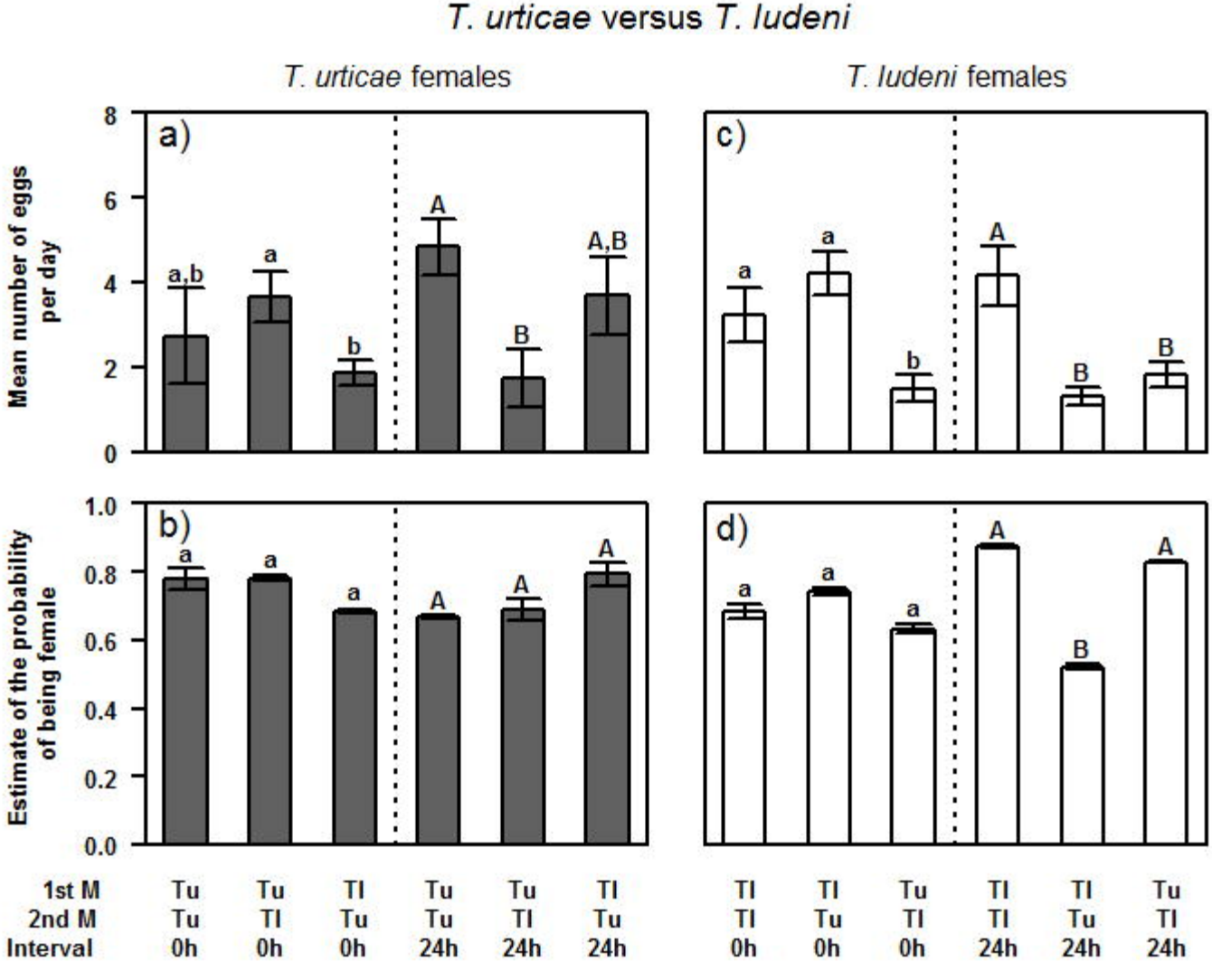
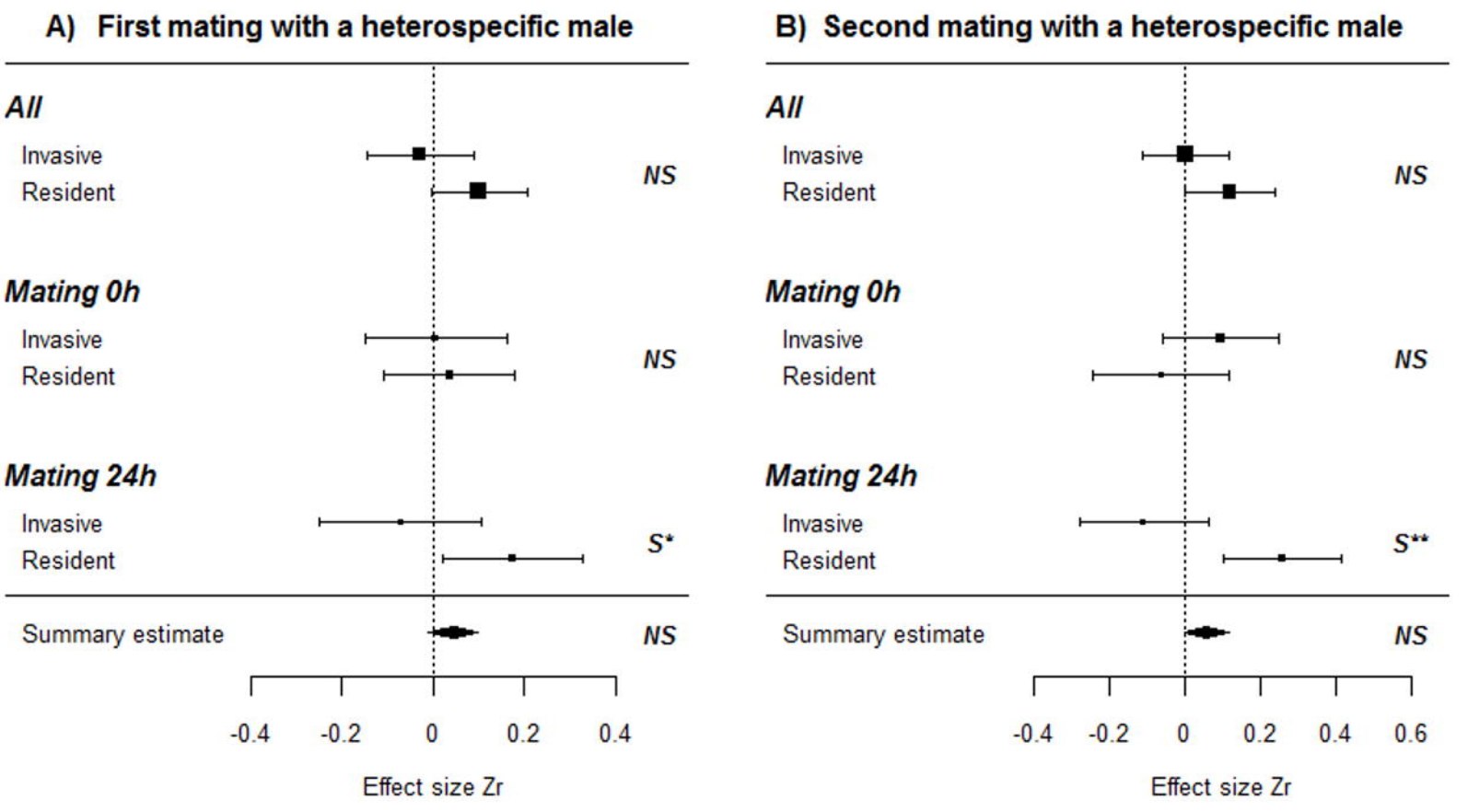
Tu


T. urticae versus T. evansi
T. urticae females
T. evansi females
a)
b)
c)
d)
Mean number of eggs per day
Estimate of the probability of being female
1st M Tu Tu Te Tu Tu Te Te Te Tu Te Te Tu
2nd M Tu Te Tu Tu Te Tu Te Tu Te Te Tu Te
Interval 0h 0h 0h 24h 24h 24h 0h 0h 0h 24h 24h 24h


T. urticae versus T. ludeni
T. urticae females
T. ludeni females
a)
c)
b)
d)
Mean number of eggs per day
Estimate of the probability of being female
8
6
4
2
0
1.0
0.8
0.6
0.4
0.2
0.0
a,b
a
b
A
B
A,B
1st M Tu Tu Tl Tu Tu Tl Tl Tl Tu Tl Tl Tu
2nd M Tu Tl Tu Tu Tl Tu Tl Tu Tl Tl Tu Tl
Interval 0h 0h 0h 24h 24h 24h 0h 0h 0h 24h 24h 24h


A) First mating with a heterospecific male
All
Invasive
Resident
NS
Mating 0h
Invasive
Resident
NS
Mating 24h
Invasive
Resident
S*
Summary estimate
NS
-0.4
-0.2
0
0.2
0.4
Effect size Zr
B) Second mating with a heterospecific male
All
Invasive
Resident
NS
Mating 0h
Invasive
Resident
NS
Mating 24h
Invasive
Resident
S**
Summary estimate
NS
-0.4
-0.2
0
0.2
0.4
0.6
Effect size Zr