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ORIGINAL ARTICLE

Incomplete species recognition entails few costs in spider mites, despite first-male precedence

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Abstract
The consequences of heterospecific matings may hinge on interspecies interactions, but also on characteristics of the intraspecies mating system, namely sperm precedence. Indeed, first-male precedence may entail costs of heterospecific matings that are usually overlooked in other systems, such as fertilization of oocytes that become unavailable to subsequent conspecific males or a decrease in female receptivity. Here, we used a system composed of two co-occurring haplodiploid spider-mite species with first-male precedence, *Tetranychus urticae* and *Tetranychus evansi*, to investigate (a) the potential costs of heterospecific matings and (b) whether mites avoid heterospecific mates. We found that heterospecific matings did not result in fertilized offspring (i.e. females). Moreover, fecundity (i.e. male offspring) of heterospecifically mated females did not differ from that of virgins, indicating that oocyte viability was not affected by heterospecific males. Furthermore, heterospecific matings did not trigger behavioural changes that typically derive from conspecific matings, namely reduced female receptivity for subsequent matings. In avoidance tests, we found that *T. evansi* females and *T. urticae* males mated as often with conspecifics as with heterospecifics, whereas *T. evansi* males and *T. urticae* females mated assortatively more often. Also, latency to copulation in virgin and mated females did not differ between conspecific and heterospecific matings, but matings between *T. urticae* individuals lasted longer than heterospecific matings. Therefore, heterospecific matings result in few costs despite first-male precedence and, concomitantly, species discrimination is low. Still, this study highlights the need to account for intraspecific mating systems in tests of the reproductive consequences of mating with heterospecifics.

Significance statement
In species where the first male fertilizes all the offspring (first-male precedence), mating with individuals from other species often yields few benefits and entails potential costs in terms of future mating events. Yet, several species exhibit incomplete recognition of conspecifics. We here show that this is the case among two spider-mite species that co-occur under natural conditions. However, we also demonstrate that the cost of mating with the 'wrong' species is low, even though they exhibit first-male precedence.

Keywords Tetranychidae · Species interactions · Mating behaviour · Sperm precedence · Species recognition

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Introduction

The ability to recognize conspecifics may enable individuals to interpret heterospecific signals as coming from low-quality mates (or non-mates) and hence allow them to avoid engaging in heterospecific interactions that are often costly (Mendelson and Shaw 2012). Nonetheless, species recognition systems are

often incomplete, leading to the occurrence of interspecific copulations between individuals from closely related species (Burdfield-Steel and Shuker 2011).

Failures of recognition systems entailing the occurrence of interspecific mating events can be due to weak selective pressures for discriminating abilities when the overlap between species distributions is occasional or recent (Coyne and Orr 2004; delBarco-Trillo and Johnston 2010; Abbott et al. 2013). Additionally, even in primary sympatry, selection for discriminating ability might not suffice to prevent heterospecific interactions. Indeed, mistaken evaluation of the quality of potential mates can be maintained due to higher costs of missing reproductive opportunities than those of mating less optimally (Pfennig 2007; Mendelson and Shaw 2012; Scharf and Martin 2013; Burdfield-Steel et al. 2015). This suggests that when heterospecific matings result in high costs—in terms of female sexual receptivity, female fecundity and offspring viability—they should occur only rarely.

Heterospecific matings may modify the physiological status of females and consequently diminish the rate of subsequent conspecific matings (Mclain and Pratt 1999; Valero et al. 2008; Burdfield-Steel and Shuker 2011). However, this may not always be the case. For instance, sperm transfer by conspecifics can result in changes in female behaviour and attractiveness (Wirmer et al. 2010), but these post-mating effects may not be properly triggered by heterospecific sperm. Hence, females that have mated heterospecifically may be as receptive as virgins.

Within-species sperm precedence (Parker 1970) may also affect the outcome of the interaction between females and heterospecific males. In particular, in species with first-male precedence, if the first mating is heterospecific, it can hamper subsequent successful fertilizations by conspecific sperm, as well as female receptivity and attractiveness to conspecific males, entailing strong costs by reducing both the fertilization success and the fecundity of the involved individuals. Such effects are expected to be less conspicuous in species with second-male precedence, as females that mate with heterospecifics first can always compensate later on by mating with a conspecific. Therefore, if costs of mating first with heterospecifics in species with first-male precedence are expected to be higher, then this is likely to affect the behaviour of individuals engaging in such matings. If, however, the first mating occurs with a conspecific male, a second heterospecific mating might have negligible effects.

Here, we investigate the occurrence, characteristics and consequences of heterospecific matings between two haplodiploid phytophagous spider mite species: *Tetranychus urticae* and *Tetranychus evansi*. These species share part of their host range, with *T. urticae* being a generalist and *T. evansi* occurring mostly on solanaceous plants (Migeon et al. 2011; Sarmento et al. 2011). Moreover, a recent *T. evansi* expansion has led to new distribution overlaps, mainly in Europe (Boubou et al. 2012). In the field, they are frequently found on the same plant (Ferragut et al. 2013). First-male sperm precedence has been found in *T. urticae*, although dependent on the mating interval (Boudreaux 1963; Helle 1967): a second mating occurring within 24 h after the first can still produce some offspring. Moreover, heterospecific matings have been observed between *T. urticae* and *T. evansi*, and indirect evidence suggests that reproductive interference may affect the outcome of competitive dynamics between these species (Sato et al. 2014).

Given the abovementioned characteristics of these spider mites, they represent an ideal system to address the physiological/behavioural effects of heterospecific matings in species with first-male precedence. We hypothesize that, due to first-male precedence, spider mites pay a high cost of mating with heterospecifics. Specifically, we expect that females that mate with heterospecifics first will (a) have reduced offspring resulting from arrested development of oocytes and that (b) they will be less receptive to subsequent matings and will hence lose future mating opportunities. As a consequence, we expect that (c) spider mites avoid mating with heterospecifics.

Material and methods

Maintenance of populations

Populations of both mite species used in this study were collected in Carregado, Portugal (Quinta do Outeiro). A laboratory population of *T. urticae* was established in May 2010 from 300 adult females collected from tomato plants (*Solanum lycopersicum*). The population of *T. evansi* was established from 300 adult females collected from *Physalis* spp. in May 2012.

Even though adult females from each species are easily identifiable, species identity was further 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 GenElute™ Mammalian Genomic DNA Miniprep Kit. We followed the manufacturer instructions, except for the elution volume, which we set to 20 μL RNase free water (Qiagen NV, Venlo, The Netherlands), in order to increase the concentration of DNA obtained from this very small animal (c.a. 300 μm long).

One hundred adult females from each species were screened for *Wolbachia* using the primers *wsp* (*Wolbachia*-specific primers) 81F and 691R (Braig et al. 1998). 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. Absence of *Wolbachia*

was then confirmed using the same protocol as above. This was done to avoid potential effects of *Wolbachia* on the behaviour of their host (Vala et al. 2004). Since then, both populations have been screened for *Wolbachia* on a regular basis, always with negative results.

Spider mite populations were maintained on trays with six to ten 3-week-old bean (*Phaseolus vulgaris*) or tomato plants at 25 °C and with a 16L : 8D photoperiod. Plant trays were changed every 2 weeks, placing old leaves on top of the new plants. Bean plants were planted every week and grown in a herbivore-free greenhouse, being watered two to three times a week.

The developmental period from egg to adult of both spider mite species used is around 10 days under optimal conditions. The offspring sex ratio is female biased and varies between 2:1 and 3:1 (Oku 2014; Sato et al. 2014). Mating occurs when the male slips under the female from behind and holds her legs with his front legs (Oku 2014). Both cases of 'male choice' (Oku et al. 2005) and 'female choice' (Tien et al. 2011) have been reported (though they may also be the outcome of female and male competition, respectively).

Experimental procedure

All experiments were performed in an acclimatized room at approximately 25 °C. Blinded methods were used to minimize inadvertent observer biases (cf. below).

1) Are there reproductive costs resulting from mating with heterospecifics?

   1a) Does mating with a heterospecific male affect offspring viability?

   When females encounter heterospecific males and accept to mate with them, they may or not produce viable offspring. Given that, in haplodiploids, fertilized eggs produce females and unfertilized eggs result in males, and the degree of hybridization was assessed through the offspring sex ratio of heterospecific matings. If no female descendants are produced (in the absence of successful hybridization), heterospecific matings can still result in the aborted development of heterospecifically fertilized eggs, thus compromising the fertility of females that mated with a heterospecific male. To test this, we compared the fecundity of 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 in the quiescent stage that precedes their last moult before reaching adulthood and kept in groups of approximately 15 females in leaf discs until emergence, to ensure their virginity. Adult males were collected from the same populations and kept isolated in leaf discs for at least 24 h before the assay, to ensure sperm replenishment (Krainacker and Carey 1990). Females were placed with either a conspecific or a heterospecific male and observed continuously until copulation occurred. Subsequently, females were isolated on a leaf disc (2 cm^2) and transferred to a new disc every 3 days until the female's death. The number of eggs was recorded before transferring the female to a new leaf disc, and eggs were left to develop until adulthood such that offspring sex ratio could be determined. The observers were unaware of the treatment they were assessing, as leaf discs were numbered and the association between number and treatment had been performed by other colleagues while setting up the experiments.

   1b) Does mating first with a heterospecific male modify the behaviour of virgins?

   When females encounter heterospecific males and accept to mate with them, a second important question is whether these females modify their physiological status or if they are still receptive to subsequent matings with conspecifics.

   First, as a control, we measured the latency to copulation and copulation duration of virgin females with conspecific males (treatment A; see Table 1). For that, a virgin female and a sperm-replenished male were placed on a leaf fragment (1 cm^2) and their behaviour was continuously observed until a mating event was completed. The time elapsed until a first mating occurred (latency to copulation), as well as the duration of copulation, were measured with a stopwatch. These experiments had the maximum duration of 2 h. If no mating occurred within this time, latency time was considered maximum, 7200 s. We did not remove these females from the analysis to avoid the assumption that they will never mate, which is not necessarily true. Removing these females would also give the wrong impression that the latency times were shorter than they really were, and that the females were accepting to mate with all kinds of males much more easily than they actually did. By considering a latency of 100 %, we avoid making these assumptions and bring all observations to the reference of the 2 h of the experiment (but see more details in the "Statistical analysis" section below and in the "Results" section). On the other hand, when a mating did occur, only those that lasted at least 1 min were included in the analysis. This restriction has a biological meaning, as matings with less than 1 min are not effective in species of the genus *Tetranychus*, as described by Boudreaux (1963).

   Second, to assess the effect of heterospecific matings in the subsequent mating behaviour of females, we

Table 1 Type of matings included in the experimental design for the no-choice tests, their use in the questions addressed (cf. "Material and methods"), number of replicates for each and number of unmated females at the end of the 2-h observation period (maximum latency)

Treatment	First male	Second male	Remating interval	Question	Replicate numbers (number of unmated females)			
					T. evansi		*T. urticae*	
					Lat	Cop	Lat	Cop
A	Con	–	–	1b) and 2b)	53 (3)	49	56 (5)	50
B	Het	–	–	2b)	31 (5)	25	34 (5)	28
C	Het	Con	0 h	1b)	30 (3)	23	24 (2)	22
D	Het	Con	24 h	1b)	47 (6)	39	40 (9)	31
E	Con	Con	0 h	2c)	40 (16)	21	40 (11)	28
F	Con	Con	24 h	2c)	61 (42)	19	45 (33)	12
G	Con	Het	0 h	2c)	14 (5)	8	15 (6)	9
H	Con	Het	24 h	2c)	30 (17)	13	30 (16)	13

In the treatments with two mating events, behaviour was observed in the second

Con conspecific, *Het* heterospecific, *Lat* latency duration, *Cop* copulation duration

measured their latency to copulation and copulation duration in a mating with a conspecific male that followed a heterospecific mating. The interval between matings was of either 0 or 24 h (treatments C and D; see more details in Table 1). The observers were unaware of the treatment they were assessing, as leaf discs were numbered and the association between number and treatment had been performed by other colleagues while setting up the experiments.

2) Do spider mites avoid mating with heterospecifics?

2a) Mating outcomes in the presence of both species

When females and males encounter, at the same time, conspecific and heterospecific sexual partners, they may or may not reject mating with heterospecifics. Discrimination among species is predicted if there are costs to female fertility and receptivity. To test this, we performed experiments with one focal individual from each species, placed with a conspecific and a heterospecific mate. These individuals were placed in leaf fragments of circa 1 cm^2 and observed continuously until copulation occurred. In the experiments that involved a female from one species and a male from each species, these were previously dusted with powder of different colours (randomized between replicates). This dust does not affect mating outcomes (Magalhães et al. 2009). In the experiments that involved one male from one species and a female from each species, the dusting of the females was not necessary because they are easily distinguishable visually. In all experiments, if copulation did not take place within 2 h, the observations were interrupted and such individuals were not included in the final sample size. Fifty replicates for each sex and species were performed. Blinded methods were used again, as males were dusted by a different author than that performing the experiments. For the male choice, and because females are distinguishable by the eye, the species of the male that performed the choice was unknown to the observer.

2b) Does mating behaviour differ according to species identity?

In those cases where females encounter heterospecific males only, the question is how different their mating behaviour will be, when compared with single encounters with conspecific males. If discrimination exists, female receptivity should be higher in the presence of conspecific males. To test this, a virgin female was placed on a leaf disc with either a conspecific or a heterospecific male, and latency to copulation and copulation duration were recorded (treatments A and B; see more details in Table 1).

2c) Does species identity affect behaviour in second matings that are preceded by conspecific matings?

When females mate first with conspecifics, the question is whether they are still receptive to subsequent matings with heterospecifics. To assess this, we performed double matings where a conspecific mating was followed by a heterospecific one (treatments G and H; Table 1) and conspecific double matings (treatments E and F; Table 1) as a control. In both cases, the mating interval was of either 0 or 24 h.

In both 2b) and 2c), the observers were unaware of the treatment they were assessing, as leaf discs were numbered and the association between number and treatment had been performed by other colleagues while setting up the experiments.

Statistical analysis

All analyses were carried out using R (version 2.15.3, R Development Core Team 2013). Differences among the fecundity of virgin females and females mated conspecifically and heterospecifically (treatments A and B) were analyzed using linear models within each species, with the type of mating (conspecific or heterospecific) as a fixed factor (question 1a, cf. Table 1).

We present the data comparing the behaviour of heterospecifically mated females to that of virgins separately from that of the other no-choice tests, because it answers a different question (question 1b). However, we analyzed this data together with all no-choice tests in a single analysis, to minimize the familywise error rate. Hence, we will here present the analysis of 1b, 2b and 2c together.

Since the time interval was shown to affect the degree of sperm precedence (Helle 1967; Satoh et al. 2001), we compared C vs D, E vs F and G vs H to test if behaviour in double matings differed according to the mating interval. When this was the case, the subsequent comparisons were done among crosses with the same interval. When no effect of the mating interval was detected for any mating sequence involved in subsequent comparisons, we grouped the observations involving different mating intervals for each mating sequence. We then used linear models with the type of mating as a fixed factor. If the factor was significant, this analysis was followed by planned contrasts corrected for multiple comparisons using the sequential Bonferroni correction. Question 1b was addressed by comparing treatments A vs C + D. A comparison between A vs B tested differences in behaviour between single conspecific and heterospecific matings (question 2b). Finally, we compared double conspecific matings with double matings with a heterospecific as the second mating (question 2c). The compared treatments were E vs G and F vs H for the latency in both species, and E + F vs G + H for copulation duration. For latency to copulation and to investigate the effect of the inclusion of data with 100 % latency (when matings did not occur after the 2-h period of observation), we performed (a) the same analysis as described above in a data set without these data and (b) a survival regression analysis, in which data referring to 100 % latency are coded as censored. Censored observations provide an information on the status of the male and female (i.e. not mated) to the analysis between time 0 and the end of the observation period but they do not provide any information to the analysis beyond that moment (see Supplementary material). Mating outcome in the presence of both species was analyzed with chi-square tests (question 2a).

Results

1a) Do heterospecific matings affect offspring viability?

Heterospecific crosses resulted in 98 and 100 % male offspring for crosses involving *T. urticae* and *T. evansi* females, respectively. Hybrid production between these species is thus negligible (and the few hybrid females produced eggs that did not hatch). Moreover, the fecundity (i.e. male offspring) of females that mated heterospecifically was not significantly different from that of virgin females or from that of females mated with a conspecific male, (Fig. 1; $F_{2,57} = 1.249$, $p = 0.294$ for *T. urticae* and $F_{2,73} = 0.238$, $p = 0.789$, for *T. evansi*). Therefore, mating with heterospecifics does not result in the aborted fertilization of oocytes.

1b) Does mating with a heterospecific male modify the behaviour of virgins?

In the general model, we found a significant effect of the mating sequence in the latency to copulation in both species (*T. urticae*, $F_{7,\ 276} = 14.55$, $p < 0.0001$; *T. evansi*, $F_{7,\ 298} = 19.31$, $p < 0.0001$). For *T. urticae* females, copulation duration was also affected by the mating sequence ($F_{7,185} = 6.453$, $p < 0.0001$), whereas no effect was found for *T. evansi* females ($F_{7,189} = 1.76$, $p = 0.098$). Hence, for *T. evansi*, we did not perform planned comparisons for the latter trait.

The mating interval did not significantly affect latency to copulation with conspecific males following heterospecific matings (C vs D: $|t| = 0.547$, $p = 0.584$ for *T. urticae* and $|t| = 0.174$, $p = 0.862$ for *T. evansi*; Fig. 2a, c). Latency to copulation of the second conspecific mating was significantly lower when it occurred immediately after the first mating than 24 h later, for both species (E vs F, $|t| = 5.41$, $p < 0.0001$ for *T. urticae* and $|t| = 3.32$, $p = 0.001$ for *T. evansi*; Fig. 4a, c). For both species, latency to copulation with heterospecific males following conspecific matings did not differ significantly

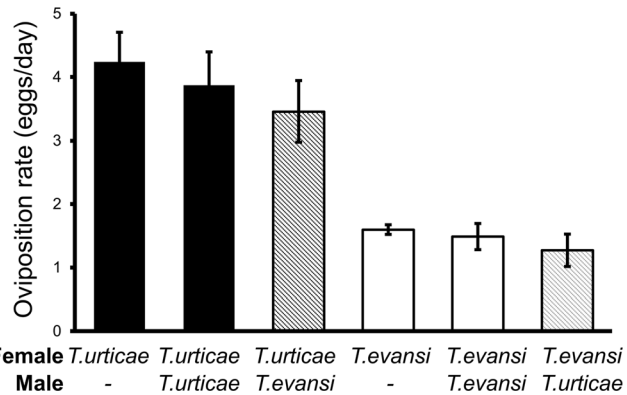

Fig. 1 Average daily fecundity of virgin, single conspecific and single heterospecifically mated *T. urticae* (*dark bars*) and *T. evansi* (*light bars*) females. *Full bars* correspond to conspecific matings and *striped bars* to heterospecific matings. *Error bars* represent the standard error of the mean

according to the mating interval (G vs H, $|t|=0.841$, $p=0.401$ for *T. urticae* and $|t|=2.161$, $p=0.0315$, $\alpha_c=0.0083$ for *T. evansi*; Fig. 4a, c). Therefore, the mating interval affected latency in the second conspecific mating, but not in mating sequences involving heterospecific matings.

In all cases, the mating interval did not significantly affect copulation duration in *T. urticae* (second conspecific crosses, $|t|=0.038$, $p=0.970$; heterospecific crosses following conspecific ones, $|t|=1.458$, $p=0.147$; conspecific crosses following heterospecific ones, $|t|=0.728$, $p=0.467$; cf. general model for *T. evansi*; Figs. 2b, d and 4b, d).

In both species, the latency of conspecific matings following a heterospecific one was similar to that of single conspecific matings (A vs C + D, $|t|=1.49$, $p=0.137$ for *T. urticae* and $|t|=0.232$, $p=0.817$ for *T. evansi*; Fig. 2a, c). Similarly, there was no difference in the copulation duration of both species under the same conditions ($|t|=1.548$, $p=0.123$ for *T. urticae*; cf. general model for *T. evansi*; Fig. 2b, d). Therefore, females that mate with a heterospecific male behave as virgins in subsequent matings.

Overall, the additional analysis performed regarding latency to copulation did not yield different results from the one presented here, and are thus presented in the supplementary material (Table S1).

2a) Mating outcomes in the presence of both species

Four out of 54 *T. urticae* females failed to mate with any male after 2 h of observation; all of the 50 *T. evansi* females mated in this period. Females of *T. urticae* mated more often with conspecific than with heterospecific males ($\chi^2_1=3.92$, $p=0.048$), whereas *T. evansi* females showed no preference ($\chi^2_1=1.285$, $p=0.258$; Table 2). Seven out of 57 *T. urticae* males and 12 out of 62 *T. evansi* males did not mate after 2 h. While *T. urticae* males did not discriminate between females of the two species ($\chi^2_1=2$, $p=0.157$), males of *T. evansi* mated assortatively ($\chi^2_1=3.92$, $p=0.048$; Table 2).

2b) Does mating behaviour differ according to species identity?

Latency to copulation did not differ between single conspecific and heterospecific matings (A vs B, $|t|=0.693$, $p=0.489$ and $|t|=0.694$, $p=0.488$, for *T. urticae* and *T. evansi*, respectively; Fig. 3a). The duration of copulation was significantly longer in *T. urticae* conspecific matings than in heterospecific ones ($|t|=4.217$, $p<0.0001$; cf. general model for *T. evansi*; Fig. 3b). Therefore, mating behaviour of virgins toward conspecifics differs from that toward heterospecifics in *T. urticae*, but not in *T. evansi*.

2c) Does species identity affect behaviour in second matings that are preceded by conspecific matings?

In both time intervals, latencies of *T. urticae* conspecific and heterospecific second matings did not differ significantly (E vs G, $|t|=1.13$, $p=0.26$; F vs H, $|t|=2.409$, $p=0.017$, $\alpha_c=0.0083$; for the 0- and 24-h intervals, respectively; Fig. 4a). Similarly, the latency to copulation of *T. evansi* heterospecific matings following conspecific ones did not differ significantly from that of conspecific double matings, for both mating intervals (E vs G, $|t|=0.318$, $p=0.751$ and F vs H, $|t|=0.342$, $p=0.732$ for the 0- and 24-h intervals, respectively; Fig. 4c).

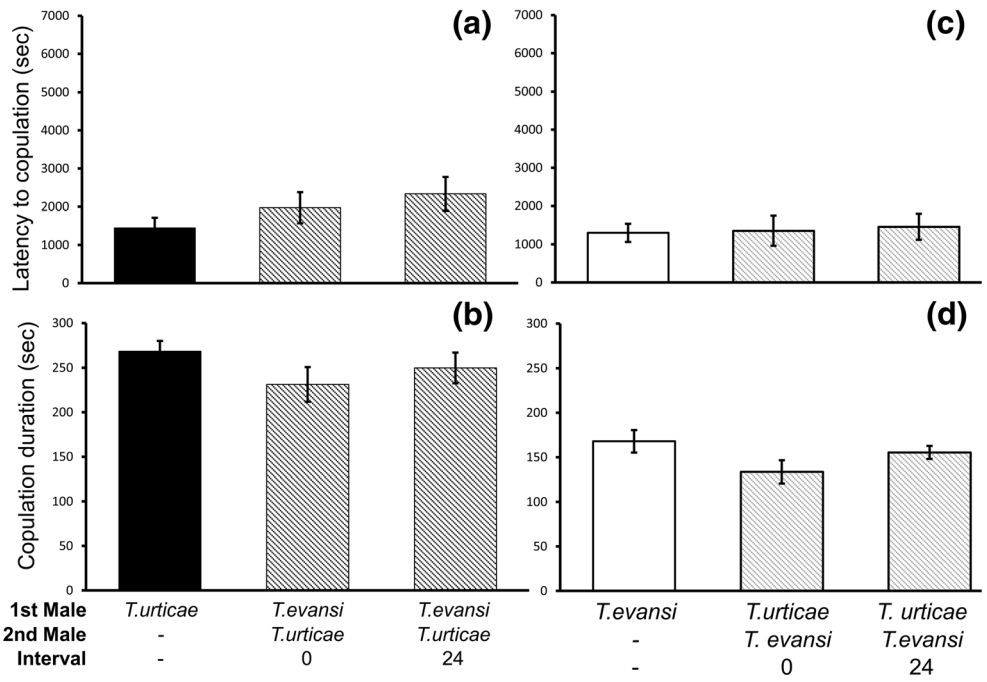

Fig. 2 Latency to copulation (**a**, **c**) and copulation duration (**b**, **d**) of single conspecific and double heterospecific matings of *T. urticae* (**a**, **b**) and *T. evansi* females (**c**, **d**). *Full bars* correspond to conspecific matings and *striped bars* to treatments in which the first mating was heterospecific and observations were made on the second, conspecific, mating. *Error bars* represent the standard error of the mean

Table 2 Mate choice experiments

Chosen mate	Female choice		Male choice	
	T. urticae	*T. evansi*	*T. urticae*	*T. evansi*
Conspecific	32	29	30	32
Heterospecific	18	21	20	18

Number of conspecific and heterospecific mates chosen by individuals after having been introduced in a leaf arena with one *T. urticae* and one *T. evansi* individuals of the opposite sex

No differences were found in copulation duration between conspecific and heterospecific matings that follow conspecific ones (*T. urticae*, EF vs GH $|t| = 1.271$, $p = 0.205$; cf. general model for *T. evansi*; Fig. 4b, d). Therefore, overall, species identity did not affect the mating behaviour of females that have previously mated with a conspecific.

Discussion

We investigated the occurrence of species recognition and its consequences for fertilization and mating behaviour in two spider mite species, with first-male sperm precedence. We found that heterospecific matings resulted in very few female offspring, confirming earlier results in this system (Sato et al. 2014). In spider mites, the production of females seems to be more costly than that of males (Macke et al. 2012). Hence, the production of male-only offspring removes the cost of producing potentially inviable females. Furthermore, since virgins and heterospecifically mated females produce the same number of unfertilized eggs (i.e. males), it seems unlikely that heterospecific sperm fertilizes oocytes, resulting in their arrested development. Therefore, our findings indicate that heterospecific crosses result in weak, if not absent, costs in terms of egg fertilization. Moreover, females that have mated with a heterospecific male remain as receptive to conspecific males as virgins (i.e. there were no significant differences in latency and copulation duration between these females). Hence, costs in terms of lowering the likelihood of future matings with conspecific sexual partners are also absent.

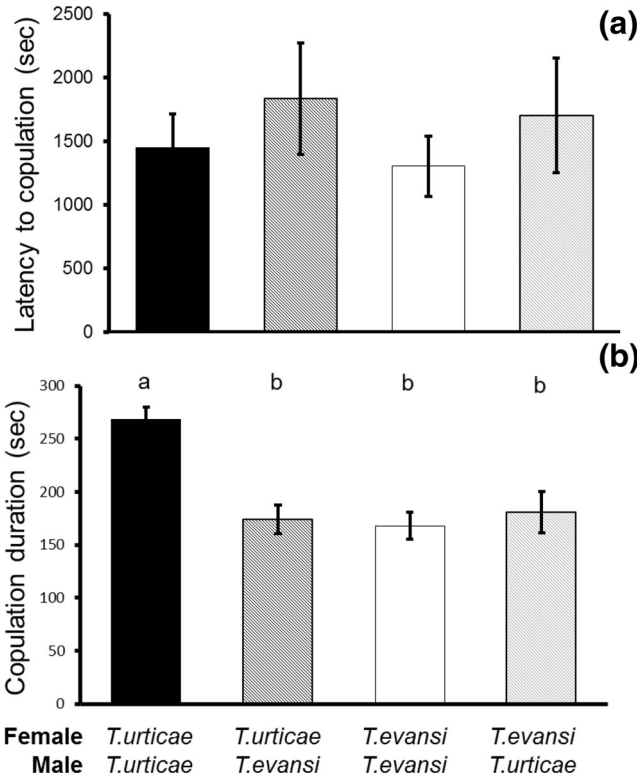

Fig. 3 Latency to copulation (**a**) and copulation duration (**b**) of single conspecific and single heterospecific matings of *T. urticae* (*dark bars*) and *T. evansi* females (*light bars*). *Full bars* correspond to conspecific matings and *striped bars* to heterospecific matings. *Error bars* represent the standard error of the mean

Given these reduced costs, we predict weak discrimination among these spider mite species. Indeed, mate-choice tests revealed that *T. urticae* males and *T. evansi* females did not discriminate between conspecific and heterospecific mates. Moreover, even *T. evansi* males and *T. urticae* females, which did mate assortatively, often chose to mate with heterospecifics. It must be noted that the experiments performed here (as most experiments on mate choice) do not allow discrimination between the effects of female preference and male–male competition on female mate choices (Wagner 1998). However, this does not affect the conclusion that heterospecific matings are likely to be common whenever populations of these two species co-occur. Furthermore, latency to copulation was similar for conspecific and heterospecific single matings in both species. *T. urticae* conspecific single matings, however, lasted longer than heterospecific single matings, which, in agreement with the mate choice results, points to some degree of discrimination in *T. urticae* females. These results do not fully reproduce the observation that both *T. urticae* and *T. evansi* males prefer *T. urticae* females (Sato et al. 2014). This difference can be due to variations in the protocols used or reflect variability among populations for this trait. In any case, the weak discrimination observed could result from the fact that the two species were only recently in contact, as the populations used were collected in areas where *T. evansi* has only recently invaded (Boubou et al. 2012) Possibly, this weak discrimination is due to a lack of specificity of chemical compounds that act as sexual attractants in each species, as shown to occur in arachnids (Gasket 2007), or to an inability of mates to distinguish between these attractants. It should be noted, however, that spider mites do perceive intraspecific differences and act accordingly. For example, *T. urticae* males show a clear preference for virgin over mated females (Oku 2010, 2014).

Despite the fact that virgins are equally receptive to males of both species, their remating behaviour may still be affected

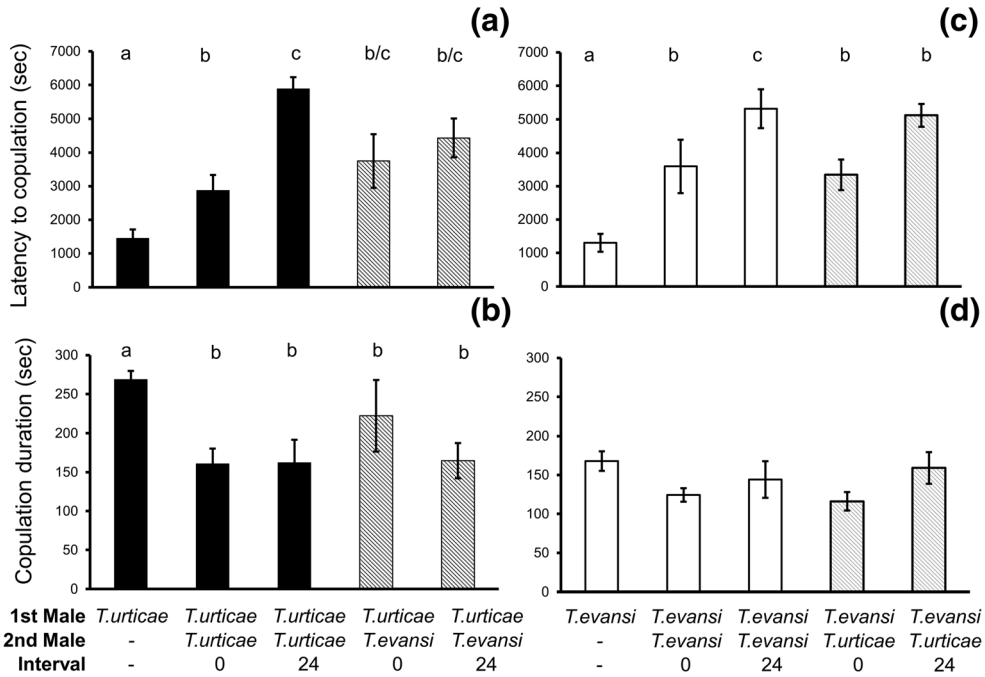

Fig. 4 Latency to copulation (**a**, **c**) and copulation duration (**b**, **d**) of single conspecific, double conspecific and double heterospecific matings of *T. urticae* (**a**, **b**) and *T. evansi* females (**c**, **d**). In treatments involving two males, *bars* represent matings by the second male. *Full bars* correspond to conspecific matings and *striped bars* to heterospecific matings. *Error bars* represent the standard error of the mean

if females modify their physiological status after a first mating. Given the occurrence of first-male precedence in *T. urticae*, once mated, females are expected to decrease their receptivity to males, and this may even be exacerbated if such males are heterospecific. Indeed, in both species, when females mate with a heterospecific male following a conspecific mating, latency to copulation in the second mating is higher than that in matings involving virgins. Furthermore, copulation duration is lower in the second conspecific mating than in the first, in *T. urticae*. These results are congruent with the occurrence of first-male precedence in *T. urticae* (Helle 1967) and lend support to the hypothesis that this is also the case in *T. evansi*, as suggested by Sato et al. (2014).

Moreover, when females mate with a heterospecific male following a conspecific mating, the behavioural traits observed are not different from those of females that mate twice with conspecific males. Hence, experience with conspecifics does not affect mate discrimination between conspecifics and heterospecifics. Furthermore, when the first mating is heterospecific, latency and copulation duration of second matings are similar to those of single conspecific matings. This finding is in agreement with studies on wolf spiders (Rutledge and Uetz 2014) and sticklebacks (Kozak et al. 2013), in which previous experience with either con- or heterospecific males did not modify female receptivity. These results suggest the existence of a cue informing about the success/failure of the first mating and that the first mating with a heterospecific might be perceived as an unsuccessful one. This putative cue may also underlie differences in the aerial dispersal of *T. urticae* females, according to their mating history. Indeed, this behaviour has been shown to occur with the highest frequency in conspecifically mated females, lowest in virgin females and with intermediary values in females that mated with a heterospecific male (Collins and Margolies 1991).

Together, these results indicate that heterospecific matings fail to trigger changes in behaviour that are normally induced by conspecific matings. Possibly, heterospecific males do not transfer sperm, or this sperm does not reach the oocytes. In any case, this may result in a lower selection pressure for traits involved in species recognition.

In more ecologically realistic environments, a cost of misrecognition may arise. In fact, Macke et al. (2012) showed that, in *T. urticae*, females that mate multiple times had a lower total reproductive investment. Possibly, mating with several heterospecific males may also entail some cost. In line with this possibility, Sato et al. (2014) found that virgin females placed on plants with conspecific and heterospecific males had lower fecundity than females that had mated conspecifically before being placed on such plants. This suggests that (a) mated females, being less receptive, mated less often with heterospecific males than virgins, and that (b) repeatedly mating with heterospecific males entails a cost. Hence, the question remains as to why discrimination has not evolved. It could be that these species rarely encounter each other in the field. However, field data show that this is not the case (Ferragut et al. 2013). Still, given that invasion of Europe by *T. evansi* is recent (Boubou et al. 2012), it may be that contact has not occurred for sufficient time for adaptation to the presence of the competitor to occur. Once both species meet in the field, severe costs resulting from the low species discrimination level in this system should only be expected when a virgin female arrives to a patch where conspecific

density is very low, where she will mate indiscriminately with heterospecifics, as in Sato et al. (2014). This situation, however, is probably not very common in nature, as in these species, female dispersal occurs mainly after a mating event (Mitchell 1970; Collins and Margolies 1991).

In sum, our results indicate that an accurate assessment of the potential costs of mating with heterospecifics necessitates knowledge on the intraspecific mating system, namely sperm precedence. This information, corroborated with knowledge on the ecology and evolutionary history of the spider mites species studied here, allowed us to conclude that mating with heterospecifics is expected to result in low, if any, costs. Therefore, the lack of discrimination we also find here is not surprising. Whether costs will be low in other spider mite species is unknown, but one could speculate that they may be higher in species that are more closely related to each other and hence produce hybrids. Indeed, many examples of cryptic speciation exist in mites, among which isolation is not complete (Skoracka et al. 2015). In such cases, mating with a heterospecific may modify the future receptivity of females, especially in the case of first-male precedence. Moreover, being haplodiploid allows addressing the costs of heterospecific matings even in the absence of fertilization, given the production of haploid males from unfertilized eggs. Spider mites can, thus, be seen as good models to study the evolution of reproductive isolation, regarding both pre- and post-mating barriers.

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Author contributions SM conceived the study; SM and SAMV designed the experiments; SHC, LRR and RP collected the data; SHC and SAMV analyzed the data; and SHC, SAMV and SM wrote the manuscript, with significant input from all authors.

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