

Male spider mites use chemical cues, but not the female mating interval, to choose between mates

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Abstract The choice of the partner an individual will mate with is expected to strongly impact its fitness. Hence, natural selection has favoured the evolution of cues to distinguish among mates that will provide different fitness benefits to the individual that is choosing. In species with first-male sperm precedence, this is particularly important for males, as mating with mated females will result in no offspring. In the spider mite *Tetranychus urticae* only the first mating is effective, except if the interval between first and second copulations is shorter than 24 h. In line with this, males prefer to mate with virgin over mated females. They do not, however, choose between females that have mated at different time intervals. Here, we tested which type of cues males use to distinguish between females with different mating status (virgin versus mated). To do so, we firstly confirmed that males prefer virgins over mated females and that they do not select females on the basis of their age or mating interval. Next, we tested whether contact and volatile compounds or chemical trails affected male discrimination between mated and virgin females, by systematically varying the exposure of males to these cues. We found that volatile compounds and chemical trails were sufficient to induce discrimination between virgin and mated females in males. Direct contact with females, however, does not seem to play a role in this discrimination. The composition of such chemical cues (trails and volatiles) remains to be identified.

Keywords First-male sperm precedence · Mating interval · Mating behaviour · Chemical cues · *Tetranychus urticae*

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Introduction

Patterns of sperm precedence range from last-male to first-male sperm precedence (Danielsson 1998). Expected mating behaviours depend on these patterns. In species with last-male precedence, selection in males should favour increased mating frequency as a result of adaptation to sperm competition (Ridley 1989a). In species with first-male precedence, however, males do not benefit from mating with females that have already mated. Moreover, females cannot choose to use the sperm of subsequent males, which should favour monandry. Hence, both sexes are expected to invest all resources in matings that involve virgin females only, except if the pattern of first-male precedence is incomplete and some copulations with mated females are effective. In males, first-male precedence should also favour the ability to distinguish virgin from mated females, as well as pre-copulatory mate guarding, which sometimes extends to immature females (Ridley 1989a, b).

The existence of cues that enable a male to discriminate females with different mating status depends on the balance between costs and benefits conferred by this discrimination. In monogamous species, the benefits are clearly very high and thus such discrimination is likely to evolve. Indeed, several studies show that males from monogamous species prefer virgin over mated females (Thomas 2011). Moreover, even in species with multiple mating, males can evolve the ability to discriminate female mating status, depending on the species mating rate and sperm precedence patterns (Engqvist and Reinhold 2006). In particular, mated females of species with first-male sperm precedence, as well as of some species with mixed sperm precedence, provide low fertilization opportunities to males, if any. Accordingly, males frequently evolve the ability to discriminate female mating status, preferring virgins (e.g. Stoltz et al. 2007; Yasui 1994).

To distinguish females of different mating status, males can use several types of cues. In arthropods, these cues are usually chemical (Johansson and Jones 2007; Thomas 2011) and can be perceived by contact (e.g., cuticular hydrocarbons, CHCs), left in the substrate (i.e., chemical trails) or released into the environment (i.e., volatile compounds). For example, *Drosophila melanogaster* uses CHC's to distinguish mated from virgin females (Friberg 2006), whereas males of *Agelenopsis aperta*, a monogamous spider, use volatiles to identify virgin females (Riechert and Singer 1995). In the mealworm beetle (*Tenebrio molitor*), males prefer virgin over mated females when only chemical trails are present in the arena (Carazo et al. 2004), indicating that such cues are sufficient for male discrimination. Identifying the type of cue used in mate choice adds to our understanding of mating behaviour, as it allows determining the ecological conditions in which they are perceived. For example, the minimal distance necessary for two individuals of the opposite sex to perceive each other's mating status will vary depending on which cues are used. This in turn may have consequences for species conservation and management.

In spider mites, the first male fertilizes most of the female's eggs (in *Tetranychus urticae* Koch, Helle 1967; in *Tetranychus kanzawai* Kishida, Oku 2008), meaning that this species follows a first-male sperm precedence pattern. Consequently, it is expected that males only attempt to mate with virgin females to avoid unnecessary costs, such as loss of other mating opportunities or reduced survival. This requires that males have access to cues allowing the perception of the female mating status. Indeed, males actively guard quiescent females and mating occurs as soon as females emerge as virgin adults (Potter et al. 1976). In addition, both prolonged copulation and post-copulatory guarding behaviours are observed in these species, which ensures first-male sperm precedence (Satoh et al. 2001). Moreover, when males outnumber virgin females, they actively fight for their possession (Potter et al. 1976). In accordance

with these observations, recent studies showed that males prefer to mate with virgin rather than mated females (Oku 2010). In addition, females pay a cost of multiple matings, having lower fecundity and a smaller proportion of fertilized eggs, when compared with once-mated females (Macke et al. 2012). Together, these results lead to the expectation that in *T. urticae* both sexes should invest all resources in the first mating event. However, surprisingly, copulations between males and mated females have been observed (Oku 2010; Clemente et al. 2016).

This puzzling behaviour could be adaptive if first-male sperm precedence in *T. urticae* is incomplete. In fact, the mating interval has been shown to affect the extent of first-male sperm precedence in this species, as found in other invertebrates (Danielsson 1998). In spider mites, if the interval between first and second copulations is shorter than 24 h, the second male can still sire some offspring (Helle 1967). However, a recent study has shown that, in an arena with females that have mated 6 or 30 h before the test, males first touch each female type indiscriminately (Oku 2013). Several possibilities may explain this result: (a) the female that is touched first does not fully represent mate choice, (b) the result is specific to the population/test details used, or (c) the result is a general feature of *T. urticae* populations, which seems to be maladaptive.

In this study, we set out to test which type of cues males use in their mate choices. We tested the role of tactile stimuli (contact compounds), volatile compounds and the presence of chemical trails, the three main chemical cues involved in female spider mite attraction (Penman and Cone 1974). To this aim, we first confirmed that males do distinguish virgin from mated females. Moreover, to limit the choices under test to these two types of females, we also verified that males do not distinguish between females mated at different time intervals in a population other than that used in Oku (2013).

Materials and methods

Spider mite populations and rearing conditions

Spider mites (*T. urticae*) were reared in large numbers (>2000) on whole cucumber plants [varieties *Ventura*, provided by Rijkzwaan, France], for experiment (a); and on whole bean plants [*Enana*, provided by Germisem, Portugal], for experiment (b); see details of the experiments below, under controlled conditions (25 °C; photoperiod of 16L:8D). The population of spider mites used for experiment (a) was originally collected from a cucumber greenhouse in Pijnacker, the Netherlands, in May 1994, and kept in the same rearing conditions at the University of Amsterdam (the Netherlands). This population was then established at the University of Montpellier (France) in 2007 from approximately 5000 individuals sampled from the Amsterdam stock. The population used for experiment (b) was originally collected from tomato plants in Carregado, Portugal, in August 2010 and has since then been kept on bean plants at the University of Lisbon (Portugal). Both experiments were done under controlled conditions (25 °C; photoperiod of 16L:8D).

Experimental setup

Male discrimination ability: female status, age and mating interval

To address male discrimination between females with different mating status, males were given the choice between virgin and mated females, the latter being mated more or less

than 24 h before the test. This interval has been shown to influence the extent of first-male precedence in *T. urticae* (Helle 1967). Moreover, male mating preferences in several species have been correlated with female age, with older females being less attractive than younger ones (Johansson and Jones 2007; Delisle and Royer 1994). In order to account for these differences, we controlled the age of the females tested, in addition to their mating interval.

In total, 300 quiescent females were randomly allocated to different conditions of age (“Y”, young or “O”, old, i.e., 2 days older), mating status (“V”, virgin or “M”, mated) and mating interval [“12” or “60”, with first mating having occurred 12 (± 1) or 60 (± 1) h before the test, respectively]. The combination of conditions resulted in five types of females, corresponding to five treatments (Fig. 1). To establish such treatments, males and quiescent females were selected from the base population. Males and females were separately placed in groups of 10 on 2 cm² leaf squares on water-saturated cotton. Twenty-four hours later (day 1), adult virgin females, 1 day old since the last moult, were placed on 0.8 cm² leaf discs. One third of the leaf discs received one male (for the OM60 treatment), whereas in the other two thirds, females were left alone (OV and future OM12 treatments). New males and quiescent females were selected from the base population on day 2 and, on day 3, the females were either placed with males (YM12 treatment) or left alone (YV treatment). In addition, half the females kept as virgins in day 1 were allowed to mate on day 3, by adding one male to each leaf (OM12 treatment). Finally, on day 4, 12 h after the establishment of the last three types of females, the preference of males was tested. Two females, each from a different treatment, were placed on a 0.8 cm² leaf square and randomly painted using pigment ink—blue or red—to allow for their discrimination by the human observer. These females were left alone for 10 min, after which one male was placed on the leaf disc and the first female the male touched and mated with was recorded as his mating choice. Simultaneously, time until the beginning of copulation (latency to copulation) and copulation duration were measured. If after 30 min the male had not touched any of the females, the group was discarded.

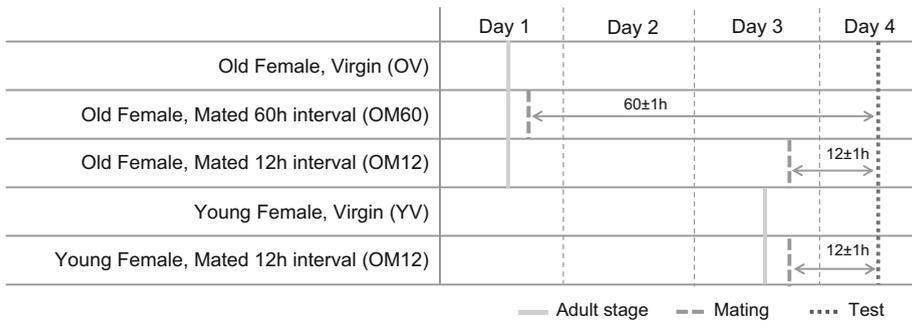


Fig. 1 Experimental procedure to test *Tetranychus urticae* male choice. To test whether males prefer females having mated at different intervals, females were randomly allocated to different conditions of age, mating status and mating interval. After emergence (solid line), virgin females were left alone on day 1 and tested (dotted line) on day 4 (OV); or they were placed with males (dashed line) on day 1 and tested with new males (dotted line) on day 4 (OM60); or they were left alone, placed with males (dashed line) on day 3 and tested with new males (dotted line) on day 4 (OM12). On day 3, after emergence (solid line), new virgin females were left alone and tested (dotted line) on day 4 (YV); or they were placed with males (dashed line) and tested with new males (dotted line) on day 4 (YM12). Hence, the preference of males for each female type was tested always on day 4 (dotted line), but females had different ages and mating histories

In total, 6 preference tests were done (OM60 × OM12, OM12 × YM12, OV × OM60, OV × OM12, OV × YV and YV × YM12), with 50, 51, 48, 51, 50 and 53 replicates (i.e., focal males), respectively. All males used throughout the experiment were isolated—kept without females—for more than 24 h before being used, so that sperm replenishment was guaranteed and mating was more likely to occur.

Type of chemical cues used in male discrimination

To disentangle the role of tactile stimuli (contact compounds), volatile compounds and the presence of chemical trails in male discrimination ability, male choice between virgin and mated females was tested while varying the type of cues available to males (Fig. 2). To this aim, quiescent females were collected from a subset of the base population (approximately 500 individuals) and they were placed in a patch of approximately 10 cm²—isolation patch—, either with an average of two males per female—mated females—, or left alone, remaining virgins. The next day, males were removed. Two thirds of the females were isolated on the leaf discs used for the test the next day (treatments “alive” and “absent”). One half of those females was removed from the patch immediately before the test, so that only chemical trails would remain (treatment “absent”), whereas the females of the other half were left intact (treatments “alive”). In addition, on the day of the test, the females remaining on the isolation patches were frozen at −4 °C for 1–2 h prior to the beginning of the tests (treatments “dead”). These females were then added to empty leaf discs. Immediately before adding the focal male, a bridge (3 × 0.5 cm) was added, either connecting (“contact”) or not (“no contact”) the two leaf discs (0.64 cm²). This setup, made entirely of bean leaf, was used to perform the choice experiment (Fig. 2).

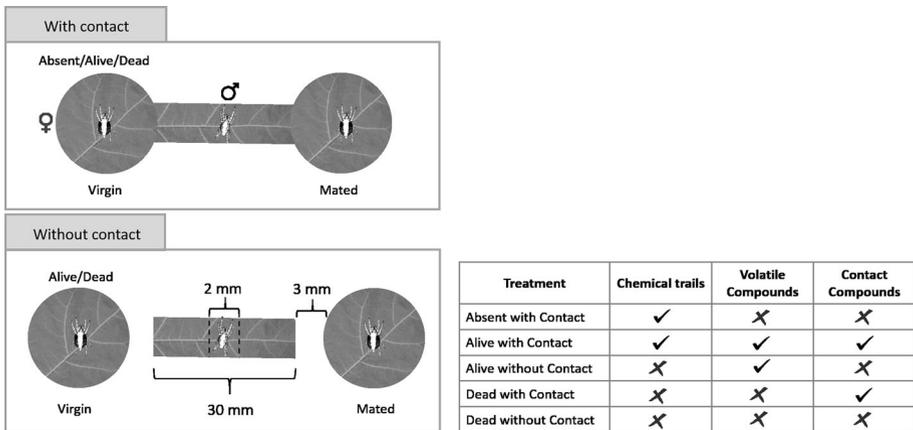


Fig. 2 Experimental setup to address cues used by *Tetranychus urticae* males to distinguish virgin from mated females. To test which type of chemical cues males use to discriminate among females with different mating status, male choice between virgin and mated females was tested under various combinations of type of arena (contact/no-contact) and female condition (alive, absent, dead). A dumbbell-shaped arena, consisting of two bean leaf discs of 0.64 cm² connected to each other by a bridge (3 × 0.5 cm), was used to perform the choice experiment. In treatment “alive with contact”, males had access to all types of cues, and in treatment “dead without contact” no cue could be perceived. In treatments “alive without contact”, “absent with contact” and “dead with contact”, the focal male could only perceive volatile compounds, chemical trails or direct contact cues, respectively

Combinations of type of arena (contact/no-contact) and female condition (alive, absent, dead) were chosen in order to allow the focal males to perceive only one, all or none of the three types of cues. Treatments “alive with contact”, in which males had access to all types of cues, and “dead without contact”, in which no cue could be perceived, were used as positive and negative controls. In treatments “alive without contact”, “absent with contact” and “dead with contact”, the focal male could only perceive volatile compounds, chemical trails or direct contact, respectively (Fig. 2). In every test, a virgin and a mated female from the same treatment were placed in opposite sides of the arena.

Focal males were collected at the quiescent stage and kept isolated for 1 day, in order to ensure their sperm was not depleted. The tests began with the placement of the focal male on a neutral zone (a 2 mm wide strip in the centre of the bridge). Each test lasted for 1 h and total time spent by a male on either side of the neutral zone was recorded. A test was considered invalid if a male drowned or a female left her leaf disc. In total, 55 replicates of “alive with contact” and “alive without contact” and 54 replicates of “absent with contact”, “dead with contact” and “dead without contact”, were analysed. We only performed this experiment for virgin vs mated females because in experiment (a) we found no difference in preference for recently-mated versus earlier-mated females (cf. Results).

To enhance the amount of volatile compounds, and of chemical trails in the females’ leaf discs, live females were placed in the discs 24 h before the tests. Dead females, however, were placed on discs immediately before the beginning of the tests as, in that case, we wanted to minimize the loss of contact cues—due to tissue decomposition—following the females’ death. As such differences in the time females were placed on a patch could influence male behaviour, we first tested if male preference for live virgin over live mated females did not differ when females were placed in the leaf discs 1 h before the test or 24 h earlier, in a setup without contact.

Statistical analyses

Male discrimination ability: female status, age and mating interval

All statistical analyses were performed with the software R (version 3.0.3, R Development Core Team 2014). In order to test if touch and copulation were correlated, we did a Fisher exact test for each preference test. A strong correlation was found between first female touched and mated in every preference test (cf. Results). We thus conducted two different analyses of male choice: we considered that a male chose a female (a) when the female was touched and then mated, or (b) when the female was touched. As the results of the two analyses were similar, we only present the results of (a).

To test for a correlation between male choice and female pigment ink colour we used a Pearson’s Chi squared test. This test was done to determine if there was an overall effect of colour on male choice behaviour. Since no correlation was found ($\chi^2 = 2.08$, $df = 4$, $P = 0.72$), colour was not included in the subsequent statistical models. We then tested male choice within each preference test, again using a Pearson’s Chi squared test.

To test for differences in mating behaviour between treatments (i.e., types of females), we used latency and duration of copulation as the response variables. To approximate both models to the assumptions of normality, we transformed the response variables using a boxcox transformation (package MASS, boxcox procedure). Treatment (of the chosen female), which was our variable of interest, was included as a fixed factor, and day and preference test as random factors. However, since none of the random factors was significant in both analyses [package lmerTest, rand() procedure], they were removed from

the final models. We analysed both latency to copulation and copulation duration using a general linear model (lmer, lme4 package), with a gaussian distribution. A multiple comparison of means using a Tukey Contrast (multcomp package, glht procedure), test was done to compare duration and latency to copulation among female types.

Type of chemical cues used in male discrimination

To determine if the time spent by females on the leaf discs could influence male attraction, we used a generalized linear model with a quasibinomial error distribution (glm), as the response variable—the proportion of time spent with virgin females—was overdispersed. Time spent by females in the leaf discs—24 or 1 h—and day were used as fixed factors. No differences were found between 24 and 1 h in time spent with virgin females ($F_{1,54} = 0.0012$, $P = 0.97$).

A Wilcoxon signed rank test with continuity correction was used to discard preference for one side of the arena—left or right, and no preference was found ($V = 17,169.5$, $P = 0.33$). The same test was used to analyse our variable of interest—the proportion of time spent by the males with a virgin female. Each treatment was analysed separately and compared to a proportion of 0.5, i.e., to a situation with no preference.

Results

Male discrimination ability: female status, age and mating interval

A significant correlation was found between first female touched and first female mated in every preference test (OV \times YV: $\chi^2 = 21.5899$, $P = 4.59e-07$; YV \times YM12: $\chi^2 = 17.249$, $P = 5.99e-05$; OV \times OM60: $\chi^2 = 14.1959$, $P = 0.0005$; OV \times OM12: $\chi^2 = 13.5807$, $P = 0.0003$; YV \times YM12: $\chi^2 = 17.249$, $P = 5.99e-05$; OM12 \times OM60: $\chi^2 = 17.8969$, $P = 5.75e-06$; all $df = 1$). Every time a preference test was composed of females that were either both virgin or both mated, males showed no preference, irrespective of the female age or mating interval (OV \times YV: $\chi^2 = 2$, $P = 0.16$; OM60 \times OM12: $\chi^2 = 0.08$, $P = 0.78$; OM12 \times YM12: $\chi^2 = 0.49$, $P = 0.48$; all $df = 1$; Fig. 3). When males were offered the choice between a virgin and a mated female, they always preferred virgins, again, irrespective of the female age or mating interval (OV \times OM60: $\chi^2 = 18.75$; OV \times OM12: $\chi^2 = 18.843$; YV \times YM12: $\chi^2 = 23.113$; all $df = 1$, $P < 0.001$; Fig. 3).

Both the time until copulation (i.e., latency to copulation) and the time males spent copulating with a female (i.e., copulation duration) were significantly different between treatments (i.e., types of females) ($F_{4,296} = 5.37$, $P < 0.001$ and $F_{4,292} = 24.3$, $P < 0.001$, respectively). Pairwise comparisons (Tukey Contrasts) revealed that treatments can be grouped into two groups: virgin females (OV and YV) and mated females (OM60, OM12 and YM12) (Table 1; Fig. 4). The two groups do not overlap in time of copulation but differences between OV and YM12 were only marginally significant in latency to copulation ($t = 2.624$, $P = 0.067$). Overall, latency to copulation was lower and copulation duration higher when males mated with virgins, as compared to when they copulated with mated females. Moreover, and confirming the choice analysis, neither the mating interval nor the age of the female affected significantly latency and copulation duration.

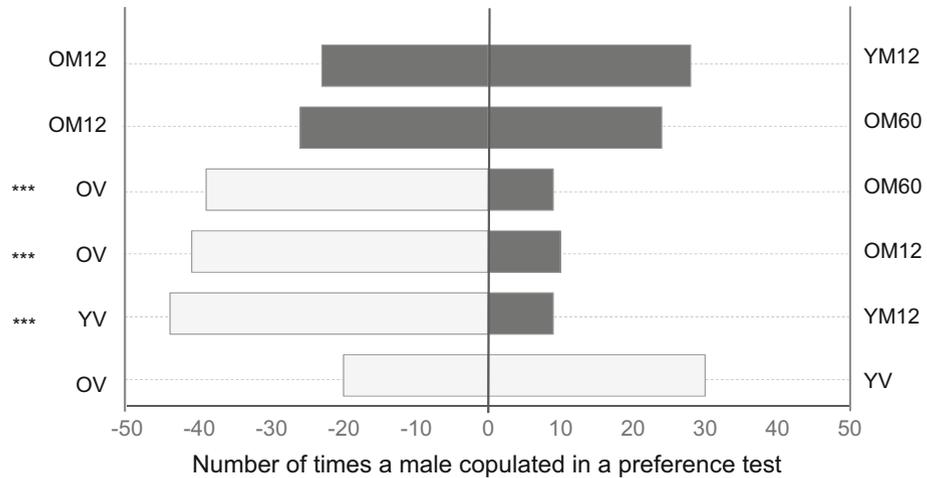


Fig. 3 Male *Tetranychus urticae* preference between different types of females. Each bar represents the number of times a male mated first with a certain type of female (i.e., treatment) within a preference test. Each preference test was done by placing two females of two different treatments (e.g. Preference test: OV × YV) with a male. Treatments (i.e., type of female) were: young virgins (YV); old virgins (OV); young females that mated 12 h before the choice test (YM12); old females that mated 12 h before the choice test (OM12); and old females that mated 60 h before the choice test (OM60). Dark grey plots mated females, light grey plots virgin females

Type of chemical cues used in male discrimination

Males did not show a preference for virgins in treatments where females were dead, independently of the type of design used (contact or no contact) (treatment “dead without contact”: $V = 787.5$, $P = 0.53$; treatment “dead with contact”: $V = 915.5$, $P = 0.14$; Fig. 5). In contrast, males from all other preference tests spent more time with virgins than expected by chance (treatment “absent with contact”: $V = 974.5$, $P = 0.045$; treatment “alive with contact”: $V = 1268$, $P < 0.001$; treatment “alive without contact”: $V = 1288$, $P < 0.001$; Fig. 5).

Discussion

In this study, we set out to investigate which type of cues *T. urticae* males use to distinguish between females with different mating status (virgin or mated). To determine whether males’ choice would also involve females with different mating intervals (mated 12 or 60 h before the test), we first tested whether males distinguish between these female types, while controlling for their age. We found that males preferred virgins over mated females but did not discriminate between females that had mated at different time intervals (Fig. 3), supporting earlier results (Oku 2010, 2013). Latency to copulation was shorter and copulation duration longer when males mated with virgins relative to when they mated with mated females (Table 1; Fig. 4), which supports the hypothesis that they are more attracted to the former than to the latter. In addition, no differences were found in latency to copulation and copulation duration of mating events with mated females. Finally, no effect of female age on male choice was found.

Table 1 Multiple comparisons of latency to copulation and copulation duration between treatments using Tukey contrasts

	Comparison ^a	Estimate	SE	t	P
Latency to copulation	OM12 × OM60	−0.250	0.682	−0.366	0.996
	OV × OM60	−1.714	0.624	−2.746	0.048
	YM12 × OM60	−0.019	0.787	−0.024	1.0
	YV × OM60	−2.050	0.678	−3.023	0.022
	OV × OM12	−1.465	0.513	−2.852	0.036
	YM12 × OM12	0.231	0.702	0.328	0.997
	YV × OM12	−1.800	0.578	−3.115	0.017
	YM12 × OV	1.695	0.646	2.624	0.067
	YV × OV	−0.335	0.508	−0.660	0.96
	YV × YM12	−2.030	0.698	−2.908	0.031
Copulation duration	OM12 × OM60	−1.798	3.557	−0.505	0.99
	OV × OM60	19.141	3.255	5.880	<0.001
	YM12 × OM60	4.638	4.137	1.121	0.79
	YV × OM60	19.742	3.567	5.534	<0.001
	OV × OM12	20.938	2.678	7.820	<0.001
	YM12 × OM12	6.435	3.700	1.740	0.40
	YV × OM12	21.540	3.050	7.063	<0.001
	YM12 × OV	−14.503	3.411	−4.252	<0.001
	YV × OV	0.601	2.692	0.223	0.999
YV × YM12	15.104	3.710	4.071	<0.001	

^a Treatments (i.e., type of *Tetranychus urticae* female): young virgins (YV), old virgins (OV), young females that mated 12 h before the choice test (YM12), old females that mated 12 h before the choice test (OM12) and old females that mated 60 h before the choice test (OM60)

In several species, older females were found to be less attractive than younger ones (Johansson and Jones 2007; Delisle and Royer 1994), most likely due to the correlation between age and female fertility. In *T. urticae*, Krainacker and Carey (1990) showed that female spider mites produce a smaller proportion of daughters when mated 5 or 10 days after emergence, compared to females mated immediately after emergence. As age differences used in our experiments were smaller than these, differences in fertility were unlikely to occur among females, and hence to affect male choice. In addition, as shown by Rasmy and Hussein (1994), the sex pheromone released by *T. urticae* females and its perception by males are both affected by age, but only when both sexes are 7 days old, whereas our females were never older than 4 days.

We also found that male choice is not affected by the female mating interval, a result that is consistent with those of Oku (2013), suggesting that this behaviour is not population-specific nor condition-dependent. Three alternative hypotheses may explain these discrimination patterns. A first possibility is that the mating interval does not play a role in determining the degree of first-male precedence. In this case, the fertilization success of males mating with recently-mated and earlier-mated females would be similar. This, however, is not in agreement with the results presented in Helle (1967). The second possibility is that the mating interval plays a role in sperm precedence, but males are not

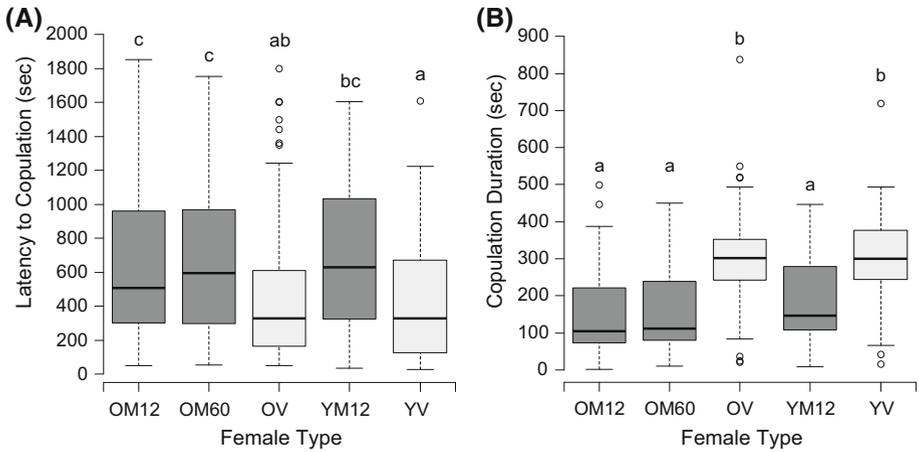


Fig. 4 Mean **a** latency to copulation and **b** copulation duration in matings involving *Tetranychus urticae* females of different ages and mating histories. Treatments (i.e., type of female): young virgins (YV); old virgins (OV); young females that mated 12 h before the choice test (YM12); old females that mated 12 h before the choice test (OM12); and old females that mated 60 h before choice the test (OM60). Different letters indicate significant differences in contrast analysis done using a Tukey test. Dark grey plots mated females, light grey plots virgin females

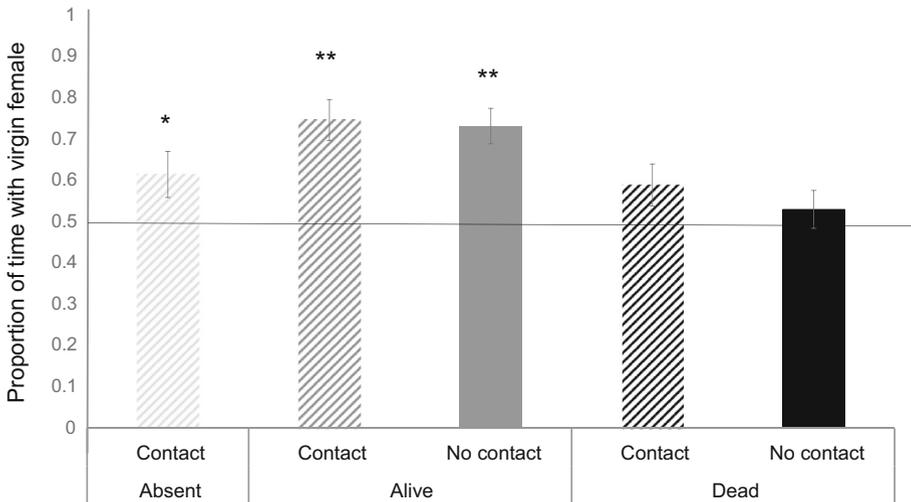


Fig. 5 Proportion of time each focal *Tetranychus urticae* male spent with virgin females during the choice test. Virgin and mated females were allocated to different combinations of type of arena (contact, no-contact) and female condition (alive, absent, dead). Dashed bars contact, solid bars no contact, light grey absent females, dark grey alive females, black bars dead females. A Wilcoxon signed rank test with continuity correction was used to analyse the proportion of time spent by the males with a virgin female. Each treatment was analysed separately and compared to a proportion of 0.5, i.e., to a situation with no preference. * $P < 0.05$; ** $P < 0.001$

able to perceive differences between females mated at different time intervals. The third possibility is that mating with mated females occurs for a reason different than fertilizing offspring, for example, to limit the effectiveness of fertilization by the first male (Macke

et al. 2012). Indeed, in certain species, males are able to increase their relative fitness by removing the sperm from a previous male, or transmitting seminal components that neutralize the sperm of competitors (Chapman et al. 1995). Even without neutralizing the sperm of another male, if the act of re-mating is more costly to females than to males, males are decreasing the fitness of females and, hence, indirectly, that of other males. The extent of such effects could still depend on the mating interval—being probably larger for recently mated females. However, the benefits of evolving a discrimination ability between recently-mated and earlier-mated females could be negligible for males. In order to disentangle these three hypotheses and to establish if the second mating results in viable offspring or reduced fertility of the first male, paternity and fertility tests should be performed in future studies, as well as analyses of female chemical profiles at different mating intervals.

Because males did not discriminate females that had mated at different time intervals, we excluded this choice from the experiment where chemical cues were manipulated. In this experiment, male discrimination was not found in treatments where females were dead (Fig. 5). Contact cues, such as CHCs (cuticular hydrocarbons), of adult females have been implicated in male arrestment in *T. urticae* (Royalty et al. 1992), but no discrimination between mated and virgin females had been tested. Possibly, CHC's do not play a role in male discrimination of females with different mating status in this species. Alternatively, such compounds disappear at a fast rate in spider mites. As experiments with other arthropods show that dead females still contain enough CHCs to elicit male discrimination (e.g., Xue et al. 2016), the former hypothesis seems more likely. Nevertheless, we cannot rule out that female behaviour and contact compounds, though apparently not necessary, may contribute to male choice.

Our results clearly indicate that chemical trails and volatile compounds trigger male preference for virgins. Volatile pheromones have been implicated in male searching behaviour for quiescent females, in distances similar to the ones we tested here (Penman and Cone 1972). In addition, in the same study, when males had access to both pheromones and web, male searching behaviour was exacerbated (Penman and Cone 1972). Possibly, the chemical trails used by males in our experiment also include web. This suggests that virgin and quiescent female detection by males may rely on the same type of cues. Although volatile cues are usually considered to trigger mite dispersal from plants (e.g., Pallini et al. 1997), it is not very likely that males use such cues to distinguish virgins from mated females over long distances. This is because females are the most dispersing sex in *T. urticae* (Li and Margolies 1993), hence males will probably always have access to females within their closer environment.

Unlike in the case of attraction towards quiescent females where web alone is not sufficient to trigger a response (Penman and Cone 1972), volatile compounds and chemical trails alone are sufficient to elicit female mating status discrimination in males. The redundancy in mate choice cues is predicted to increase mate-choice costs but also to decrease mate-choice errors. If multiple cues increase the amount of information obtained and/or reduce the energy spent on mate assessment, such redundancy is expected to be selected (Candolin 2003). In addition, different cues may be differentially perceived depending on environmental conditions or distances, hence relying on multiple cues maximizes the probability of a correct mate choice in most environments (Bro-Jørgensen 2010). The fact that spider mite males are able to distinguish between females with different mating status, and that they rely on multiple cues to do so, reinstates that sperm precedence patterns affect the selection pressures on mating traits. Identifying the chemical composition of the cues involved in this behaviour will open the way to manipulative

experiments allowing to go further in our understanding of the ecology and evolution of the mating behaviour of spider mites.

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