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# Males mate with females even after sperm depletion in the two-spotted spider mite

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## Abstract

Generally, males increase their reproductive success by mating with as many females as possible, whereas females increase their reproductive success by choosing males who provide more direct and indirect benefits. The difference in reproductive strategy between the sexes creates intense competition among males for access to females, therefore males spend much energy and time for competition with rival males for their reproduction. However, if they do not need to engage themselves into male competition and females are in no short supply, how many females can a male mate with and fertilize? We address this question in the two-spotted spider mite, *Tetranychus urticae* Koch. In this study, we investigated how many females a young, virgin male mated in 3 h, and checked whether the mated females were fertilized. We found that on average males mated with 12–13 females (range: 5–25). As latency to next mating did not change with the number of matings, the males are predicted to engage in even more matings if the mating trial were continued beyond 3 h. Copulation durations decreased with the number of matings and typically after 11 copulations with females any further copulations did not lead to fertilization, suggesting that males continued to mate with females even after sperm depletion. We discuss why spider mite males continue to display mating and copulation behaviour even after their sperm is depleted.

**Keywords** Haplodiploidy · Male reproductive potential · Mating strategy · Multiple mating

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Hisaho Kobayashi and Yukie Sato have contributed equally.

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## Introduction

Generally, males increase their reproductive success by mating with as many females as possible, whereas females increase their reproductive success by choosing males who provide more direct benefits (e.g., nutrients, superior territories with resources, and parental care) and/or indirect benefits (e.g., good genes) (Andersson 1994). The difference in reproductive strategy between the sexes creates variation in mating success much greater among male individuals than among female individuals, resulting in intense competition among males for access to females (Bateman 1948). The male competition can be strong enough to lead to evolution of weapons to win the male fights (e.g., horns and tusks), exaggerated characters in males to attract females (e.g., colour patterns, ornaments, vocalizations and display behaviour) and alternative reproductive tactics in males (Andersson 1994; Gross 1996; Andersson and Simmons 2006; Emlen 2008). Thus, males spend much energy and time on male competition for their reproduction. However, if they do not need to compete with other males and females are in no short supply, how many females can a male mate with and fertilize?

In arthropods, not much is known about male reproductive potential (Kelly and Jennions 2011). The two-spotted spider mite, *Tetranychus urticae* Koch, is arguably one of the best-studied model species. Reproductive strategies of the mite have been studied for a long time, both from the perspectives of males and females (Helle 1967; Potter et al. 1976a, b; Potter and Wrensch 1978; Satoh et al. 2001; Macke et al. 2012; Sato et al. 2013, 2014, 2016; Schausberger and Sato 2019; Schausberger et al. 2019; Rodrigues et al. 2020; Morita et al. 2020). For example, it is known that females often mate with several males but they use the sperm only from the first male for egg fertilization as long as the copulation was not disturbed (Helle 1967; Potter and Wrensch 1978; Satoh et al. 2001). Therefore, males compete for virgin females and often show premating guarding behaviour: males mount teleiochrysalis females—the stage just before adulthood—and chase rival males away by fighting until the guarded female ecloses as adult (Potter et al. 1976b). This indicates that male competition for access to females is strong and males spend much energy and time on the competition for their reproduction in *T. urticae*.

Several studies have addressed reproductive potential of *T. urticae* males with varying results. One paper on male reproductive potential in *T. urticae* reported that males mated with 7–9 females in 2–2.5 h and fertilized 5–7 of them (Helle 1967), whereas another paper reported that a 1-day-old male mated on average with 15 females in 12 h and fertilized on average 15 females in a day (Krainacker and Carey 1989). However, none provide data on male reproductive success per mating. Besides, it is unclear if males continue to mate even after sperm shortage or depletion as reported in several species (Ramadan et al. 1991; King 2000; Jacob and Boivin 2004; Garcia-Gonzalez 2004; Damiens and Boivin 2005; Steiner et al. 2008; Pérez-Lachaud 2010; Abe 2019).

In this study, to investigate male reproductive potential in *T. urticae*, we measured how many virgin females a male mates with and fertilizes in 3 h. We also checked the relationships of copulation duration, and of latency to mate, with the number of matings.

## Materials and methods

### Mites

*Tetranychus urticae* (strain ‘Houten-1’) was obtained from the Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, in 2014, and reared at the University of Tsukuba. The mites were reared on detached leaves of common bean (*Phaseolus vulgaris* var. Naga-Uzura), which were placed on wet cotton wool in styrofoam and plastic trays. The colony started from more than 50 individuals. The colony size changed while being reared, and the colony was kept in climate chambers at 18–25 °C and L15:D9 h photoperiod. At least 1 month before the experiments took place (roughly two generations), the colony was kept at 25 °C and L15:D9 h photoperiod, and with a large enough size for collecting more than 500 teleiochrysalis females.

To obtain virgin males and females, teleiochrysalis males and females were collected from the mite cultures. Collected males were placed individually onto bean leaf discs (1.5 cm diameter) on wet cotton wool in insect breeding dishes (5 cm diameter, 1.5 cm high; SPL Life Sciences, Gyeonggi-do, Korea). We kept the males individually isolated until observation of mating behaviour started because experience of other male presence might affect the number of matings. Collected females were placed onto the bean leaf discs together (20–30 females per leaf disc). These mites were left inside climate chambers at  $25 \pm 1$  °C and L15:D9 h photoperiod, and the adult mites that emerged within 24 h were used in the experiment.

### Observation of mating behaviour

We introduced a virgin male and three virgin females onto a bean leaf disc (1.5 cm diameter) on wet cotton wool in an insect breeding dish (hereafter, called ‘mating arena’), then observed the male behaviour under a stereo microscope. In order to copulate, the male manoeuvres under the female, curls his abdomen up, and extends and inserts his aedeagus into the female genital orifice (Cone 1985). We recorded the time of aedeagus insertion and removal as the start and finish of copulation. After copulation finished, the mated female was immediately transferred onto a bean leaf disc 2.5 cm in diameter on wet cotton wool in an insect breeding dish (hereafter, called ‘oviposition arena’), then a new virgin female was introduced onto the mating arena to ensure ad libitum mating opportunities with virgin females in the mating arena. We continued the observation and replenishment of virgin females for 3 h after the male mite was introduced on the mating arena. The observations were carried out in a room with air conditioner set at 25 °C on November 18, 2019 (series A) and August 25, 2020 (series B). We observed mating behaviour of five males in series A and 11 males in series B, although observation of one male in series A and of three males in series B ceased before 3 h because the males got stuck in wet cotton wool during observation.

We counted the matings (i.e., we assessed the number of females a male mated) in 3 h ( $n = 12$ ). We also assessed the cumulative number of matings for 1, 2 and 3 h ( $n = 16$ ), and used them for estimation of the number of females a male can mate in 3 h. We calculated copulation duration and latency to mate (time from the end of previous copulation to start of next copulation). Sometimes, males re-copulated with the same females very quickly before female transfer. In that case, we did not force them apart because there was a risk to injure them. Most of the recopulations were much shorter than the first

copulation. We regarded them as sequences of one copulation and therefore used the sum of durations in the analysis.

### Check of fertilization status of mated females

Spider mites are haplodiploid, i.e., females develop from fertilized eggs and males develop from unfertilized eggs. Therefore, fertilization status of mated females can be checked by the production of daughters. To see if the mated females were fertilized, we allowed the mated females to oviposit for 4 days in oviposition arenas in climate chambers at  $25 \pm 1$  °C and L15:D9 h photoperiod. We recorded the number of eggs and survival of females every day. After 4 days of oviposition, females were removed from the oviposition arenas, and the eggs were reared until they developed into adults in the same climate chambers. The numbers of daughters, sons and dead immatures were recorded.

Females that produced at least one daughter were regarded as fertilized, and females that produced only sons were regarded as unfertilized. We calculated female ratio in offspring, and used the ratio for the evaluation of female fertilization status. We treated 16 males in mating observation, but we failed to check fertilization status of all females who mated with a single male in series B. We failed to check fertilization status in several females which mated with other males if they died, got stuck in the wet cotton wool barrier or did not lay eggs during 4 days possibly by getting injured. In total, we succeeded in checking fertilization status of 141 females for 15 males.

### Statistical analysis

We used the software R v.4.0.2 for the statistical analysis (R Core Team 2020) with the *glmmTMB* package for model construction (Brooks et al. 2017) and the *performance* package for test of overdispersion in the models (Ludecke et al. 2021). In the analyses of the cumulative number of matings, we constructed a generalized linear mixed models (GLMM) with time (h), series (A and B) and the interaction as fixed factors, and with male individual as a random factor. In the GLMM, the Poisson distribution (link = *log*) was applied as the error distribution, as the data were significantly different from normal distribution especially in the data of 1 h (Shapiro–Wilk normality test:  $P < 0.001$ ). In the analyses of latency to mate, female ratio in offspring, and female fertilization status (fertilized or unfertilized), we constructed GLMMs with mating order (sequential number of matings), series (A and B) and the interaction as fixed factors, and with male individual as a random factor. In the GLMM of latency to mate, gamma distribution (link = *log*) was applied as the error distribution, as the data were significantly different from normal distribution (Shapiro–Wilk normality test:  $P < 0.001$ ). In the GLMM of female ratio in offspring, we applied beta-binomial distribution (link = *logit*) as the error distribution, because overdispersion was detected when binomial distribution was applied. In the GLMM of female fertilization status (fertilized or unfertilized), binomial distribution (link = *logit*) was applied as the error distribution. For the analysis of copulation duration, we constructed a linear mixed model (LMM; Gaussian distribution (link = *identity*) was applied as the error distribution) with mating order, series (A and B) and the interaction as fixed factors, and with male individual as a random factor. For the comparisons of the copulation duration and latency to mate between mating with and without sperm transfer, we constructed a LMM [Gaussian error-distribution (link = *identity*)] and a GLMM

[gamma error-distribution (link = log)] with female fertilization status, series (A and B) and the interaction, in which male individual was incorporated as a random variable.

We did not focus on the effect of series; however, we incorporated series not as a random variable but as a fixed variable in the models, because it is pointed out that models tend to underestimate the standard error of the fixed effect covariate’s slope when there are too few levels of the random effect (Silk et al. 2020). The number of series was two and sufficiently low to concern the problem caused by too few levels of a random effect. We incorporated male individual as a random variable, as measurements were taken repeatedly on each male individual (repeated measures). We dealt with mating order as numeric and series as categorical in the models. When the effects of interactions between mating order/female fertilization status and series (A and B) were not significant in the GLMMs, we removed them from the explanatory variables (GLMMs were reconstructed only with time as the fixed factor). For the estimations of the number of matings in 3 h, mating order in which almost all offspring (95–99%) are male, and mating order in which females are not fertilized with 95–99% probability, we reconstructed the GLMMs without the series. In the LMMs for the analysis of copulation duration, the interaction between mating order/female fertilization status and series (A and B) had a significant effect. Therefore, the LMM was reconstructed with mating order in each series.

## Results and discussion

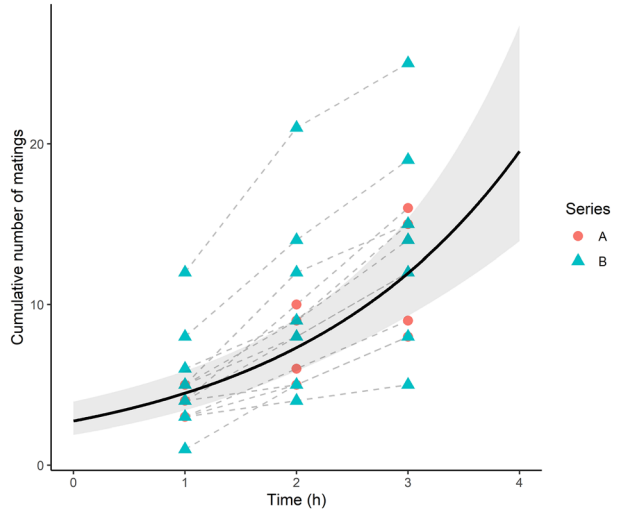
A young, virgin male of *T. urticae* on average mated with 13.2 females in 3 h (n = 12). The number of females a male mated varied strongly among male individuals; it ranged from 5 to 25. The cumulative number of matings over time was not significantly different between series (Table 1a, b). The number of matings in 3 h predicted by the model of the cumulative number of matings was 12.0 (Table 1c; Fig. 1). Latency to mate fluctuated and did not clearly decrease with the sequential number of matings (gamma GLMM, mating order: estimate = 0.031 ± 0.017, z = 1.814, P = 0.070; series: estimate = -0.042 ± 0.385, z = 0.342, P = 0.37; Fig. 2a and Fig. S1a). It suggests that males might continue to mate if

**Table 1** Generalized linear mixed models of the cumulative number of matings (the number of females a male mated) analysed with time, series and the interaction (a), without the interaction (b), and only with time (h) (c) as fixed factors

	Estimate	SE	z	P
<i>a</i>				
(Intercept)	0.729	0.356	2.051	0.040
Time	0.573	0.133	4.312	< 0.001
SeriesB	0.390	0.417	0.936	0.35
Time: SeriesB	- 0.111	0.156	- 0.714	0.48
<i>b</i>				
(Intercept)	0.908	0.245	3.702	< 0.001
Time	0.493	0.070	7.078	< 0.001
SeriesB	0.145	0.233	0.623	0.53
<i>c</i>				
(Intercept)	1.007	0.187	5.396	< 0.001
Time	0.491	0.070	7.058	< 0.001

Male individual is incorporated as a random factor in the models, as measurements were taken repeatedly on each male individual (repeated measures)

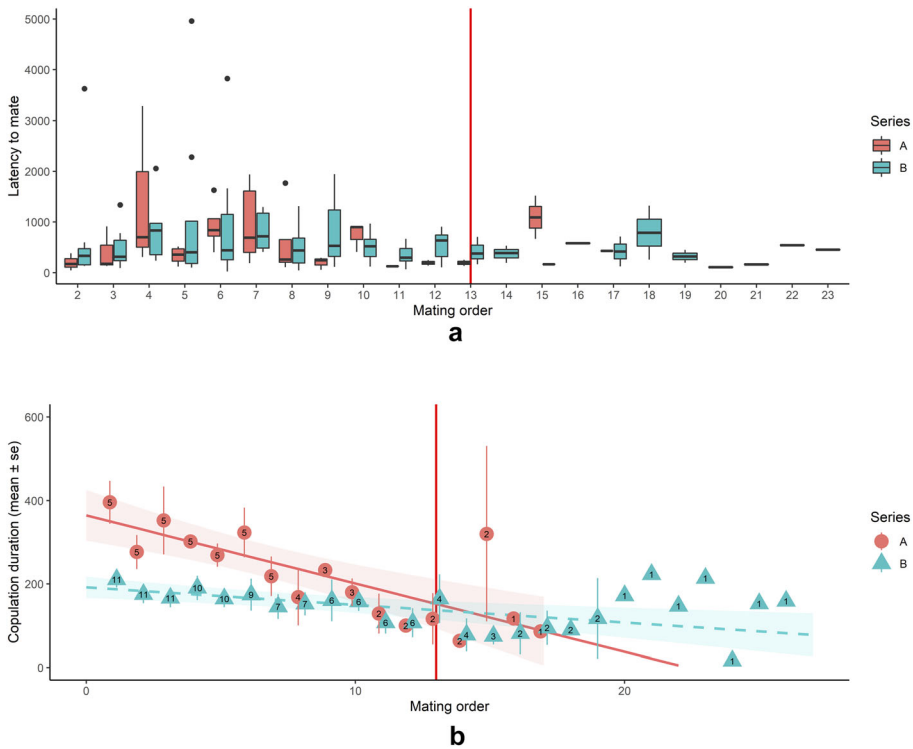
**Fig. 1** Cumulative number of matings in 3 h in each male of *Tetranychus urticae*. The experiment on male mating behaviour was carried out in two series: series A (n = 5) and B (n = 11). Symbols from the same male are connected by grey dashed lines along time (h). The black bold line and the grey filled area along the line indicate the predicted number of matings and the 95% confidence interval by the model in Table 1c. The total number of males is 16 in 1 h, 14 in 2 h, and 12 in 3 h



the mating trial would be extended. Considering that a previous study reported 15 females in 12 h (Krainer and Carey 1989), the number of matings in this study might be larger than predicted. However, in both series, copulation duration decreased as the sequence number of matings increased (Table 2; Fig. 2b and Fig. S1b). Most copulation durations were longer than 40 s, the threshold time for fertilization in *T. urticae* (albeit estimated in a different strain and form) (Satoh et al. 2001). However, male fertilization success also decreased as the sequential number of matings increased.

We used two indices for evaluation of the number of fertilized females: one is female ratio in offspring and the other is fertilization status of females. In the former index, female ratio in offspring was not significantly different between series, and decreased with the sequential number of matings of males (Table 3; Fig. 3a and Fig. S2a). The number of females in which almost all offspring were males was estimated as 13–20 (95–99%; Table 3c). In the latter index, females were judged as fertilized if the females produced at least a daughter in 4 days oviposition. Female fertilization status was not significantly different between series, and significantly changed with the sequential number of matings of males (Table 4; Fig. 3b). The number of females in which females are not fertilized was estimated as 16–21 (95–99%; Table 4c). These numbers seemed to correspond to the number of matings (Fig. 3a, b). However, these numbers are average values, and many males mated with females without sperm transfer especially in the later matings (Fig. 3 and Fig. S2), suggesting that males continued to mate with females even after sperm depletion in *T. urticae*.

Copulation duration was significantly shorter in the mating without sperm transfer than that with sperm transfer in both series (LMM, mating order in series A: female fertilization status =  $130.183 \pm 33.542$ ,  $z = 3.881$ ,  $P < 0.001$ ; mating order in series B: estimate =  $-49.827 \pm 15.790$ ,  $z = 3.156$ ,  $P < 0.01$ ; Fig. 4a). Latency to mate in the matings without sperm transfer tended to be shorter than that in the matings with sperm transfer, but the difference was marginal (gammaGLMM, female fertilization status: estimate =  $0.414 \pm 0.222$ ,  $z = 1.864$ ,  $P = 0.062$ ; series: estimate =  $-0.927 \pm 0.662$ ,  $z = 1.401$ ,  $P = 0.16$ ; Fig. 4b). Mating without sperm transfer does not result in offspring for the male and can be costly because it takes time and possibly energy. However, the behaviour of males continuing to mate even after sperm depletion has been found in



**Fig. 2** Latency to mate (s) **(a)** and mean ( $\pm$  SE) copulation duration (s) **(b)** along sequential number of matings (mating order) in males of *Tetranychus urticae*, carried out in two series, A and B. Vertical lines show the average number of matings in 3 h. **a** The boxplots comprise the following information: the upper and lower box indicate the first and third quartiles, the thick line between the boxes indicates the median, the whiskers indicate  $1.5 \times$  the interquartile range, and the dots are outliers. No line is shown because mating order did not have a significant effect on latency to mate. **b** Circles and triangles indicate means in series A and B, respectively, whereas the numbers in the symbols show the number of males. Bold lines and filled areas along the lines indicate the predicted copulation durations and the 95% confidence interval by the models for series A and B in Tables 2b, c

**Table 2** Linear mixed models of copulation duration analysed with mating order (sequential number of matings), series and the interaction in all data (a), with mating order in series A (b) and with mating order in series B (c) as fixed factors

	Estimate	SE	z	P
<b>a (All data)</b>				
(Intercept)	365.297	23.583	15.490	< 0.001
Mating order	- 16.563	2.842	- 5.827	< 0.001
SeriesB	- 175.792	27.670	- 6.353	< 0.001
Mating order: SeriesB	12.433	3.168	3.925	< 0.001
<b>b (Series A)</b>				
(Intercept)	365.644	28.933	12.637	< 0.001
Mating order	- 16.679	3.601	- 4.362	< 0.001
<b>c (Series B)</b>				
(Intercept)	189.613	12.661	14.976	< 0.001
Mating order	- 4.120	1.246	- 3.308	< 0.001

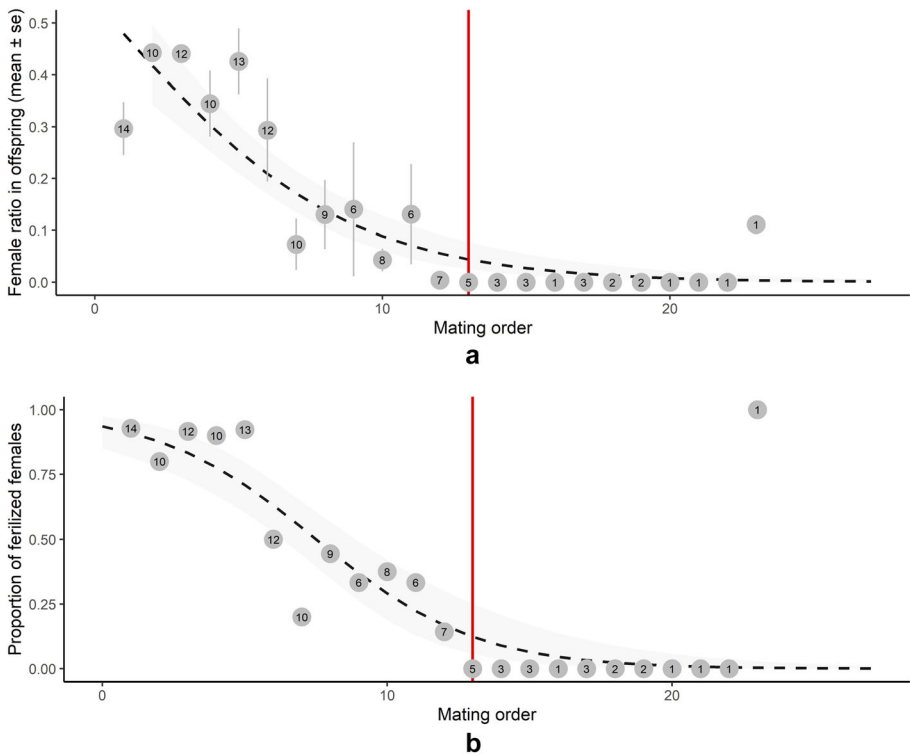
Male individual is incorporated as a random factor in the models, as measurements were taken repeatedly on each male individual (repeated measures)



**Table 3** Generalized linear mixed models of female ratio in offspring analysed with mating order (sequential number of matings), series and the interaction (a), without the interaction (b), and only with mating order (c) as fixed factors

	Estimate	SE	z	P
<i>a</i>				
(Intercept)	0.678	0.390	1.738	0.082
Mating order	- 0.326	0.064	- 5.060	< 0.001
SeriesB	- 0.743	0.466	- 1.597	0.11
Mating order: SeriesB	0.109	0.075	1.444	0.15
<i>b</i>				
(Intercept)	0.286	0.273	1.047	0.30
Mating order	- 0.251	0.035	- 7.251	< 0.001
SeriesB	- 0.177	0.253	- 0.700	0.48
<i>c</i>				
(Intercept)	0.166	0.213	0.781	0.44
Mating order	- 0.249	0.034	- 7.267	< 0.001

Male individual is incorporated as a random factor in the models, as measurements were taken repeatedly on each male individual (repeated measures)



**Fig. 3** Mean ( $\pm$  SE) female ratio in offspring (a) and proportion of fertilized females (b) along sequential number of matings (mating order) in males of *Tetranychus urticae*. Vertical lines show the average number of matings per male in 3 h. The dashed lines indicate the female ratio in offspring and proportion of fertilized females predicted by the model in Tables 3c and 4c, respectively

**Table 4** Generalized linear mixed models of female fertilization status (fertilized or unfertilized) analysed with mating order (sequential number of matings), series and the interaction (a), without the interaction (b), and only with mating order (c) as fixed factors

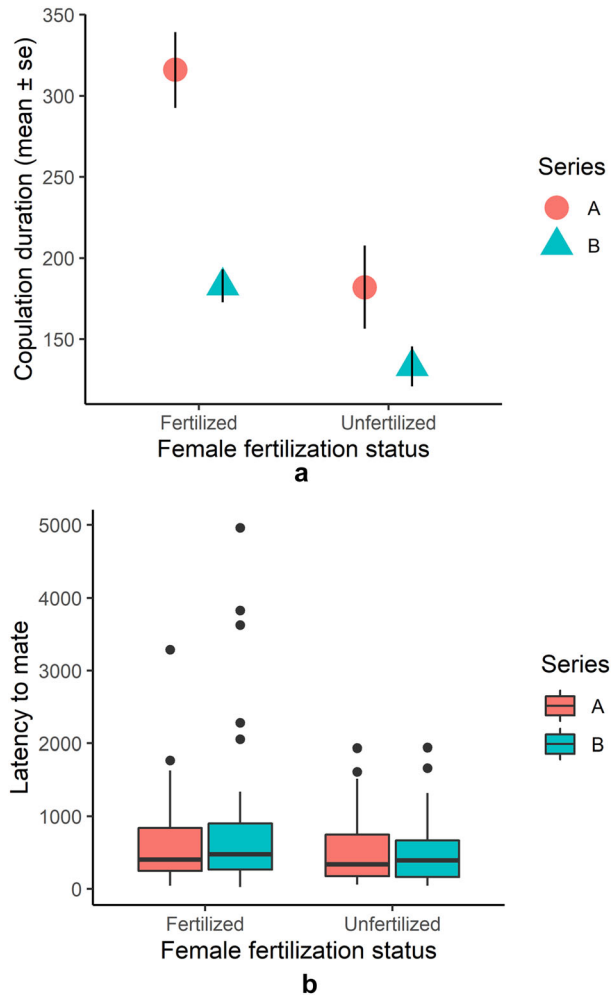
	Estimate	SE	z	P
<i>a</i>				
(Intercept)	4.673	1.363	3.427	< 0.001
Mating order	− 0.631	0.187	− 3.375	< 0.001
SeriesB	− 2.482	1.455	− 1.706	0.088
Mating order:SeriesB	0.337	0.198	1.706	0.088
<i>b</i>				
(Intercept)	2.773	0.556	4.985	< 0.001
Mating order	− 0.357	0.062	− 5.725	< 0.001
SeriesB	− 0.154	0.442	− 0.350	0.73
<i>c</i>				
(Intercept)	2.674	0.474	5.647	< 0.001
Mating order	− 0.356	0.062	− 5.737	< 0.001

Male individual is incorporated as a random factor in the models, as measurements were taken repeatedly on each male individual (repeated measures)

several taxa, especially in parasitoid wasps (Ramadan et al. 1991; King 2000; Jacob and Boivin 2004; Damiens and Boivin 2005; Steiner et al. 2008; Pérez-Lachaud 2010; Abe 2019). In parasitoid wasps, males may gain benefit by mating without sperm transfer, because mating with sperm-depleted males does not prevent female remating but decreases the amount of sperm from the remating stored in females (Damiens and Boivin 2006). This effect may increase relative fitness of the sperm-depleted male by decreasing the number of offspring of other males (Damiens and Boivin 2006). In *T. urticae*, it is known that females often remate even after mating with virgin males who may have plenty of sperm and that eggs can be fertilized by sperm from remating if the first mating is incomplete (Helle 1967; Potter and Wrensch 1978; Satoh et al. 2001; Morita et al. 2020). However, remated females produce on average fewer fertilized offspring (Rodrigues et al. 2020). Therefore, mating without sperm transfer may benefit a *T. urticae* male, not because it prevents the female from remating or egg fertilization, but it may reduce the fitness of competing males that remate such females if sperm storage is reduced or if production of fertilized offspring is reduced. In both cases, it is necessary to investigate the effect of mating without sperm transfer on the amount of sperm stored in *T. urticae* females.

Males may continue to mate after sperm depletion possibly because sperm is replenished in a very short time. In this study, a few daughters were produced in the 23rd mating of a male, after absence of a daughter from the 13th to 22nd mating. It could happen that sperm was replenished while the males continued to mate without sperm transfer. How long it takes on average for sperm replenishment is unknown in *T. urticae*. This would be important to figure out not only for understanding male reproductive potential but also for understanding male reproductive strategy. For example, if it takes a very long time or sperm is not replenished well after depletion, as in several species of parasitoid wasps, sperm would be a very limited resource. If so, mating history may affect male mating strategies and males possibly choose females for sperm transfer (cryptic male choice) after ensured paternity by several matings (Puurtinen and Fromhage 2017). A few daughters in the 23rd mating in a male may suggest that it does not take a very long time for sperm replenishment in the spider mite, but it would be worth to investigate how long it takes on average for sperm replenishment and how much sperm can be replenished.

**Fig. 4** Mean ( $\pm$  SE) copulation duration (s) (**a**) and latency to mate (s) (**b**) when a male mated with fertilized or unfertilized females in *Tetranychus urticae*, carried out in two series, A and B. **a** The numbers of fertilized and unfertilized females were 27 and 20 in series A, respectively, and 47 and 46 in series B. **b** Left and right boxplots show latency to mate in series A and B. The upper and lower box indicate the first and third quartiles, the thick line between the boxes indicates the median, the whiskers indicate  $1.5 \times$  the interquartile range. The numbers of fertilized and unfertilized females were 21 and 20 in series A, and 39 and 45 in series B



So far, we discussed why spider mite males continue to display mating and copulation behaviour even after their sperm is depleted, with the implicit assumption that males recognize their own sperm depletion. However, we should also consider the alternative possibility that spider mite males do not recognize sperm depletion; therefore, they continue their mating activities as just programmed to mate with as many females as possible in order to increase their reproductive success. Spider mite males show severe male competition for access to females as described before (e.g., Potter et al. 1976a, b), therefore males may rarely experience sperm depletion in *T. urticae*. Although decrease in copulation duration may support the possibility that males recognized sperm exhaustion and depletion, it would be worth to investigate how often males experience sperm exhaustion and depletion in nature.

The number of matings in this study was larger than predicted from the previous study (Krainacker and Carey 1989). The strain used in this study often shows male-biased sex ratio (Schausberger et al. 2019); therefore, male competition in the strain is likely more severe than other strains and populations. Perhaps male reproductive potential differs

among strains and populations, dependent on their evolutionary history. Variation in life-history traits among populations and artificially selected lines in *T. urticae* has been reported (Koveos et al. 1993; Yano and Takafuji 2002; Macke et al. 2011; Scranton et al. 2013; Golizadeh et al. 2017). It would be important to investigate variation in male reproductive potential among lines, strains and/or populations and also the relationships with life-history traits.

Alternative male reproductive tactics were reported in the strain used in this study (Sato et al. 2013). Males usually fight with other males for access to females, but some males display a non-fighting, sneaking tactic. Sneakers mount teleiochrysalis females as fighters do, but never engage themselves into male fights and are rarely detected as rival males by fighters. Which tactics a male displays can be determined by artificial disturbance (Sato et al. 2013). We can disturb a mounting male artificially by making the legs of a male on a wet fine brush touch it. If the mounting male responds actively toward the male on the brush and shows a fighting posture, the male is classified as a fighter. If the mounting male does not show any response to the male and stays quietly, the male is classified as a sneaker. Which tactics a male displays depends on its age and on male density: young males tend to be sneakers and the proportion of sneaker males shows a dome-shaped relationship with male density (Sato et al. 2014). It is thought that young males tend to be sneakers because fighting is risky and costly, therefore reduces the opportunity of future reproduction. However, when alone with a female, young males never display the sneaking tactic. In this study, we did not check whether the males were fighters or sneakers. We found large difference in reproductive potential among males, some of which may possibly be explained by the difference in male reproductive strategies. It would be interesting to investigate whether male reproductive potential is associated with male reproductive tactic.

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**Author contributions** YS and HK contributed to the study conception and design. Material preparation, data collection and analysis were performed by HK and YS. The first draft of the manuscript was written by YS and ME and all authors read and approved the final manuscript.

## Declarations

**Conflict of interest** The authors have no conflict of interest to declare.

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