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The effect of male density on guarding timing in spider mites (*Tetranychus urticae)*

Report on bachelor project

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Abstract

Spider mite females commonly only use the sperm from their first mating to sire offspring throughout their entire life, leading to intense intrasexual male mating competition. To increase chances of being the first male to copulate with a female, spider mite (*Tetranychus urticae*) males exhibit precopulatory mate guarding. Precopulatory mate guarding consists of mounting the dorsum of quiescent females in the last stage before molting into adults (teleiochrysalis stage). In this study, we investigated whether male density affects the timing of this precopulatory mate guarding behavior. We hypothesized that higher male density would be associated with earlier guarding time and higher guarding prevalence. Arenas were created each containing one teleiochrysalis female and 2-6 males. After placement of the female, a timer was started to initiate a 3.5 hour monitoring period. Observations were noted on the presence of guarding behavior, male-male interactions and distance to female in time intervals. Guarding presence and time elapsed before guarding behavior occurrence were analyzed using a Fisher's exact test (P = 0.081) and a cox proportional hazard model (reference level male density 1:2: 1:3 NA, 1:4 P = 0.077, 1:5 P = 0.91, 1:6 P = 0.067) respectively, and both yielded no significant results. Due to limitations in our experimental setup, we suggest a duplication of this study with a larger sample size and a higher number of males per arena to investigate whether a trend can be observed.

Introduction

To get access to females, males must compete with each other, which drives sexual selection. Intense intrasexual competition drives the evolution of (precopulatory) mate guarding behavior, which is widespread amongst the animal kingdom. Mate guarding occurs when one member of a pair prevents other mates from approaching, or prevents the other from seeking additional mates (Breed, 2016). It is particularly common in species with short time windows for fertilization, as is the case in the two-spotted spider mite (*Tetranychus urticae*). Spider mites are commonly known as pests, feeding on various crops. Hence, this organism's mating behavior has been studied quite extensively (Sato et al, 2013). Although female spider mites often mate with several males, commonly only the sperm from the first mating is used for egg fertilization throughout their life. Therefore, precopulatory guarding of teleiochrysalis females (quiescent phase in females of last stage before molting into adult) by mounting their dorsum is crucial for males. This, to increase chances of copulating with the female immediately after emergence and being the first male to do so (Sato et. al, 2023).

To get access to teleiochrysalis females, the primary male mating tactic is fighting. Fighter males, when approached by competitor males, will attempt to drive them away by engaging in combat, after which they usually return to the female. However, not every male displays this aggressive behavior when competing for a female. An alternative reproductive tactic (ART) that has evolved in spider mites is the sneaker tactic. Sneaker males ignore their competitors and do not engage in fights (Sato et. al, 2013). Additionally, sneaker males are not attacked by their competitors, possibly due to chemical camouflage, although the proximal mechanisms for their crypsis are not yet discovered (Sato et al., 2023). Aside from their different mating strategies, no support has been found for morphological differences between fighter and sneaker males (Sato et al., 2023).

Specifically in spider mites, ARTs are flexible and might be associated with various environmental and physiological factors (Sato et al., 2023). Firstly, mate tactic choice likely depends on age, as support was found for the late-hawk strategy. This entails that young males are more likely to adapt the sneaker tactic, whereas older males are more aggressive, and adopt the fighter tactic. This is presumably because adopting a sneaker tactic reduces risk of injury associated with fighting. In older males the importance of current reproductive success exceeds possible future reproductive success, especially if they do not have offspring yet, leading them to be more aggressive when pursuing a female. Age plays into ART choice along with male density: for young males it might increase fitness advantages to be sneakers when male density is high, whereas older males would be better off as fighters (Sato et al., 2016). Overall, the percentage of sneaking males is a saturating function of male density (Sato et al., 2023). Additionally, it was shown that the maternally experienced OSR (operational sex ratio) affects the time it takes before sneakers start to guard teleiochrysalis females. When maternal OSR is female-biased, females anticipate intense male-male competition, since they will produce proportionally more sons for the next generation. Hence, it is beneficial for sneakers to reach females earlier than the fighters, to ensure copulation (Sato et al., 2023).

We were intrigued by the effect of maternal OSR on the timing of guarding in the male spider mites. Considering the effect of male density on male mating tactics, solely the ratio of sneaker and fighter males was examined, not the timing of guarding behavior (Sato et al., 2016). Therefore, we examined whether a varying intensity of male density could affect the timing of guarding behavior in spider mite males. We predicted that as male density increased, the time it takes for spider mite males to start guarding the teleiochrysalis females will decrease, as male competition is intensified when more males are present in proportion to females. Additionally, we predicted that the prevalence of male guarding would be higher when male density increased.

Due to our small sample size and the relative rarity of sneaker males, we could not distinguish the two to investigate whether male density affects sneakers and fighters differently, this is interesting subject matter for future research.

Materials and methods

Study organism

The organism used in this research is the two-spotted spider mite (*Tetranychus urticae*), which is a small (0.5 mm long) arachnid, belonging to the subclass Acari (Mahr, n.d.). Spider mites are generally considered pests that live and feed on leaf undersides of various garden plants, such as vegetables, fruits and flowers (Cranshaw et al., 2014). Spider mite males can be distinguished from females due to their comparatively smaller size, longer legs and more pointed abdomen with narrower body (Mahr, n.d.). Spider mites lay spherical eggs, from which larvae arise. Two nymphal stages follow, a protonymph and deutonymph. Finally, there is the last molting stage before becoming adults. The last quiescent stage for females before molting into an adult is called teleiochrysalis, during which they are especially attractive to males. This is the stage during which they emit pheromones to attract males and to elicit male guarding behavior (Schausberger et al., 2023). The development speed of spider mites and the number of eggs laid per day depends heavily on the temperature of their environment. In higher temperatures, development occurs significantly faster and more eggs are laid per female per day. Spider mites develop from eggs into adults in 7-8 days under warm conditions (27.5 - 32.5 degrees °C). The speed of this development declines with lower temperatures: generally, development takes 3-15 days (Mahr, n.d.). The number of eggs laid per female can vary from 1-2 eggs at 14 °C in contrast to 11-12 eggs at 30 °C (Naher et al., 1970).

Rearing the mites

Spider mites (*Tetranychus urticae*) used for experiments were offspring from mites obtained from a kidney bean population at the University of Amsterdam. This mite population was first allowed to grow in an incubator for a ''big rearing'' box, in order to have a big population to extract from for the experiments. The big rearing was formed by placing 2 or 3 bean leaves in wet cotton wool, one of which containing the population. Over time the leaves get replaced, as the leaf starts to die and the population of mites has moved to the other leaves. Mites were reared on disks (16 mm diameter) of detached kidney bean leaves (*Phaseolus vulgaris L.*), placed on wet cotton wool inside petri dishes with small holes in the top to prevent excessive condensation. These petri dishes were placed inside an incubator with constant climatic conditions (23 °C, day/night cycle of 16-8 hours). We considered the sex determination system of spider mates to arrange a large number of males: spider mites exhibit haplodiploidy (Macke et al., 2010). A male

develops from an unfertilized egg (n), whereas females develop from fertilized eggs (2n): females in teleiochrysalis stage are guaranteed to be virgins and only produce male offspring. 45 teleiochrysalis females and 10 fertilized females were collected, placed in groups of 5 or 6 on leaf disks and were allowed to lay eggs over the course of 11 days. Within a maximum 72-hour window, eggs were repeatedly collected and moved to separate leaf disks, where they were kept until sexual maturity.

Experimental setup

Arenas consisted of a leaf disk (16 mm diameter) with one female and a varying amount of males. Teleiochrysalis females were harvested either from our own rearing or from the "big rearing". This is done due to an insufficient number of females in the correct molting stage being present in our own rearing. Sexually mature males were always collected from our own egg waves. To the highest possible extent, males placed together in arenas were from different rearing leaf discs. Mites were transferred using a wet fine brush onto the leaf disc arenas: first the males, subsequently the females. As a male density measure, the following female:male ratios were used: 1:2 (0.5), 1:3 (0.33), 1:4 (0.25), 1:5 (0.2), 1:6 (0.17). The corresponding replications done were 8, 6, 5, 7, 4. A timer was activated as soon as the female was introduced into the arena, and behavior was checked through a microscope in time intervals: per 20 minutes during the first hour, per 10 minutes during the second hour, per 5 minutes during the third hour and per 10 minutes during the final 30 minutes (24 checkpoints in total). In total, behavior was monitored for 3.5 hours for the majority of arenas. During these checkpoints, it was noted down whether male mites showed guarding behavior (if yes, whether they displayed sneaker or fighter behavior if visible), what their distance from the female was and whether the males displayed interactions with each other. In order to assess whether a guarding male is a fighter or a sneaker, we used two tactics; touch the male with another male on a brush to see if it would move into a fighting posture (fighter), or sit still (sneaker); or wait for another male to walk past to see the response of the guard.

Results

Statistical analyses

For the statistical analysis the programming software R 4.2.2 was used. Firstly, a Fisher's exact test was conducted to test whether male density was significantly correlated with the presence of guarding behavior throughout the 3.5 hours of monitoring. If at any point during these 3.5 hours guarding behavior was observed, regardless of the timing or duration, guarding behavior is marked as being present. The selection of Fisher's exact test was based on the premise that our sample size was too small for a Chi Square test, in addition to some values being lower than 5.

Our hypothesis states that we expect guarding behavior to be more prevalent with increasing male density. However, a two-sided Fisher's exact test was selected to test the relationship between male density and guarding prevalence in both directions. This yielded a result of P = 0.081, which does not show a significant relationship. Therefore this result does not support the notion of a relationship between male density and prevalence of guarding behavior (Figure 1).
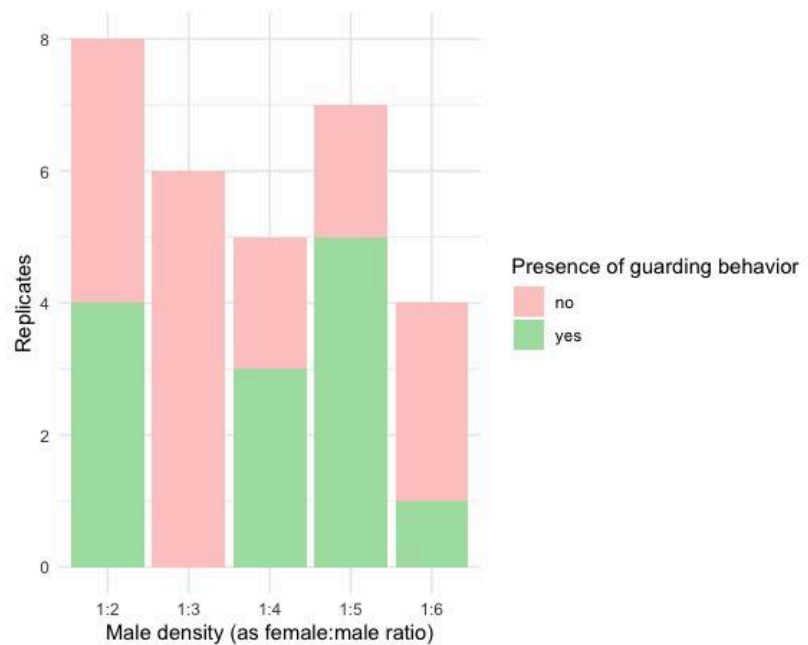

Figure 1: guarding presence (yes/no) for all variations of male density (n = 30)

To test the relationship between the time elapsed before guarding and the male density, a cox proportional hazard model was used. This model examines the time elapsed before an event occurs as a function of (a) variable(s). Our event being the time interval in which guarding is first detected and our variable being female:male ratio. In this model, the instances where no guarding behavior was detected during the 3.5 hours of monitoring were omitted from our dataset to conduct the test. By censoring these cases (n = 17 samples removed), the remaining sample size is n = 13. Censoring the instances without detected guarding is necessary because it is unknown whether guarding did or did not occur after the monitoring period. In the cox test, the reference level of 1:2 female:male ratio was used, to which 1:3 (NA: no data points), 1:4 (P = 0.077), 1:5 (P = 0.91) and 1:6 (P = 0.067) were compared. No significant correlations between male density and guarding timing were found (Figure 2).
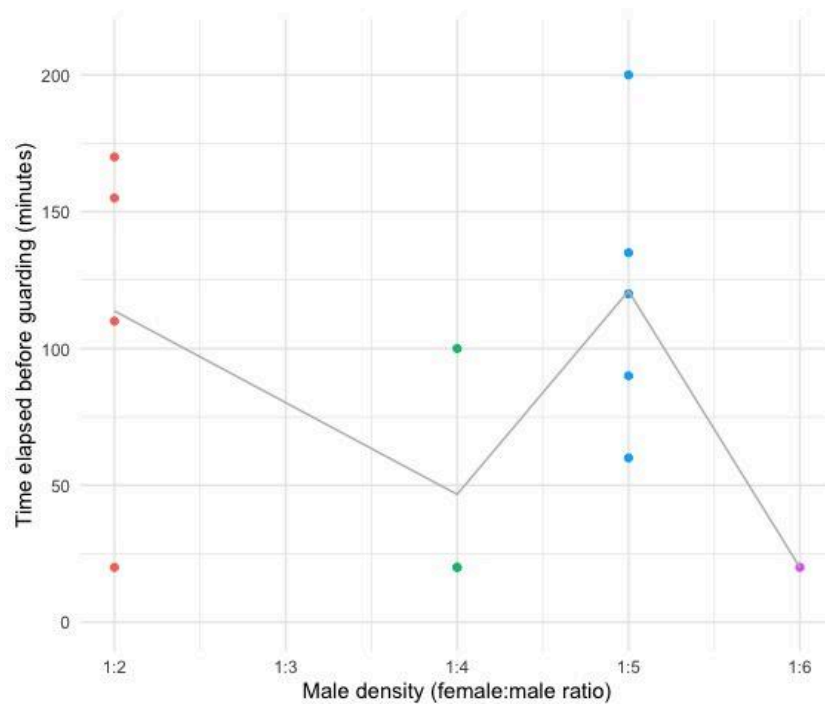
Figure 2: graph depicting the relationship between male density (female:male ratio) and time elapsed before male guarding occurred (n = 13). The gray line connects the mean values of guarding timing per male density treatment: no clear trend is visible.

Discussion

Firstly, the test conducted on the relationship between presence of guarding behavior and male density yielded no significant result. Therefore, the data from our experiment do not provide support for our hypothesis that male density influences the presence of guarding behavior. Additionally, no significant relationship was found between male density and guarding timing. Hence, our hypothesis stating that increased male density is associated with earlier guarding timing is not supported by our findings. Aside from the possible lack of the hypothesized relationship, there are various reasons why these findings could have been observed.

During our observations it was clear that a substantial amount of our males did not exhibit guarding behavior. Here, we provide possible explanations for this phenomenon and implications this might have for future research. Firstly, the exact phase of the molting stage of the female or age of the male might be crucial for guarding behavior to occur during the observational period. We selected females in the teleiochrysalis stage from the batches, but it was unknown to us when the onset of this quiescent stage was. Meaning, these teleiochrysalis females might have just

entered this stage, or might have been at the end of it. The teleiochrysalis stage lasts 24 hours on average, leaving substantial room for variation between selected females and their attractiveness to our male subjects. Females are most attractive to males during the last 2 hours before ecdysis due to the abundant release of attractive pheromones, during which they have a silvery appearance (Schausberger et al., 2023). Another study found that males preferred older teleiochrysalis females over younger ones, but only when the time lag between them was 23 hours. Moreover, fewer males guarded the younger females than the older females in non-choice situations, suggesting a stronger attraction of males to older females (Oku & Saitô, 2013). Possibly, the pheromones emitted by females who were not in this final phase are often not strong enough to elicit a response from males within the timeframe of our observations. The significance of this difference between teleiochrysalis females could be of interest for future research.

Additionally, many factors contributing to individual differences amongst males could affect guarding behavior. Although we corrected as much as possible for the effect of age differences in males by creating multiple egg waves and consequently selecting males in the same phase, other individual factors are more difficult to control for. One relevant factor is past mating history, which can affect a male's guarding behavior (Royalty et al., 1992). All of our males were reared completely isolated from females, keeping them virgins and depriving them of pursuing mating behavior before the start of our observational trials. We attempted to minimize socialization effects by assembling males from different rearing patches in the arenas, but the lack of socialization of males with females during rearing was not considered beforehand. Therefore, the effects of socialization amongst males and females on future guarding behavior in males might be of interest for future research.

Moreover, we manipulated the male densities after the males had already emerged into their adult form. They were reared on leaf discs with all other males from the same egg waves, and each leaf disc had a different amount of males on them. Some leaf discs only contained a few males, whereas some contained around 20 males. A study on the effect of male density experience on guarding behavior in the *Tetranychus kanzawai*, a relative of *Tetranychus urticae*, showed that male density experience after adult emergence had a significant effect on whether males showed guarding behavior (Oku, 2008). Hence, there is a possibility that the male density effect during the rearing period overshadowed the male density effect during our observations.

Finally, we have considered the possibility of having timed our observations too early. We started the monitoring period of 3.5 hours immediately after introducing the female to the arena, very

shortly after introducing the males. At that point, the males might have not yet familiarized themselves with their surroundings, and could be unaware of the amount of other males present on the leaf disc. If we were to duplicate this research setup in the future, we would have allowed the males to wander around on the disc for a set period of time before introducing the female and starting the timer. Moreover, this allows the males time to recover from being moved with the brush. Interfering with organisms in such a way can potentially give them stress, which might affect the behavior they exhibit during the observational period.

Conclusion

The statistical tests performed on our observations did not yield significant results from which can be concluded that male density affects male guarding presence and/or timing. However, considering the limitations of our research (further elaboration below in "limitations" sections), we believe a duplicate study that resolves these limitations could yield intriguing and more reliable results. This study would demand a significantly larger sample size, to allow for larger variations in male densities (e.g. exploring 1:10 - 1:20 female:male ratios) and a higher number of duplicates to control for variation between males. Additionally, further investigation of the intense fighting behavior we observed amongst males who did not show guarding behavior during the trails could be a promising future challenge for spider mite research.

Limitations

Rearing live organisms can pose issues pertaining to ideal sample sizes. When constructing our research method, we considered established research on the life cycle and reproductive behavior of *Tetranychus urticae*. However, organisms will often not exhibit expected behavior, especially under laboratory conditions. A portion of our teleiochrysalis females died before emerging, possibly due to effects of manipulation by our wet brushes, or due to natural or other unforeseen circumstances. The females also laid substantially less eggs than what was expected on the basis of our literary research beforehand. These factors combined lead to an eggwave that was too small to conduct a large portion of our research proposal. We lacked the amount of males necessary to run experiments of up to 10 males per female with a reasonable amount of repeats. Hence, we were only able to run experiments of up to 6 males maximum per female. In a study on Kanzawa spider mites, a species from the same genus as two-spotted spider mite, a similar limitation was considered. The proportion of males that mount females was investigated in relation to the degree of male density (high or low): in high densities, a low proportion of males mounted. The researcher's criterion of a 'high density' was ten individuals per leaf disc, which was likely not enough to incite all males to mount (Oku, 2008). If our research is comparable to

this study, we have likely used insufficiently low male densities for our experiments to see any effect.

Acknowledgements

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