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## RESEARCH ARTICLE

# Do *Tetranychus urticae* males avoid mating with familiar females?

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## ABSTRACT

The two-spotted spider mite, *Tetranychus urticae*, usually lives in kin groups under common webs. Because only the first mating results in fertilisation in female *T. urticae*, adult males guard quiescent deutonymph females, those at the stage immediately before maturation, to ensure paternity. Therefore, the cost of precopulatory guarding time seems considerable for males. Moreover, the fitness indices of daughters from intra-population crosses were significantly lower than those of daughters from inter-population crosses, indicating that inbreeding depression exists in *T. urticae*. Therefore, we hypothesised that *T. urticae* males should be choosy in guarding familiar females to avoid inbreeding depression. Furthermore, webs should be a key element of the environment shared by familiar individuals. In this study, we demonstrated the inbreeding avoidance mechanism of *T. urticae* males in relation to webs produced by familiar females (known webs) or unfamiliar females (unknown webs). Regardless of surrounding webs (known or unknown), males preferred unfamiliar to familiar females. We further examined whether males detect unfamiliar females by their webs. When males had experienced a female's web without encountering that female, they subsequently preferred females that did not produce the surrounding webs in which the choice experiment was conducted. Results suggest that putative kin recognition for inbreeding avoidance in *T. urticae* males is based on the relationship between webs and females, and not on the discrimination of webs in shared environments.

KEY WORDS: *Tetranychus urticae*, Inbreeding depression, Mate choice, Precopulatory guarding, Familiarity, Webs

## INTRODUCTION

As inbreeding depression is universal in sexually reproducing animals, a means of avoiding inbreeding between closely related individuals should have developed (Pusey and Wolf, 1996). In group-living animals, relatedness among familiar individuals should be relatively high. Thus, mating with familiar individuals is avoided through dispersal (Pusey, 1987) or by kin recognition based on the following attributes: prior association (familiarity), learned shared phenotypic traits (phenotype matching), and cue detection in a shared environment (context-based recognition) (Schausberger, 2007).

Wild populations of the group-living spider mite *Tetranychus urticae* Koch experience frequent inbreeding (Carbonnelle et al., 2007). Thus, familiar individuals of the opposite sex are likely to be close kin. However, inbred offspring of *T. urticae* have been reported to mature more slowly than outbred offspring (Tien, 2010), and inbred adult females have lower reproductive output (Perrot-

Minnot et al., 2004; Tien, 2010). Therefore, *T. urticae* may have evolved the means to discriminate familiar individuals. Putative kin recognition based on familiarity has been reported in the predatory mite *Phytoseiulus persimilis*, which preferentially cannibalises unfamiliar individuals (Schausberger, 2007). However, inbreeding avoidance based on familiarity has not been explicitly reported in group-living spider mites.

In female *T. urticae*, only the first mating results in fertilisation (Helle, 1967). Therefore, adult males guard quiescent deutonymph females (those at the stage immediately before maturation) to guarantee paternity. Tien et al. (Tien et al., 2011) suggested that *T. urticae* can recognise kin because adult virgin females are more likely to mate with unrelated males. However, because *T. urticae* females usually mate with guarding males immediately after maturation (Cone, 1985), the author's experimental condition, where an adult virgin female had a choice of multiple males, seems questionable. Moreover, although the cost of copulation seems negligible to *T. urticae* males (Krainacker and Carey, 1990), the cost of precopulatory guarding time, usually lasting several hours (Potter et al., 1976), seems considerable compared with their short generation time of ~10 days at 25°C. Therefore, we hypothesised that *T. urticae* males should be choosy and guard unfamiliar females.

*Tetranchus urticae* constructs complicated irregular webs on leaf surfaces (Saito, 1983), usually invisible even under a microscope (Clotuche et al., 2009). Because the mite usually lives under webs that protect it from rain (Davis, 1952) and predators (McMurtry et al., 1970), webs should be a key element of a shared environment among familiar individuals. The term web, as used here, refers to silk threads and other associated attributes of the inhabitant mites.

In this study, we confirmed inbreeding depression in *T. urticae*, and demonstrated their inbreeding avoidance mechanism by examining whether males prefer to guard unfamiliar females, and whether *T. urticae* males that had previously experienced a female's web avoid guarding that female.

## RESULTS
### Do males prefer unfamiliar females?

With known webs of a familiar female (a female that males had encountered previously; Fig. 1A), significantly more unfamiliar females (females that males had not encountered previously) were guarded by males than familiar females (27:15, *P*=0.044; Fig. 2A). With unknown webs of an unfamiliar female (Fig. 1B), significantly more unfamiliar females were guarded by males than familiar females (29:15, *P*=0.024; Fig. 2B). However, without webs (Fig. 1C), males showed no preference (9:8, *P*=0.50; Fig. 2C).

### Do males remember female webs?

With known webs of a weaver female (a female that males had not previously encountered but whose webs they had experienced; Fig. 3A), significantly fewer weaver females were guarded by males than control females (13:28, *P*=0.014; Fig. 4A). With unknown webs

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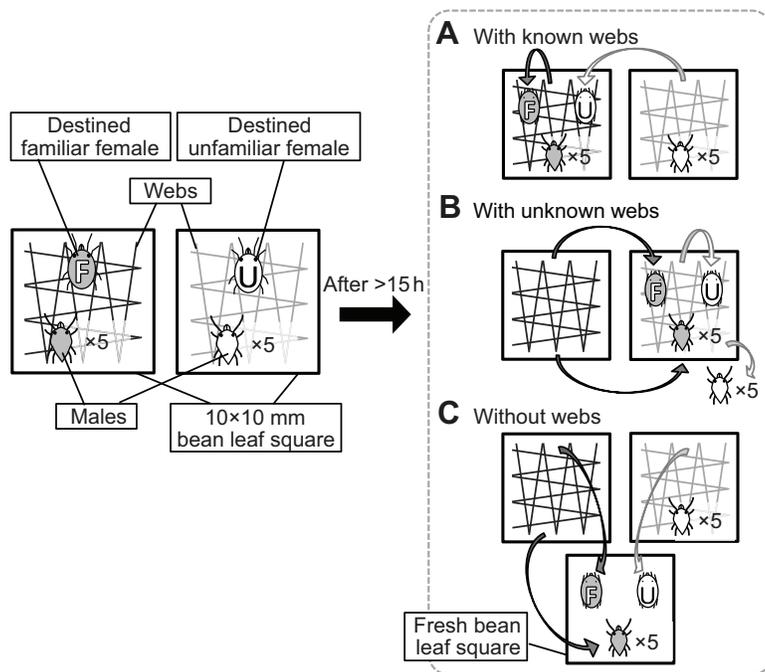*Author for correspondence (yoshioka.tsubasa.24s@st.kyoto-u.ac.jp)

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**Fig. 1. Experimental design used to examine male preference for unfamiliar females.** The three experimental treatments were: (A) with known webs, (B) with unknown webs and (C) without webs. F, familiar female; U, unfamiliar female.

of a control female (non-weaver females; Fig. 3B), significantly more weaver females were guarded by males than control females (15:6, $P$=0.039; Fig. 4B). Without webs (Fig. 3C), males did not distinguish between weaver and control females (18:24, $P$=0.22; Fig. 4C).

### Inbreeding depression in *T. urticae*

To examine inbreeding depression in *T. urticae* populations, we conducted intra- and inter-population crosses between Susaka (S) and Kakegawa (K) populations. The number of eggs (mean ± SE) produced within 5 days by daughters from the S♂×S♀ cross (22.3±1.9, $n$=23) was significantly lower than that produced by daughters from the S♂×K♀ cross (44.7±1.9, $n$=23, $P$<0.0001, Mann–Whitney $U$-test). Similarly, the number of eggs produced by daughters from the K♂×K♀ cross (40.1±1.5, $n$=24) was significantly lower than that produced by daughters from the K♂×S♀ cross (47.4±2.0, $n$=20, $P$=0.0019, Mann–Whitney $U$-test). That is, the fitness indices of daughters from inbred crosses were consistently lower than those of daughters from outbred crosses, indicating inbreeding depression in *T. urticae*.

### DISCUSSION

As the fitness indices of daughters from inbred crosses were consistently lower than those of daughters from outbred crosses,
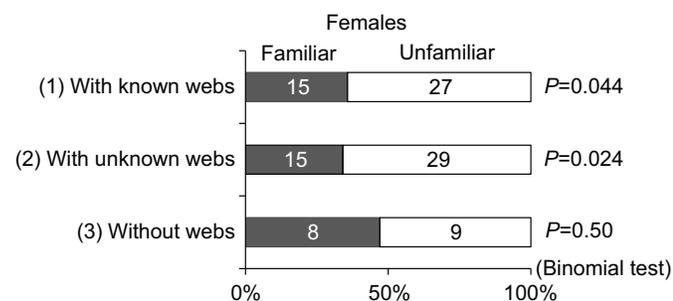
males that preferentially guard familiar females are more likely to be at a disadvantage in terms of daughter fecundity compared with males that preferentially guard unfamiliar females.

The results of our choice experiment support our hypothesis that *T. urticae* males preferentially guard unfamiliar quiescent females. Regardless of whether surrounding webs are known, *T. urticae* males preferred to guard unfamiliar females, suggesting that putative inbreeding avoidance in *T. urticae* males is based on prior associations with females, and not by the discrimination of webs in shared environments. Without webs, however, this preference was absent. It is possible that this is due to males remembering a set of habitat features, including webs, along with familiar females. However, conditions without webs may also simply be unnatural.

To determine whether male preference for unfamiliar females is only based on memory of webs, a second choice experiment was conducted. When males had previously experienced a female's web without encountering that female, they responded differently to familiar females. That is, males preferred unfamiliar females with known webs and familiar females with unknown webs. This suggests that males preferred females that did not produce surrounding webs, probably based on the relationship between females and webs. Because *T. urticae* females deposit abundant faeces on their webs (Clotuche et al., 2009), such discrimination would be possible if the female body and her webs carry common chemical compounds. We predict that *T. urticae* males that failed to remember familiar females in the wild would prefer females unrelated to the surrounding webs. Without webs, however, this preference was absent, either because there were no surrounding webs to be compared with females, or because the web-free condition was unnatural. Thus, it is likely that males do not have the ability to discriminate between familiar females only by their webs. In other words, to remember familiar females, males need to experience their substance, e.g. chemical compounds of the female body.

Mated females are considered the dispersing stage of *T. urticae* (Margolies and Kennedy, 1985); therefore, males should mate with familiar females if they live in stable environments. However, *T. urticae* is an agricultural pest that lives in disturbed environments
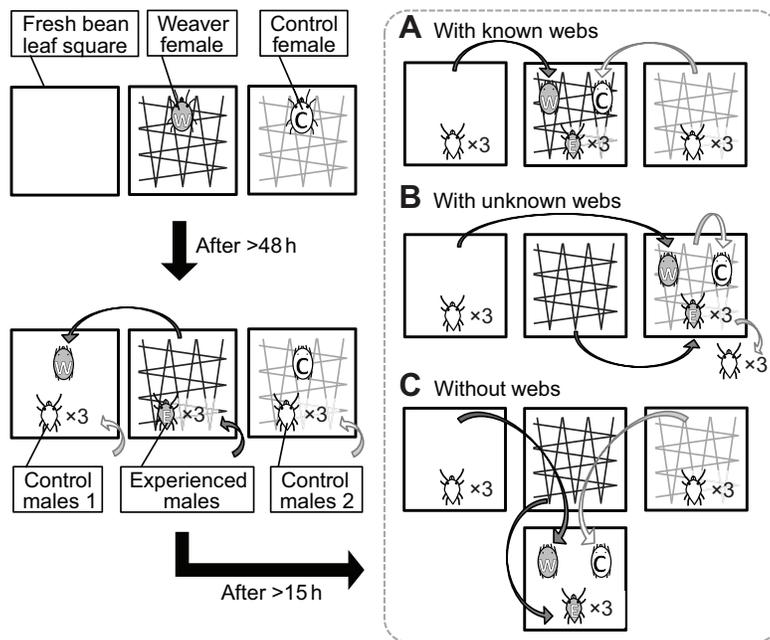


**Fig. 2. Preference of adult males for unfamiliar females.**

**Fig. 3. Experimental design used to examine male preference for females that produce unknown webs.** The three experimental treatments were: (A) with known webs, (B) with unknown webs and (C) without webs. W, weaver female; C, control female; E, experienced male.

(Van de Vrie et al., 1985). Moreover, larvae of *T. urticae* readily join conspecific webs (Clotuche et al., 2013), which may promote outbreeding after maturation. Hence, opportunities for mating with unfamiliar individuals should be extensive.

The female-biased sex ratio in *T. urticae* (Wrensch and Ebbert, 1993) suggests frequent sibling mating during local mate competition (Hamilton, 1967), so the degree of inbreeding depression in this species should have been minimised through natural selection. However, the presence of inbreeding depression and inbreeding avoidance even in such species suggests that this mechanism is universal among groups of living arthropods, a topic that remains to be examined. Many additional questions also remain unaddressed. For example, we do not know whether males in developmental stages other than that examined here (15 h after maturation) can remember females. Furthermore, the number of females that males can memorise simultaneously, the amount of time it takes for males to memorise familiar females, how long this memory can persist, and whether the memory is reset by successful mating remain unknown.

## MATERIALS AND METHODS
### Mites
In September 2011, a study population of *T. urticae* was collected from apple trees in Susaka, Nagano Prefecture, Japan. The population was reared
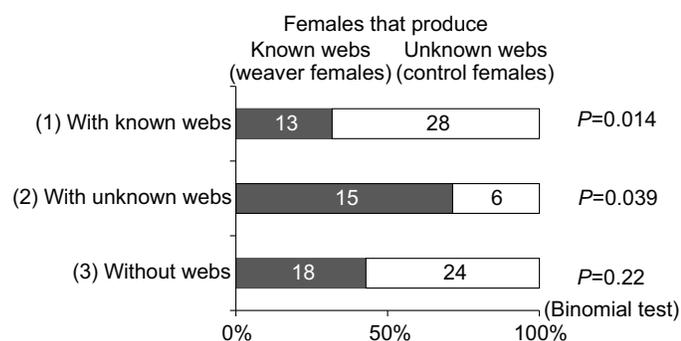
on kidney bean (*Phaseolus vulgaris*) leaf discs pressed onto water-saturated cotton in Petri dishes (90 mm in diameter; 14 mm deep). The population, as well as individuals used in the mate choice experiment, was maintained under climate-controlled conditions (25°C, 16 h:8 h light:dark).

The life cycle of *T. urticae* consists of egg, larva, quiescent larva, protonymph, quiescent protonymph, deutonymph, quiescent deutonymph and adult stages. *Tetranchus urticae* is arrhenotokous, which means that virgin females only produce male offspring. Because this species has a female-biased sex ratio (e.g. Sabelis, 1991), it is difficult to obtain a sufficient number of male offspring from mated mothers. Therefore, we used virgin females for this purpose. Quiescent deutonymph females were randomly selected from the study population and transferred onto leaf discs. Following adult emergence, they were allowed to oviposit under laboratory conditions, where eggs were kept until adult emergence. All adult males were obtained in this manner.

To examine inbreeding depression in *T. urticae*, a second population of the mites was collected in Kakegawa, Shizuoka Prefecture, Japan, in December 2012. Kakegawa is ca. 210 km from Susaka, the origin of our first study population. The Kakegawa population was maintained in the same manner as above.

The experiments described below comply with the current laws of Japan.

### Do males prefer unfamiliar females?
To examine a male's preference for unfamiliar, quiescent females, fresh 10×10 mm bean leaf squares were placed individually on water-saturated cotton in Petri dishes. One deutonymph female and five adult males were introduced to each leaf square (Fig. 1). We used more than one male because the guarding of a quiescent female occurs primarily in the presence of competitors. After >15 h, when females had become quiescent deutonymphs, females and males were assigned to one of three treatments.

In treatment 1, with known webs, a quiescent deutonymph female from the initial leaf square was placed on another portion of the same square (familiar female) so that webs around the female (Penman and Cone, 1972) would not bias male preference. A female from a second leaf square was then transferred to a position axially symmetrical to that of the familiar female on the initial leaf square (unfamiliar female, *n*=42; Fig. 1A). In treatment 2, with unknown webs, a quiescent deutonymph female from the initial leaf square was transferred to a second square (familiar female). A female from the second leaf square was then placed in a position axially symmetrical to that of the familiar female on the same square. Five males from the second square were replaced with males from the initial square (*n*=44; Fig. 1B). In treatment 3, without webs, a quiescent deutonymph female (familiar female) and five males from an initial leaf square were



**Fig. 4. Preference of adult males for females that produce unknown webs.**

transferred to a fresh leaf square. A female from the second leaf square was also transferred (without delay) to a position axially symmetrical to the familiar female on the fresh leaf square (*n*=17; Fig. 1C). In treatment 1, males may memorise a set of environmental features, including webs, and habitat features along with a familiar female. Treatments 2 and 3 were designed to exclude this possibility. We recorded quiescent females first guarded by one of five males. If no guarding took place within 90 min, the replication was excluded from analysis. The number of excluded replicates was six (treatment 1), seven (treatment 2) and three (treatment 3).

To determine whether *T. urticae* males prefer unfamiliar quiescent females under the above treatment conditions, experimental outcomes were analysed using binomial tests with a null hypothesis that males would choose either of the two quiescent females with an equal probability (i.e. 0.5).

### Do males remember female webs?

To determine whether males memorise female webs, one protonymph female (weaver female) was introduced to a leaf square and allowed to produce webs (Fig. 3, middle square). After more than 48 h, when the female became a deutonymph or quiescent deutonymph, she was replaced with three males (experienced males), which were allowed to experience webs produced by the weaver female. The weaver female and three additional males (control males #1) were transferred to a fresh leaf square until the next experiment (Fig. 3, left square). Another protonymph female (control female) was introduced to a fresh leaf square (Fig. 3, right square). After 48 h, three males (control males #2) were transferred to the square until the next experiment. Compared with the previous experiment, we added one extra group (three groups in total) of males for each replication. Because we had to complete the experiments during limited working hours, we reduced the number of males per group to three. Control males were used to detect any effects of associated males on weaver and control females. After more than 15 h since males were transferred, when females had become quiescent deutonymphs, females and males were assigned to one of three treatments.

In treatment 1, with known webs, weaver and control quiescent females were transferred to the leaf square harbouring experienced males and webs of a weaver female (*n*=41; Fig. 3A). In treatment 2, with unknown webs, a weaver female was transferred to a leaf square harbouring a control female and her webs. Control males #2 were then replaced with experienced males (*n*=21; Fig. 3B). In treatment 3, without webs, experienced males and weaver and control females were transferred to another fresh leaf square (*n*=42; Fig. 3C). In treatments 1 and 2, experienced males may distinguish females by examining the putative relationship between females and webs. Treatment 3 was intended to examine whether experienced males were able to distinguish chemical compounds in the absence of webs. We recorded quiescent females first guarded by one of the experienced males. If no guarding took place within 90 min, the replication was excluded from analysis. The number of excluded replicates was six (treatment 1), four (treatment 2) and 16 (treatment 3).

Numbers of guarded females were compared using binomial tests in the same manner as above.

### Inbreeding depression in *T. urticae*

To confirm inbreeding depression in *T. urticae* in the Susaka study population, we conducted intra- and inter-population crosses using the Susaka (S) and Kakegawa (K) populations. Thirty randomly selected quiescent deutonymph females from the Susaka or Kakegawa populations were separately placed onto 10×10 mm bean leaf squares, along with an adult female of either population. After 24 h, females of each cross were gathered onto a bean leaf disc so that we could obtain their daughters. After 10 days, 25 randomly selected quiescent deutonymph females from each leaf disc were introduced separately onto 20×20 mm bean leaf squares. After 5 days, the number of eggs produced by each female on a leaf square was recorded as the fitness index. The number of eggs produced within a certain period after maturation is often used as the most sensitive fitness index in mites (e.g. Agrawal, 2000; Yano et al., 2001). Females that failed to moult were excluded from analysis. The number of excluded females was one (S♂×S♀), one (S♂×K♀) and four (K♂×S♀). To examine inbreeding depression from the point of view of males, fitness indices were compared between daughters from crosses with males of the same population, i.e. between S♂×S♀

(inbred) and S♂×K♀ (outbred) crosses and between K♂×K♀ (inbred) and K♂×S♀ (outbred) crosses, using the Mann–Whitney *U*-test.

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