Journal of Invertebrate Pathology 128 (2015) 6–13

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Fatal attraction: Male spider mites prefer females killed by the mite-pathogenic fungus *Neozygites floridana*

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ARTICLE INFO

Article history:
Received 25 November 2014
Revised 7 April 2015
Accepted 9 April 2015
Available online 15 April 2015

Keywords:
Behaviour
Mate choice
Neozygites floridana
Tetranychus urticae
Two-spotted spider mite
Pathogen transmission

ABSTRACT

Exploring prospective mates can be risky. Males of the spider mite *Tetranychus urticae* approach and guard immobile (quiescent) female nymphs to increase their chances of fathering offspring, this being a first-male sperm priority species. We investigated the behaviour of male *T. urticae* towards females killed by the mite pathogenic fungus *Neozygites floridana*, letting them choose between a fungal killed and a healthy quiescent female. The dead female (called cadaver) was in one of three stages: (1) non-sporulating; (2) sporulating with primary conidia (non-infective); (3) surrounded/partly covered by secondary capilliconidia (infective). When the cadaver was in stage 1 or 2, males were significantly more often observed near the cadaver than near the healthy female. When the cadaver was in stage 3 (infective capilliconidia), males preferred the vicinity of healthy females. The frequency of two male behaviours, touching and guarding, was also recorded. Touching the cadaver tended to decrease as cadaver developed, whereas touching the healthy females increased. Guarding of non-sporulating cadavers and healthy females was equally common, while guarding of sporulating cadavers was only observed once (stage 2) or not at all (stage 3). To differentiate between the effect of fungal infection and sex, we also let males choose between a non-sporulating cadaver of each sex. Males then preferred to approach the female cadaver. Touching behaviour followed the same pattern, and guarding of male cadavers was not observed. Our results indicate that *T. urticae* males are more attracted to non-infective female cadavers than to healthy females, only detecting their mistake when very close. Moreover, males approach and explore cadavers surrounded by infective conidia. Whether the results of host manipulation by the pathogen or just sensory constraints in the host, this inability to detect unsuitable and indeed infective mates promotes transmission of the pathogen.

1. Introduction

Approaching and courting prospective mates often entails an increased exposure to natural enemies, leading to a trade-off between reproductive efforts and avoidance of natural enemies such as predators, parasitoids and pathogens (Magnhagen, 1991; Knell and Webberly, 2004; Scharf et al., 2013; Beltran-Bech and Richard, 2014). To avoid pathogens, only uninfected mates should be approached. There are many obvious benefits in doing so, not the least because reproductive effort in itself often impairs the immune system (Scharf et al., 2013). However, there are also costs associated with avoidance, like developing the ability to detect the pathogens and spending time on the assessment. Moreover, the pathogen will be under selection to escape detection, and a host–pathogen association can thus be viewed as an evolutionary arms race, in which a diverse array of adaptations interacts and counteracts to maximize reproductive output and fitness of both host and pathogen (May and Anderson, 1990; Roy et al., 2006).

The ability to detect an infective conspecific will depend on the sensory capabilities of the approaching individual, as well as the degree of successful crypsis or host manipulation exerted by the pathogen. All three possible outcomes: avoidance, indifference and attraction, have been observed in hosts encountering infected conspecifics, and according to a recent review, avoidance is the most common outcome in studies of mate choice and disease risk (Beltran-Bech and Richard, 2014). In arthropods, avoidance of conspecifics infected with entomopathogenic fungi have been noted in a number of soil-dwelling insects, complicating the use of these fungi as control agents (Baverstock et al., 2010), and in insects and mites infected with bacteria of the genus *Wolbachia*

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http://dx.doi.org/10.1016/j.jip.2015.04.002
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(Beltran-Bech and Richard, 2014). Indifference, or an inability to distinguish between infected and non-infected individuals, has also been demonstrated in several cases, including females of the ladybird *Adalia bipunctata* (Coleoptera: Coccinellidae) not discriminating between males with and without *Coccipolipus hippodamiae* (Acarina: Podapolipidae), a highly pathogenic parasitic mite (Webberley et al., 2002), and pea aphids, *Acyrthosiphon pisum* (Hemiptera: Aphididae), colonizing new plants without regard to the presence of cadavers with the fungal pathogen *Pandora neoaphidis* (Entomophthoromycota: Erynioideae) (Baverstock et al., 2005). Categories such as avoidance and indifference are rarely clear-cut, however, as there will be differences among individuals and through pathogenic development (Moore, 2013). Hountondji et al. (2009) discovered that cassava green mites, *Mononychellus tanajoa* (Acari: Tetranychidae), were indifferent to conspecifics killed by the mite-pathogenic fungus *Neozygites tanajoae* (Entomophthoromycota: Neozygitaceae) as long as no spores were produced.

A well-known case of infective individuals being attractive, and as such a potential example of host manipulation by the pathogen, is found in the domestic fly (*Musca domestica*). Males of this species will try to mate with female cadavers producing infective spores of the fungal pathogen *Entomophthora muscae* (Entomophthoromycota: Entomophthoraceae), and are more attracted to those than to non-infective females dead from other causes (Møller, 1993). The males are able to distinguish between fungal cadavers of males and females, but neither changes in sex pheromone production nor size can explain the phenomenon alone (Zurek et al., 2002). More recently, George et al. (2013) reported that the mosquito *Anopheles stephensi* (Diptera: Culicidae) is attracted to spores of two entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana* (Hypocreales), and to conspecifics as well as lepidopteran larvae killed by these fungi.

A system allowing detailed studies of behaviour in relation to disease risk is the two-spotted spider mite *Tetranychus urticae* (Acari: Tetranychidae) and its fungal pathogen *Neozygites floridana* (Entomophthoromycota: Neozygitomycetes). *T. urticae* is a well-studied species due to its worldwide status as a serious pest of many crops (Greco et al., 2005) and suitability as a model species (Oku, 2014). *N. floridana* is an obligate pathogen of spider mites (Keller, 1997), developing inside its host as hyphal bodies. After killing the host, which is then called a cadaver, the fungus penetrates the cuticle and produces spores. The spores, called primary conidia, are ejected from swollen cadavers. These conidia germinate to form capilliconidia that infect new mites (Carner, 1976; Elliot, 1998; Delalibera et al., 2006) by adhering to legs or other parts of hosts passing by (Elliot et al., 2002). The capilliconidia germinate and penetrate the host cuticle to gain entrance to the host interior before developing into hyphal bodies and starting a new fungal life cycle. A single capilliconidium of *N. floridana* is sufficient to kill and infect an adult female spider mite (Oduor et al., 1997).

T. urticae has five developmental stages, three of them ending with a quiescent phase (period of inactivity and slow metamorphosis): egg, larva, quiescent larval stage, protonymph, quiescent protonymph, deutonymph, quiescent deutonymph, and adult (Crooker, 1985). *T. urticae* is a precopulatory mate guarding species. Adult males guard females in the last phase before adult emergence (the quiescent deutonymph) in order to mate immediately after ecdysis (shedding of old exoskeleton). This behaviour can be explained by the first-male sperm priority documented for this species: only sperm from the first mating will normally fertilize the eggs (Helle, 1967). Fighting or aggression among males around quiescent deutonymphs is common in *T. urticae*. These competitive interactions between males occur more frequently and last longer with an increasing male: female ratio (Potter et al., 1976).

Guarding behaviour is time-consuming and exposes spider mite males to predators, diseases and competitors, so males should be able to identify females worth guarding. In particular they should avoid females killed by *N. floridana* and other diseases, which represent both a waste of time and a potentially lethal threat. Nevertheless, observations made when culturing *N. floridana* indicate that *T. urticae* males do not keep away from female cadavers (K.W., pers. obs.). The purpose of this study was therefore to examine to what extent male *T. urticae* will approach and guard cadavers in different stages of *N. floridana* infection. We evaluated male sexual behaviour in a series of laboratory experiments where males could choose between two non-moving females: a healthy quiescent deutonymph and a cadaver infected with *N. floridana*. We also presented males with a choice between two cadavers, one male and one female. Knowledge on male sexual behaviour towards infected conspecifics in this system will help elucidate host–pathogen co-evolutionary pathways. This knowledge will also increase the understanding of the epidemic development of *N. floridana*, which in turn may contribute to improved biological control of spider mites. To our knowledge there are no previous studies on how mite pathogenic fungi affect the sexual behaviour of their hosts.

2. Materials and methods

2.1. T. urticae stock culture

T. urticae was collected in a commercial strawberry field at Ås, in southeastern Norway (59°42′ N, 10°44′ E), and maintained on bean plants (*Phaseolus vulgaris*) cv. Masai in a climate chamber at 25 ± 1 °C, 60% RH and 16 h L:8 h D.

2.2. N. floridana isolate

The *N. floridana* isolate ESALQ 1420 used in these experiments was collected in Piracicaba, São Paulo, Brazil (22°42′30″ S, 47°38′00″ W) from its natural host *T. urticae* on jack bean (*Canavalia ensiformis*).

2.3. Production of cadavers

Three dry non-sporulating cadavers of *T. urticae* (killed by *N. floridana*) were placed on a bean (*P. vulgaris* cv. Masai) leaf disc (15 mm diameter) laid upside down on 1.5% water agar in a Petri dish (5 cm diameter and 2 cm high). Six such Petri dishes with water agar, leaf discs and cadavers were placed in a plastic box (22 × 16 × 7 cm) with the lid slightly open, to provide the right RH, and wrapped in aluminium foil for darkness. The box was kept in a climate chamber at 20 ± 1 °C, 90% RH for 24 h, for the cadavers to sporulate with primary conidia which in turn germinate to infective capilliconidia. Thirty healthy adult females were then transferred to each leaf disc with cadavers and spores, and subjected to the conditions described above for 24 h to inoculate *N. floridana*. The next day, the leaf discs with inoculated *T. urticae* were transferred to a 3 week old bean plant at ambient laboratory conditions (21–25 °C, 20–35% RH and 24 h of light). When leaf discs with inoculated mites started to wilt, mites walked onto the bean plant and established there. After 8–9 days, infected *T. urticae* had died and dry non-sporulating cadavers were collected from the leaves with a small paint brush and wrapped in a cotton cloth and put in a NUNC Cryo Tube™ (1.8 ml), to be stored in a refrigerator at 3–4 °C until experiments started (25–35 days).

Three stages of cadavers were then produced for immediate use in the experiments: (1) dry non-sporulating cadavers (females, Fig. 1A, or males, 1D) were prepared by transferring cadavers from storage to the centre of leaf discs (15 mm in diameter) on 1.5%

Fig. 1. A–D: Images of *Tetranychus urticae* killed by *Neozygites floridana*. (A) Dry non-sporulating female cadaver. (B) Female cadaver with non-infectious primary conidia. (C) Female cadaver finished ejecting primary conidia and surrounded/partly covered by secondary infective conidia (capilliconidia). (D) Dry non-sporulating male cadaver. (E) Healthy quiescent deutonymph female. Scale bar (white) = 0.5 mm.

water agar in a Petri dish (5 cm diameter, 2 cm high) without lid; (2) female cadavers with primary conidia (Fig. 1B) were produced by incubating dry non-sporulating cadavers (including leaf discs and Petri dishes) for 3 h at 20 ± 1 °C and 90% RH in the dark, for the fungus to produce primary conidia on conidiophores without ejecting them or producing any secondary capilliconidia; (3) female cadavers surrounded/partly covered by capilliconidia (infective, secondary conidia, Fig. 1C) were produced by increasing the incubation time to 8 h, allowing time for ejection of primary conidia and germination to capilliconidia.

2.4. Production of healthy ♀QDs (female quiescent deutonymphs)

Healthy adult *T. urticae* females were placed individually on bean leaf discs (15 mm diameter) laid upside down on 1.5% water agar in a 30 ml plastic vial with lid. Nine holes were made in the lid with insect pin no. 2 for aeration. The vials were placed in a climate chamber at 21 ± 1 °C, 60% RH, 16 h L:8 h D for the mites to lay eggs for 24 h. The adults were then removed and eggs left to hatch and develop into quiescent deutonymphs in the climate chamber. Deutonymphs can be sexed by their form and size. Pilot studies established that ♀QDs (Fig. 1E) developed 7–8 days after oviposition (at 21 °C). They were transferred to the centre of 15 mm leaf discs and used in the experiments as soon as possible (the quiescent stage lasting only 1.4 days (Herbert, 1981). If saprophytic fungi were observed on leaf discs or water agar during the production period, mites were transferred to a new vial with fresh leaf disc and water agar.

2.5. Choice experiment 1: dry non-sporulating female cadaver vs healthy ♀QD

A leaf disc with one ♀QD was put in a Petri dish containing a leaf disc with one dry non-sporulating cadaver on 1.5% water agar (stage 1 cadaver, as described in Section 2.3). The two leaf discs were slightly overlapping (Fig. 2). Male *T. urticae*, freshly picked from the stock culture, were then introduced where the two leaf discs overlapped, allowing the males to walk freely on both discs. A few drops of water were added to the agar to prevent males from walking off the discs. Three male densities were tested: One, two and three males per Petri dish. The density of one male was replicated eight times, the other two densities had four replicates. The whole experiment was run three times at ambient laboratory conditions (21–25 °C, 20–35% RH), each run lasting from 10 a.m. to 17 a.m. and involving $N = 16$ Petri dishes. The Petri dishes were without lids throughout the experiment, the low RH in the laboratory inhibiting sporulation.

2.6. Choice experiment 2: sporulating female cadaver with primary conidia vs healthy ♀QD

This experiment was conducted in the same way as experiment 1, except that sporulating cadavers with primary conidia (stage 2 cadavers as described in Section 2.3) were used instead of dry non-sporulating cadavers. The low RH in the laboratory prevented further development of the conidia, keeping them in a non-infective stage.

2.7. Choice experiment 3: sporulating female cadaver with capilliconidia vs healthy ♀QD

This experiment was conducted in the same way as experiment 2, except using cadavers that had produced secondary conidia (capilliconidia; stage 3 as described in Section 2.3), capable of adhering to new hosts and infect them.

2.8. Choice experiment 4: dry non-sporulating male cadaver vs dry non-sporulating female cadaver

To differentiate between the effect of fungal infection *per se* and the effect of cadavers being female, we also conducted an experiment in which males were given the choice between a dry

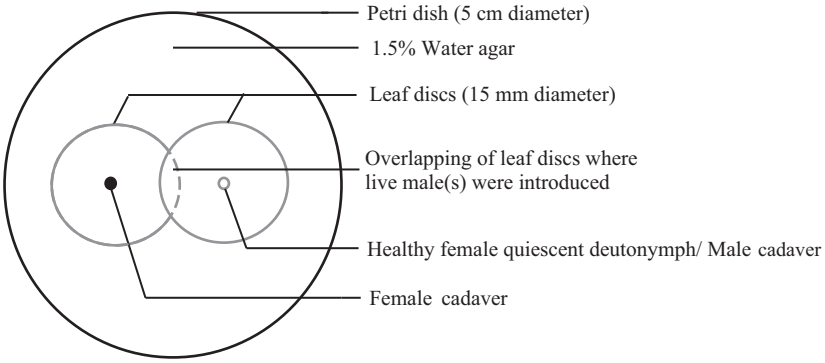

Fig. 2. Experimental set-up in the four choice experiments.

non-sporulating cadaver of each sex. This experiment was similar to experiment 1 except that a male cadaver was used instead of a healthy ♀QD. Both cadavers were produced as described in Section 2.3 (stage 1 cadavers).

2.9. Observations of male behaviour in the four choice experiments

Each Petri dish was checked in a stereo microscope (20×) once every hour for 6 h after males were introduced. On all 6 occasions, the leaf discs were observed for three male behaviours: (1) leaf disc choice, (2) touching, and (3) guarding.

2.9.1. Leaf disc choice

Each petri dish was observed for 10 s to register leaf disc choice. This was measured as the number of males present on each leaf disc.

2.9.2. Touching behaviour

A male was considered to be touching if he touched a healthy ♀QD or cadaver in any way during the 10 s period mentioned above, and the contact lasted less than 30 s, i.e., observation period was extended to distinguish between this behaviour and guarding, see below.

2.9.3. Guarding behaviour

Collins et al. (1993) considered a male to be guarding "if he remained motionless for 30 s or longer while mounted upon or within one body length of the quiescent female". In our experiments with more than one male, guarding males were sometimes disturbed, meaning they did not remain motionless. Hence, we considered it guarding behaviour when a male remained mounted upon or within one body length of the ♀QD or cadaver for at least 30 s.

2.10. Statistical analysis

All data were analysed with Minitab® Statistical Software version 17.1, using Petri dish as the experimental unit and a significance level of 0.05. An index of the overall leaf disc choice in each Petri dish was calculated by summing the number of times males were seen on the disc with the female cadaver during the six observations (maximum 6, 12 or 18 times, depending on male density) and divide this by the number of observations made (6, 12 or 18). The index thus ranged between 0 and 1, with 0.5 representing a Petri dish with equal number of observations of males on the two leaf discs, and 1.0 a Petri dish with all males present on the disc with a female cadaver in all 6 observations. In eleven dishes the ♀QD developed into an adult towards the end of experiment. The index was then based on the observations made before adult females occurred. The leaf disc choice index yielded normally distributed residuals when analysed with the general linear model tool (GLM). The four choice experiments were analysed separately, using male density (1, 2 or 3 males) and experimental run (1,2,3) as explanatory factors. A two-tailed 1-sample *t*-test was used to test if the index mean was significantly different from 0.5, i.e. to test whether males had a preference for one of the two leaf discs.

Data on guarding and touching behaviour were not normally distributed (too many zeros). These behaviours were therefore analysed with the binary logistic regression tool, letting the response '1' signify at least one occurrence of the behaviour during the 6 observations, and the response '0' an absence of the behaviour during all 6 observations. For each petri dish there were four such binary responses: male touching a female cadaver at least once, male touching a healthy ♀QD (or male cadaver in experiment 4) at least once, and the corresponding two responses for guarding behaviour. In the binary logistic regression (BLR) of each behaviour, both relevant binary responses (one for healthy and one for cadaver) were included, meaning that each Petri dish was entered twice. Again, the four choice experiments were analysed separately, the explanatory factors being: condition of object receiving the behaviour (choice experiment 1–3: healthy/cadaver; experiment 4: female/male), male density, and experimental run. If not explicitly stated, experimental run was not significant.

3. Results

3.1. Choice experiment 1: dry non-sporulating female cadaver vs healthy ♀QD

Despite a living healthy ♀QD being present, males were most often observed on the leaf disc with the cadaver (Fig. 3A; 1-sample *t*-test of disc choice index = 0.5: $T = 2.18$, $N = 48$, $P = 0.034$). Male density did not affect this pattern (GLM, male density: $F_{2,43} = 0.37$, $P = 0.69$). Touching behaviour was more frequently directed towards the cadaver than the healthy ♀QD (Fig. 3B; BLR, female condition: $X_1^2 = 5.97$, $P = 0.015$), and this behaviour increased with the number of males present ($X_2^2 = 7.45$, $P = 0.024$). Males guarded healthy ♀QD and cadavers at similar rates, unaffected by male density, but experimental run was a significant factor (BLR, female condition: $X_1^2 = 0.54$, $P = 0.46$; male density: $X_2^2 = 3.31$, $P = 0.19$; run: $X_2^2 = 7.05$, $P = 0.029$).

3.2. Choice experiment 2: sporulating female cadaver with primary conidia vs healthy ♀QD

Also when primary conidia had emerged on the cadaver, males regardless of density were more frequently found on the cadaver leaf disc than on the healthy ♀QD one (Fig. 4A; 1-sample *t*-test of

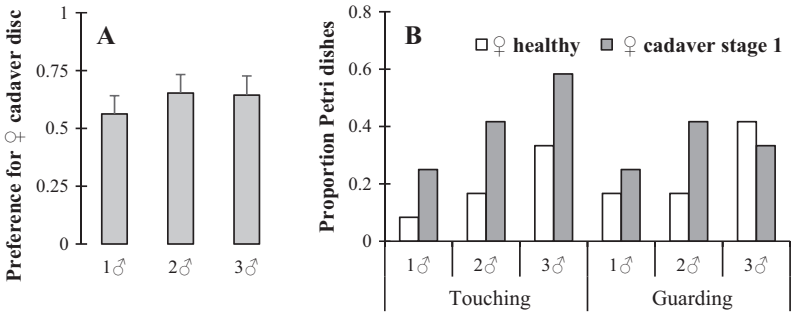

Fig. 3. Choice experiment 1. Male *Tetranychus urticae* behaviour when presented with one female (♀) cadaver in stage 1 and one healthy ♀ quiescent deutonymph on leaf discs in a Petri dish. Stage 1 = Dry non-sporulating cadaver killed by *Neozygites floridana*. (A) Leaf disc choice index (mean + SE) tested at three male (♂) densities. Index > 0.5 indicates a preference for the leaf disc with cadaver. (B) Proportion of Petri dishes in which touching and guarding the two females were observed at least once tested at three ♂ densities.

disc choice index = 0.5: $T = 2.57$, $N = 48$, $P = 0.014$; GLM, male density: $F_{2,43} = 0.05$, $P = 0.95$). Touching behaviour was not affected by any of the three explanation factors tested (BLR, $P > 0.11$ for all three). In contrast, guarding of cadavers was only observed once, which was significantly less than guarding of healthy ♀QD (Fig. 4B; BLR, female condition: $X_1^2 = 12.9$, $P < 0.0005$; male density: $X_2^2 = 2.76$, $P = 0.25$).

3.3. Choice experiment 3: sporulating female cadaver with capilliconidia vs healthy ♀QD

In the experiments with cadavers surrounded/partly covered by infectious, secondary capilliconidia, both male density and experimental run significantly affected disc choice index (GLM, male density: $F_{2,43} = 4.43$, $P = 0.018$; run: $F_{2,43} = 5.59$, $P = 0.007$). In dishes with only one male there was a significant preference for the healthy ♀QD disc (Fig. 5A; 1-sample t-test of disc choice index = 0.5: $T = -3.96$, $N = 24$, $P = 0.001$, the experimental run with a higher cadaver disc preference than the two others included). At the two higher male densities, the disc choice index was not significantly different from random ($T = -1.18$, $P = 0.26$, and $T = 0.73$, $P = 0.48$ for 2 and 3 males, respectively, $N = 12$ for each). Touching behaviour was not affected by female condition but male density was just significant (Fig. 5B; BLR, female condition: $X_1^2 = 2.13$, $P < 0.15$; male density: $X_2^2 = 6.10$, $P \approx 0.05$), the overall occurrence being comparable to the experiment with non-infective spores. However, guarding of this cadaver stage was never observed, whereas the healthy ♀QD was guarded to a high degree compared to the previous experiments. The lack of guarding behaviour towards sporulating cadavers prevented parameter estimation of these data with BLR (algorithm did not converge). Data on guarding of healthy ♀QD were therefore analysed separately, revealing no significant effect of male density (BLR, male density: $X_2^2 = 0.97$, $P = 0.33$).

3.4. Choice experiment 4: dry non-sporulating male cadaver vs dry non-sporulating female cadaver

The disc choice index of males choosing between a non-sporulating cadaver of each sex was not significantly affected by male density (GLM, male density: $F_{2,43} = 0.08$, $P = 0.92$). Males consistently preferred the leaf disc with female cadaver (Fig. 6A; 1-sample t-test: $T = 3.39$, $N = 48$, $P = 0.001$). Touching behaviour followed the same pattern, with more interest towards the female cadaver, the sex of the cadaver being the only significant factor in the binary regression (Fig. 6B; BLR, sex: $X_1^2 = 16.2$, $P < 0.0005$; male density: $X_2^2 = 3.04$, $P = 0.22$). Guarding behaviour showed an even more distinct difference: guarding of male cadavers was not observed in any of the 48 Petri dishes, while guarding of female cadavers was common (observed in 20 dishes). The guarding of female cadavers was not significantly affected by male density (BLR, excluding male cadaver data: $X_2^2 = 5.33$, $P = 0.07$).

4. Discussion

Males preferred leaf discs with female cadavers to discs with healthy ♀QD unless cadavers (and the leaf disc) had infective capilliconidia. Males also touched non-sporulating cadavers more often than healthy ♀QD, and guarded them as often as healthy ones. Infective cadavers with capilliconidia were less frequented but still touched as often as non-infective cadavers with primary conidia, the most distinct response being a lack of guarding behaviour.

These results indicate that male *T. urticae* embark on risky behaviour in their search for mates. Not only do they waste time on soliciting dead females, they even risk infection with a deadly pathogen. However, they were able to perceive that non-sporulating male cadavers and sporulating female cadavers were not worth guarding. Thus it seems that males had to experience conidia to detect the pathogen in female cadavers, whilst male cadavers elicited little interest even when not sporulating.

We are not aware of any other example of males consistently preferring dead females to living ones in choice tests. Since *T. urticae* males are adapted to search for immobile female nymphs to guard, they are probably predisposed to approach any immobile female, including dead ones. For example, *T. urticae* males will readily approach mite-sized latex pellets (Royalty et al., 1993). But this phenomenon does not explain why males approached the non-sporulating and non-infective female cadavers more often than they did healthy ♀QD or male cadavers. If they had responded equally to the three objects, not being able to distinguish them from a distance, we should have seen a random pattern, with the index of leaf disc choice not being different from 0.5. It therefore seems that males responded to cues from female cadavers, finding them more attractive than healthy ♀QDs until getting within touching distance.

Cues working from a distance could in this case be visual or chemical. Female cadavers are normally bigger than both healthy ♀QD individuals and male cadavers (Fig. 1). In the fly *M. domestica*, males are more attracted to female than male cadavers infected with the fungus *E. muscae*, but size cannot explain this as very small female cadavers are more attractive than bigger male ones (Zurek et al., 2002). The visual capacity of *T. urticae* is considerably more limited than the one of flies. Apart from the size difference, the colours of female cadavers differ from that of healthy ♀QDs, changing through cadaver development (Fig. 1)[1]. In earlier studies

[1] For interpretation of color in Fig. 1, the reader is referred to the web version of this article.

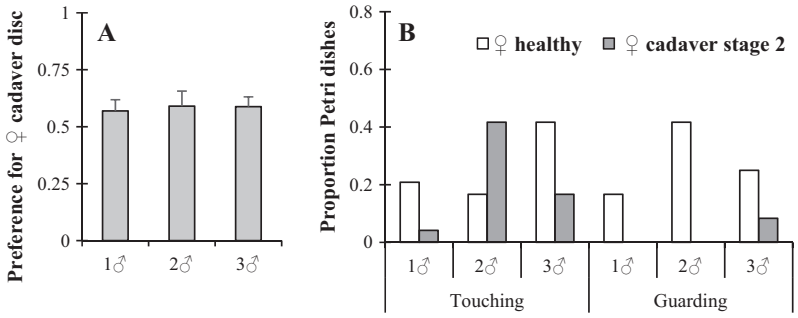

Fig. 4. Choice experiment 2. Male *Tetranychus urticae* behaviour when presented with one female (♀) cadaver in stage 2 and one healthy ♀ quiescent deutonymph on leaf discs in a Petri dish. Stage 2 = Cadaver with non-infectious primary conidia of *Neozygites floridana*. (A) Leaf disc choice index (mean + SE) tested at three male (♂) densities. Index > 0.5 indicates a preference for the leaf disc with cadaver. (B) Proportion of Petri dishes in which touching and guarding the two females were observed at least once tested at three ♂ densities.

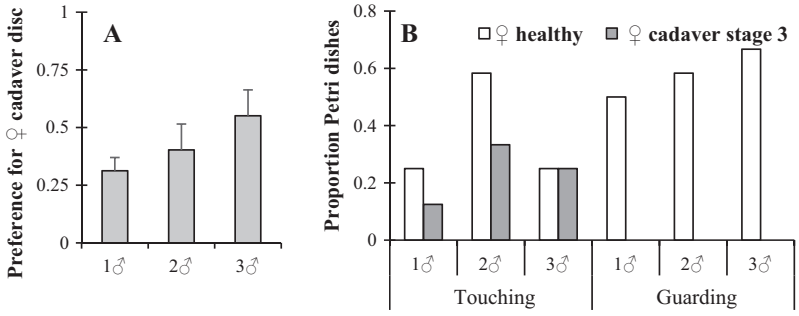

Fig. 5. Choice experiment 3. Male *Tetranychus urticae* behaviour when presented with one female (♀) cadaver in stage 3 and one healthy ♀ quiescent deutonymph on leaf discs in a Petri dish. Stage 3 = Cadavers finished ejecting primary conidia and surrounded/partly covered by secondary infective conidia (capilliconidia) of *Neozygites floridana*. (A) Leaf disc choice index (mean + SE) tested at three male (♂) densities. Index > 0.5 indicates a preference for the leaf disc with cadaver. (B) Proportion of Petri dishes in which touching and guarding the two females were observed at least once tested at three ♂ densities.

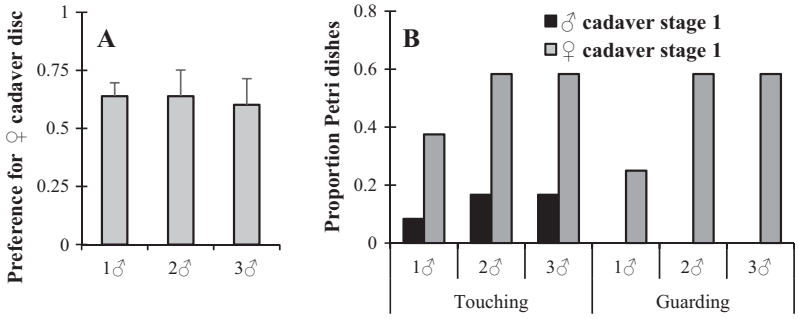

Fig. 6. Choice experiment 4. Male *Tetranychus urticae* behaviour when presented with one male (♂) and one female (♀) stage 1 cadaver on leaf discs in a Petri dish. Stage 1 = Dry non-sporulating cadaver killed by *Neozygites floridana*. (A) Leaf disc choice index (mean + SE) tested at three ♂ densities. Index > 0.5 indicates a preference for the leaf disc with ♀ cadaver. (B) Proportion of Petri dishes in which touching and guarding the two cadavers were observed at least once tested at three ♂ densities.

of *T. urticae* male behaviour, the only cue attractive from a distance was colour (yellow), whilst the right form (ovoid) or odour (extract of ♀QD) were arrestment stimuli only, eliciting and prolonging guarding once the male was within touching distance from a female (Penman and Cone, 1974; Royalty et al., 1992, 1993). From our experiments we cannot exclude the possibility that an interaction between colour and size was involved in the male preference for non-sporulating cadavers.

Concerning chemical cues, the last 40 years there have been a number of studies aiming to identify and understand sex pheromones in *T. urticae*, but the only uncontested conclusion seems to be that ♀QD do send chemical signals to males (Oku, 2014 and references therein). In a study of mating strategies in *Tetranychus kanzawai*, the results were consistent with ♀QD being able to induce male competition and thus exhibiting indirect mate choice (Oku, 2009). As in *T. urticae*, *T. kanzawai* ♀QD become sexually receptive immediately before adult emergence, and only the first mating results in fertilization. Oku (2009) hypothesized that ♀QD only release sex pheromones when a male has started guarding her. She suggested this female behaviour to be the result of a trade-off between the need to avoid predators and the need to incite male competition. We may further speculate that the healthy ♀QD in our experiment were able to detect the pathogenic fungus in the cadavers and therefore shut off their production of attractive odours completely, but we find it unlikely that immobile nymphs should be better at detecting the fungus odour than the actively searching males.

Our results could also be explained by the pathogenic fungus producing chemicals that are attractive to spider mites, thereby being an example of parasitic manipulation of the host's phenotype

(Weinersmith and Faulkes, 2014). The smaller size of male cadavers compared to female cadavers (Fig. 1) could then account for male cadavers being less attractive, since smaller cadavers (each cadaver being full of hyphal bodies) can be assumed to produce smaller amounts of fungal compounds. Alternatively, these compounds could act synergistically with a mite-produced compound leaking from female cadavers only. *N. floridana* is a specialist pathogen, having a relatively narrow host range comprising *Tetranychus* and closely related genera (Hountondji et al., 2002), and may therefore have evolved more host specific mechanisms to increase its host encounter rate than possible for generalist pathogens. In their discussion of the mechanisms behind the mosquito *A. stephensi* being attracted to two entomopathogenic fungi, George et al. (2013) consider host manipulation to be unlikely because both species, *B. bassiana* and *M. anisopliae*, have a very wide host range. Some of the hosts, especially among soil-dwelling insects, are repelled by these fungi (Baverstock et al., 2010).

At touching distance, males were better at detecting the unsuitability of sporulating cadavers as mates. In addition to visual and chemical cues, tactile stimuli could account for this. In particular the presence of spores on the body of the cadaver and on the leaf disc (around infective capilliconidia cadavers only) could have provided new cues of the pathogen presence as cadavers developed. When a primary conidium of *N. floridana* germinates into an infective capilliconidium on a long capillary, the capilliconidium will rise 60–100 μm (Keller, 1997) above the leaf surface. When it only takes one capilliconidium to kill a spider mite (Oduor et al., 1997), and one *Tetranychus* cadaver may throw more than 2000 primary conidia (Wekesa et al., 2010) which germinate into infective secondary capilliconidia, detection before getting too close to a spore-throwing cadaver would be advantageous to the mite host. However, in 40% of our observations, males ventured onto the leaf disc with infective capilliconidia (and quite a few males made it as far as the cadaver). It is therefore likely that the males in our experiment did get into physical contact with spores and only then lost their preference towards female cadavers. Hountondji et al. (2009) found that the cassava green mite *M. tanajoa* avoided spores of *N. tanajoae*, but were indifferent to non-sporulating cadavers of conspecifics. They called this ability of the pathogen to hide (not being repellent) until sporulation 'Trojan horse behaviour', acting to spread the disease into unsuspecting host colonies. They did not study mating behaviour, however, and did not investigate the effect of the stage of the fungal killed cadaver on mite behaviour. They tested the effect of leaf discs with and without *N. tanajoae* spores only (no cadavers present), however, and leaf discs with spores were avoided, but still more than 40% of the mites migrated to leaf discs with spores. It was not specified whether the spores used in the study were primary conidia or the infective secondary capilliconidia.

All our experiments were performed at three male densities, and the choice of leaf disc was affected by male density in the presence of infective conidia only. The preference for the pathogen-free leaf disc by single males suggests a trade-off between avoidance of capilliconidia and competitors. The other male density effect found, was that touching increased with the number of males in the experiments with non-sporulating cadavers. This is the expected pattern from a simple mathematical viewpoint: the probability of observing a behaviour increases with the number of individuals present. The lack of a consistent density response in the other data may be due to competitive interactions creating more complex density responses, the 0/1 dichotomy employed on touching and guarding observations being too crude, and in some cases low sample size (guarding of sporulating cadavers being rare). Guarding normally increases with male density (Potter et al., 1976), but the experimental density we employed was relatively low. An interesting feature of this system is that asymmetric competition for the most attractive females, i.e. the non-sporulating cadavers, may favour the weakest male combatants (usually the smaller males, Potter et al., 1976; Oku, 2014) by rendering them less likely to guard dead and subsequently lethal females. Such a reversed fitness pattern may help to maintain the diversity in male mating strategies observed in *T. urticae* (Sato et al., 2013).

More experiments are needed to identify how males are fooled (until spores appear) by visual or chemical stimuli emitted by cadavers. Unless the males have a strong immune response protecting them from infection, it seems the pathogen has gained an advantage in the evolutionary arms race between *N. floridana* and *T. urticae*. However, the pest status of *T. urticae* proves that other factors, like unsuitable microclimate or light conditions (de Castro et al., 2013) and fungicide use (Klingen and Westrum, 2007; Wekesa et al., 2008) work against *N. floridana* fulfilling its pathogenic potential, at least in intensive agro-ecosystems.

Acknowledgments

This research was funded by the Norwegian Foundation for Research Levy on Agricultural Products (FFL) and the Agricultural Agreement Research Funds (JA) through the project BERRYSYS (project number 190407/110). We thank Torfinn Torp, senior advisor and statistician at Bioforsk for help with the statistical analysis and Erling Fløistad for editing graphical abstract and Fig. 1.

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