Entomopathogenic fungal conidia marginally affect the behavior of the predators *Orius majusculus* (Hemiptera: Anthocoridae) and *Phytoseiulus persimilis* (Acari: Phytoseiidae) foraging for healthy *Tetranychus urticae* (Acari: Tetranychidae)

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Abstract

We determined how conidia of arthropod-pathogenic fungi on leaves affected the behavior of two predators—*Orius majusculus* (Hemiptera: Anthocoridae) and *Phytoseiulus persimilis* (Acari: Phytoseiidae)—when offered a choice between preying on two-spotted spider mites, *Tetranychus urticae* (Acari: Tetranychidae), in the presence or absence of infective conidia of *Metarhizium brunneum* (Ascomycota: Hypocreales) and *Neozygites floridana* (Entomophthoromycota: Neozygitaceae). The results indicate no significant relation between the presence of conidia and predator behavior. The only indication of interference is between the generalists *O. majusculus* and *M. brunneum*, with a trend towards more time spent feeding and more prey encounters turning into feeding events on leaf discs without conidia than on leaf discs with conidia. Our results show that the presence of fungal conidia does not alter the preying behavior of the predators, and using predators and fungi together is not limited by any interference between organisms in the short term.

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Introduction

Arthropod predators and arthropod-pathogenic fungi are important natural enemies of pests used in biological pest control (Hajek and Eilenberg 2018). Arthropod predators encounter arthropod-pathogenic fungi while foraging on plants for prey (Roy and Pell 2000) or when searching for mates (Trandem et al., 2015). Such fungi can affect predators directly through infection or indirectly by competition for prey (Roy and Pell 2000) or by reducing prey quality (Seiedy et al. 2012). Predator behavior can possibly be affected by the perceived threat from a fungus present as conidia on infected, dead target arthropods or as conidia on leaves.

Detailed studies on behavioral effects in systems combining one fungus species and one predator species have shown interesting results. The two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae), infected with its specialist biotrophic fungus *Neozygites floridana* (Entomophthoromycota: Neozygitaceae), can induce behavioral responses in predators (Trandem et al. 2016; Wekesa et al. 2007). This is also possible of prey infected with generalist fungal species, where avoidance often has been reported (Alma et al. 2010; Meyling and Pell 2006; Roy et al. 1998; Wu et al. 2016). Seiedy et al. (2012) showed that prey handling time in the Tetranychid specialist predator *Phytoseiulus persimilis* (Acari: Phytoseiidae) (McMurtry and Croft 1997) increased, while feeding rate decreased when the predator was presented with their target prey, *T. urticae,* infected with mesotrophic generalist entomopathogenic fungus *Beauveria bassiana* (Ascomycota: Hypocreales). The generalist predator *Orius albidipennis* (Hemiptera: Anthocoridae) responded in a similar way to *Thrips tabaci* (Thysanoptera: Thripidae) infected with the mesotrophic generalist entomopathogenic fungus *Metarhizium anisopliae* sensu lato (Ascomycota: Hypocreales); their searching time increased and their feeding time decreased (Pourian et al. 2011). The outcome of predator-fungus interactions in a more natural environment may be significant for successful biological control. In the field, Fischhoff et al. (2017) and Rauch et al. (2017) documented that the mesotrophic generalist *Metarhizium brunneum* (Ascomycota: Hypocreales) (Boomsma et al. 2014) aimed for pest control, did not reduce non-target arthropod abundance and diversity significantly. Actually, the interactions between a predator and a fungus may even prove beneficial for biological control attempts. A study by Azevedo et al. (2017) revealed that the combined use of *M. brunneum* and the specialist predatory gall midge *Aphidoletes aphidimyza* (Diptera: Cecidomyiidae) positively influenced aphid control compared to when either natural enemy was used alone. Though the combined use significantly reduced the number of predatory midges, the same treatment still suppressed the aphid population more than either control agent used alone.

Entomopathogenic fungi and arthropod predators are now often combined to control a complex of pests in a crop. It is therefore important to understand whether their biology and behavior will have a synergistic, antagonistic or no effect on each other. We therefore performed a comparative study on fungus-induced changes in predator behavior and chose the entomopathogenic fungi *M. brunneum* and *N. floridana* that belong to different orders of arthropod pathogenic fungi and are very different in their biology (Boomsma et al. 2014). *Metarhizium brunneum* is a mesotrophic fungus in the Hypocreales that produces large quantities of small, dry conidia in long chains, which are passively detached from dead hosts and can be readily suspended in water. It can be grown on artificial media (sabouraud dextrose agar (SDA), rice etc.), is produced commercially in large quantities, and is used as the active ingredient in several microbial control products and may be used against *T. urticae*. *Neozygites floridana* is a biotrophic fungus in the Entomophthoromycota and a

specialist of *T. urticae*. It actively discharges larger non-infective primary conidia. These primary conidia then produce secondary sticky infective conidia on long capillary tubes, so called capilliconidia (Keller 1997). It only takes one capilliconidium to kill a spider mite (Oduor et al. 1995), and one *Tetranychus* cadaver may throw more than 2000 primary conidia (Wekesa et al. 2010), which germinate into infective secondary capilliconidia. *Neozygites floridana* is an important natural enemy of *T. urticae* and may be produced in vivo on *T. urticae* but not yet for commercial use, though there has been some success of in vitro production by Leite et al. (2000) and an in vivo production patent was also made several years ago (Kennedy and Smitley 1988). We chose to expose these two very different fungal species to the specialist spider-mite predator *P. persimilis* and the generalist predator *Orius majusculus* (Hemiptera: Anthocoridae) (Fathipour and Maleknia 2016) to evaluate their behavioral changes in searching and time feeding on prey when presented with the following leaf disc choice combinations: (1) *M. brunneum* conidia vs. no conidia, (2) *N. floridana* conidia vs. no conidia. As target prey for predators we used the pest mite *T. urticae*.

Materials and methods

Fungi, plants and arthropods

Colonies of *T. urticae* were obtained from a laboratory culture kept on strawberry plants in a plexiglass cage, in a climatically controlled room at 21 °C, 60% RH and 16 h L: 8 h D. The predatory bugs, *O. majusculus,* were provided by the company EWH Bioproduction (Denmark) in bottles containing 500 individuals of all stages mixed with buckwheat. The predatory mites, *P. persimilis,* were provided by LOG (Norway) and by EWH Bioproduction (Denmark) in 100 mL bottles containing 2000 adult mites mixed with vermiculite.

The in vitro culture of *M. brunneum*, isolate KVL 99-112 (i.e. F52, BIPESCO 5) was grown on Sabouraud Dextrose Agar (SDA) at room temperature in darkness for approximately 25 days before harvesting the conidia for the experiments.

The in vivo culture of *N. floridana*, Brazilian isolate ESALQ 1420, was produced as described in Castro et al. (2013). Similar leaf disc methods have also been used by Oduor et al. (1995). The following procedure was used: adult female *T. urticae* were inoculated with conidia of *N. floridana* on bean plants (*Phaseolus vulgaris* cv. Masai). After 8–9 days, *N. floridana* infected *T. urticae* had died and dry non-sporulating cadavers were collected, wrapped in a cotton cloth and stored in Eppendorf tubes at 5 °C until used in the experiment within 30 days.

Preparation of leaf discs with fungal spores

Leaf discs were made from strawberry leaves from the same plant for each observation day. Due to the differences in biology between *M. brunneum,* and *N. floridana,* the preparation of leaf discs with fungal conidia that was used in the choice experiment was conducted in two ways.

For *M. brunneum*, leaf discs were inoculated by dipping them in a conidial suspension. This was done as follows: the *M. brunneum* isolate was taken out of the freezer and transferred to sabouraud dextrose agar (SDA), cultured for 19–25 days at ambient laboratory conditions (21–25 °C; 20–35% RH) placed in a plastic box (22 × 16 × 7 cm) and wrapped

with aluminum foil for darkness. Conidia of *M. brunneum* from SDA were then harvested with a sterile spatula, in sterile water with 0.05% Tween 80 to suspend the hydrophilic fungal conidia in water. The suspension was filtered through a 3-layer cotton cloth and adjusted to 1×10^7 conidia/mL with a Neubauer Improved hemocytometer. Strawberry leaf discs (diameter 15 mm) were then dipped in the *M. brunneum* conidial suspension before being air-dried on a tissue paper with the abaxial side up. Leaf discs with conidia were placed in Petri dishes with water agar (1.5%) at 6 °C overnight to be used in choice experiments the next day. Conidial viability of conidia suspensions were established by a standard germination test (Inglis et al. 2012), and only suspensions with > 95% germination were used in the experiment.

Since *N. floridana* is a biotrophic fungus, it is difficult to produce conidia from other substrates than the host (*T. urticae*) itself. Therefore, three *N. floridana*-killed *T. urticae* cadavers, dorsal side up, were evenly distributed onto one strawberry leaf disc and placed in darkness for 24 h at 20 °C and 90% RH for primary conidia to discharge and germinate to form infective capilliconidia (secondary conidia). For each leaf disc, sporulation of all cadavers and an even distribution of conidia were assured by observing each leaf disc under a compound microscope (X80) prior to the observations. Spore-producing cadavers were carefully removed to obtain similar conditions as for leaf discs with *M. brunneum*, i.e. presence of conidia only, before the introduction of healthy *T. urticae* (see below) and the predator species. All leaf discs, including those receiving no spores, were dipped in 0.05% Tween 80 as described above to correct for possible effects of Tween 80.

Experimental set-up of choice experiment

The experimental set-up is shown in Fig. 1. One strawberry leaf disc with fungal conidia and one strawberry leaf disc without fungal conidia were placed with a small gap between them, onto 1.5% water agar in a Petri dish (diameter 5 cm). The leaf discs were connected with a bridge of Parafilm (10×10 mm) as described by Asalf et al. (2011). Six young adult female *T. urticae* (for *O. majusculus*) or deutonymphs (for *P. persimilis*) were transferred to each leaf disc approximately 1 h before a predator was introduced. During the observation time, *T.*
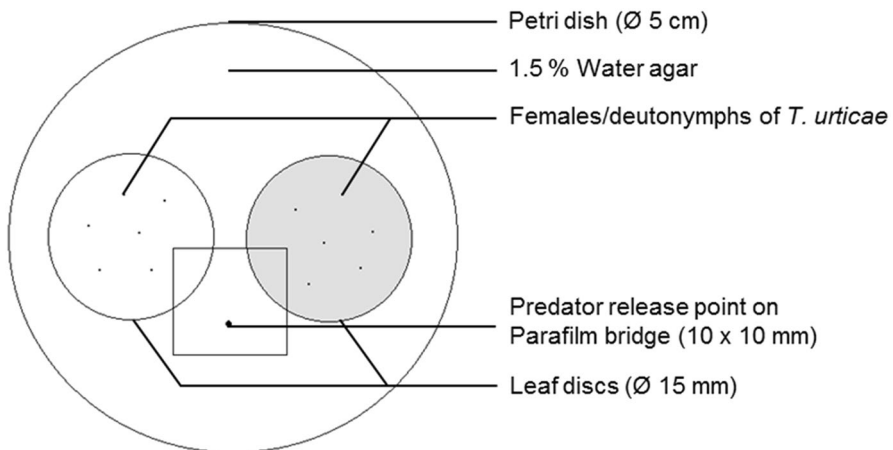

Fig. 1 The experimental arena. The white leaf disc (left) contained no fungal conidia, the grey leaf disc (right) did. The strawberry leaf discs with healthy *Tetranychus urticae* were placed on water agar in a Petri dish and were connected by a Parafilm bridge, on which the predator was released

urticae remained on the leaf discs where they had been released, no webbing was observed, and only on rare occasions did they lay eggs. In such cases, eggs were removed before the introduction of the predator. The set up of the choice experiments was as follows: *O. majusculus* (4th or 5th nymphal stage) or an adult female *P. persimilis* were offered a choice between a leaf disc with (1) *M. brunneum* conidia vs. no conidia, or (2) *N. floridana* vs. no conidia. Petri dishes with two leaf discs without any fungal conidia served as the control. All predators were starved individually in plastic vials, with moist filter paper in a climate cabinet at 23 °C, 16 h L: 8 h D and 70% RH for 24 h prior to the start of the experiment.

Observation of behavior

Each predator was placed in the middle of the Parafilm bridge, allowing it to choose between the two leaf discs. The observation time per treatment was 15 min, starting immediately after the predator was released on the bridge. The following five behaviors were recorded: (1) walking (searching), (2) encountering prey (number of events, when prey was within a body length of the predator and the latter reacted to the presence of the prey), (3) feeding, (4) resting, (5) grooming. If feeding continued after the observation time of 15 min, observation continued until feeding stopped to obtain total feeding time per prey. Furthermore, the number of prey encounters was used to assess the success rate. Searching, resting and grooming time was recorded as it affects predation, especially if conidia attach to the body and legs of the predators.

Observations were made under an even light source. All treatments were replicated three times a day, between 9 am and 4 pm, with the sequence of treatments rotated between observation days (n=9). Each observation was conducted in a new Petri dish with new leaf discs and a new predator. The position of the treated leaf disc (left/right of predator) was randomized. Observations with no feeding events were discarded and the experiment was continued until at least 20 replicates were obtained for each treatment. An average of four observations per day were discarded, mainly due to predator inactivity or it was disturbed by the water barrier surrounding the leaf discs.

Statistical analysis

Three response variables were analyzed separately for the two predators. (1) Number of prey encounter was analyzed with a Poisson regression with log-link including the logarithm of time spent searching as offset in order to correct for searching time. (2) Success of prey encounter turning into a feeding event was analyzed in a binomial regression with logit-link. (3) Feeding time per prey was analyzed in a normal regression after log-transformation. All analyses were done with conidia (none/*N. floridana*/*M. brunneum*) as fixed effect, and with arthropod id as random effect to correct for arthropods that searched on both leaves. If an overall effect of conidia was found, pairwise comparisons of the three levels (none/*N. floridana*/*M. brunneum*) were done with a Tukey correction for multiple testing. The statistical analyses were done in R v.3.2.2 (R Core Team 2015).

Results

Both predators spent the majority of the observation time feeding, followed by time spent searching (Table 1). Little time was invested in resting (0.1–1.5% of total observation time) and grooming (0.1–1.1% of total observation time), and these behaviors were therefore not considered further.

For both predators no significant relations were found between the presence of conidia and number of prey encountered, the proportion of prey encounters resulting in a feeding event, and time spent feeding per prey. However, for *O. majusculus*, there was an almost significant effect of the presence of conidia on the success rate of the predators ($p = 0.10$), and time spent feeding per prey ($p = 0.06$). The odds ratio for a successful feeding event was 3.5 times larger (95% CI 0.9–13.2) on leaf discs with no conidia relative to leaf discs with *M. brunneum* (adjusted $p = 0.07$). The feeding time per prey was 1.8 times longer (95% CI 1.0–3.2) on leaf discs without conidia compared to leaf discs with *M. brunneum* (adjusted $p = 0.06$). As expected, no differences were found between control treatments.

Discussion

The presence of entomopathogenic fungal conidia did not significantly affect the behavior of either predator species, either directly or indirectly, through changes in behavior of the prey. As described above, the conidia of the generalist fungus *M. brunneum* and the specialist fungus *N. floridana* both have the potential to influence predator behavior in different ways due to their very different biological characteristics.

The primary conidium of *N. floridana* germinates into an infective sticky capilliconidium on a long capillary that will rise 60–100 µm above the leaf surface (Keller 1997; Trandem et al. 2015). Capilliconidia easily break off and can attach to the body and legs of host and non-host arthropods (Delalibera et al. 2003). Specialist fungi cannot infect the predators and do therefore not pose a threat to them as such. This being said, we

Table 1 Predator searching time, feeding time and prey encounters by *Orius majusculus* and *Phytoseiulus persimilis*

Fungus	Predator	+/− conidia	Searching (% of total observation time)	Feeding	Prey encounters (No. of events)
Metarhizium brunneum	*O. majusculus*	+	7.7 ± 2.5	24.4 ± 7.9	1.0 ± 0.2
		−	9.2 ± 1.7	46.3 ± 8.6	1.0 ± 0.2
	P. persimilis	+	5.3 ± 1.7	31.0 ± 8.5	1.1 ± 0.5
		−	8.7 ± 2.5	51.6 ± 8.7	1.2 ± 0.3
Neozygites floridana	*O. majusculus*	+	8.1 ± 2.1	48.5 ± 8.1	1.3 ± 0.3
		−	5.8 ± 1.5	29.1 ± 8.5	0.6 ± 0.2
	P. persimilis	+	12.2 ± 3.2	42.0 ± 8.2	0.8 ± 0.2
		−	4.1 ± 1.4	37.0 ± 9.2	0.5 ± 0.2

Each treatment consisted of one fungal species and one predator species that was given a choice between a leaf disc with fungal conidia (+) and a leaf disc without fungal conidia (−)

The values are calculated averages of searching time and feeding time as percentage of the total observation time (excluding time spent on the platform and time spent resting and grooming). Prey encounters are the average number of prey encounters per observation period (no. of events) ± standard error

considered the physical presence of *N. floridana* conidia as likely to disturb the preying behavior of the predator, but this showed not to be the case. A longer observation time would perhaps reveal an interference between the specialist fungal conidia and the predators, as found by Wekesa et al. (2007).

Metarhizium brunneum produces smaller conidia (length 5.0–7.0 μm; Bischoff et al. 2009), and can induce avoidance responses, as other generalist entomopathogens do, by being perceived as a threat by predators that encounter them (Alma et al. 2010; Meyling and Pell 2006; Ormond et al. 2011). Previous studies with generalist entomopathogenic fungi have shown behavioral changes in predators (Pourian et al. 2011; Seiedy et al. 2012), but unlike in the present study, previous studies have been conducted with inoculated prey. Infected prey are likely producing a different volatile profile, ultimately increasing the likelihood of an altered predator response. Both situations are relevant and important for the understanding of the interactions between natural enemies, and both must be considered when developing strategies for pest control.

The lack of significant differences found in behavior in response to fungal conidia can also have been caused by low conidial concentrations. Because of distinct differences in life styles of the two fungal species, it was necessary to utilize two methods of applying fungal inoculum. The presence of fungal inoculum was established with agar imprints and visual observations with a microscope throughout the experiment, while the specific concentrations on the leaf discs were not known and not comparable between species.

There was a trend towards a significant effect of *M. brunneum* conidia on the behavior of *O. majusculus*. This predator spent more time feeding and had more prey encounters turning into feeding events on leaf discs without conidia than on leaf discs with *M. brunneum*, i.e. where there was no risk for the predator to engage in these behaviors. This trend needs confirmation with longer observation times. If confirmed, this would support the findings from other studies (Alma et al. 2010; Meyling and Pell 2006; Ormond et al. 2011).

Except for *N. floridana*, the organisms used in the present study are commonly used separately in augmentative biological control against various pests (Eilenberg et al. 2001; Gacheri et al. 2015; Gerson and Weintraub 2007; van Lenteren 2012). The outcome of such augmentative releases would be affected by the interaction between the released organisms. This study shows that the combined use of these natural enemies of taxonomically remote groups in augmentative releases will not interfere with each other, at least not initially. Thus, our initial expectation that the presence of entomopathogenic fungal conidia would alter the preying behavior of predators was not confirmed. Nevertheless, inoculated prey and risk of infection of predators should be investigated further as there may be long-term negative or positive effects on pest control.

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