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1 1 **Only marginal effects of entomopathogenic fungal conidia on the preying behavior of two arthropod**  
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3 2 **predators *Orius majusculus* (Hemiptera: Anthocoridae) and *Phytoseiulus persimilis* (Acari:**  
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5 3 **Phytoseiidae) preying on healthy *Tetranychus urticae* (Acari: Tetranychidae)**  
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142 **Abstract:** We determined how conidia of entomopathogenic fungi on leaves affected the behavior of two  
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343 different predators (*Orius majusculus* [Hemiptera: Anthocoridae] and *Phytoseiulus persimilis* [Acari:  
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544 Phytoseiidae]) when offered a choice between preying on two spotted spider mites (*Tetranychus urticae*  
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845 [Acari: Tetranychidae]) in the presence or absence of infective conidia of *Metarhizium brunneum*  
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1046 (Ascomycota: Hypocreales) and *Neozygites floridana* (Entomophthoromycota: Neozygitaceae). The results  
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1247 indicate no significant relation between the presence of conidia and predator behavior. The only indication of  
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1448 interference is between the generalists; *O. majusculus* and *M. brunneum*, with a trend towards more time  
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1649 spent feeding and more prey encounters turning into feeding events on leaf discs with no conidia compared  
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1950 to leaf discs with conidia of *M. brunneum*. Our results show that the presence of fungal conidia do not alter  
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2151 the preying behavior of predators, and a use in combination is initially not limited by any interferences  
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2352 between organisms.

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4860 **Keywords:** behavior, entomopathogenic fungi, predators, Hypocreales, Neozygitales, biological control  
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163 **Introduction**

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464 Arthropod predators and arthropod-pathogenic fungi are important natural enemies of pests and are used in  
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65 biological pest control (Hajek and Eilenberg 2018). Arthropod predators encounter arthropod-pathogenic  
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966 fungi while foraging on plants for prey (Roy and Pell 2000) or when searching for mates (Trandem et al,  
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1167 2015). Such fungi can affect predators directly through infection or indirectly by competition for prey (Roy  
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1368 and Pell 2000) or by reducing prey quality (Seiedy et al. 2012). Predator behavior can possibly be affected  
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1569 by the perceived threat from a fungus present as conidia on infected, dead target arthropods or as conidia on  
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1870 leaves.

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2171 Detailed studies on behavioral effects in systems combining one fungus species and one predator species  
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2372 have shown interesting results. The two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae),  
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2573 infected with its specialist biotrophic fungus *Neozygites floridana* (Entomophthoromycota: Neozygitaceae),  
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2774 can induce behavioral responses in predators (Trandem et al. 2016; Wekesa et al. 2007). This is also possible  
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3075 of prey infected with generalist fungal species, where avoidance often have been reported (Alma et al. 2010;  
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3276 Meyling and Pell 2006; Roy et al. 1998; Wu et al. 2016). Seiedy et al. (2012) showed that prey handling time  
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3477 in the Tetranychid specialist predator *Phytoseiulus persimilis* (Acari: Phytoseiidae) (McMurtry and Croft  
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3678 1997) increased, while feeding rate decreased when the predator was presented with their target prey, *T.*  
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3979 *urticae*, infected with mesotrophic generalist entomopathogenic fungus *Beauveria bassiana* (Ascomycota:  
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4180 Hypocreales). The generalist predator *Orius albidipennis* (Hemiptera: Anthocoridae) responded in a similar  
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4381 way to *Thrips tabaci* (Thysanoptera: Thripidae) infected with the mesotrophic generalist entomopathogenic  
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4582 fungus *Metarhizium anisopliae* sensu lato (Ascomycota: Hypocreales); their searching time increased and  
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4883 their feeding time decreased (Pourian et al. 2011). The outcome of predator-fungus interactions in a more  
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5084 natural environment may be significant for successful biological control. Fischhoff et al. (2017) and Rauch et  
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5285 al. (2017) documented in their field studies that the mesotrophic generalist *Metarhizium brunneum*  
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5486 (Ascomycota: Hypocreales) (Boomsma et al. 2014) aimed for pest control, did not reduce non-target  
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5687 arthropod abundance and diversity significantly. Actually, the interactions between a predator and a fungus  
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188 may even prove beneficial for biological control attempts. A study by Azevedo et al. (2017) revealed that the  
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389 combined use of *M. brunneum* and the specialist predatory gall midge, *Aphidoletes aphidimyza* (Diptera:  
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590 Cecidomyiidae) positively influenced aphid control compared to when either natural enemy was used alone.  
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891 Though the combined use significantly reduced the number of predatory midges, the same treatment still  
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1092 suppressed the aphid population more than either control agent used alone.  
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1393 Entomopathogenic fungi and arthropod predators are now often combined to control a complex of pests in a  
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1594 crop. It is therefore important to understand whether their biology and behavior will have a synergistic,  
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1795 antagonistic or indifferent effect on each other. We therefore performed a comparative study on fungus  
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2096 induced behavioral changes in predator behavior and chose the entomopathogenic fungi *M. brunneum* and *N.*  
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2297 *floridana* that belong to different orders of arthropod pathogenic fungi and are very different in their biology  
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2498 (Boomsma et al. 2014). *Metarhizium brunneum* is a mesotrophic fungus in the Hypocreales that produce  
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2699 large quantities of small, dry conidia in long chains, which are passively detached from dead hosts and can  
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3101 be readily suspended in water. *Metarhizium brunneum*, which can be grown on artificial media (sabouraud  
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4005 dextrose agar (SDA), rice etc.), is produced commercially in large quantities and used as the active  
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5110 ingredient in several microbial control products and, it may be used against *T. urticae*. *Neozygites floridana*  
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113 behavioral changes in searching and feeding time of prey when presented to the following leaf disc choice  
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114 combinations: 1) *M. brunneum* conidia vs no conidia, 2) *N. floridana* conidia vs no conidia. As target prey  
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115 for predators we used the pest mite *T. urticae*.  
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## 117 **Material and methods**

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118 *Fungi, plants and arthropods*

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119 Colonies of *T. urticae* were obtained from a laboratory culture kept on strawberry plants in a plexiglass cage,  
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120 in a climatically controlled room at 21 °C, 60% RH and 16 h L: 8 h D. The predatory bugs, *O. majusculus*,  
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121 were provided by the company EWH Bioproduction (Denmark) in bottles containing 500 individuals of all  
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122 stages mixed with buckwheat. The predatory mites, *P. persimilis*, were provided by LOG (Norway) and by  
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123 EWH Bioproduction (Denmark) in 100 mL bottles containing 2000 adult mites mixed with vermiculite.  
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124 The *in vitro* culture of *M. brunneum*, isolate KVL 99-112 (i.e. F52, BIPESCO 5) was grown on Sabouraud  
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125 Dextrose Agar (SDA) at room temperature in darkness for approximately 25 days before harvesting the  
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126 conidia for the experiments.  
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127 The *in vivo* culture of *N. floridana*, Brazilian isolate ESALQ 1420, was produced as described in Castro et al.  
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128 (2013). Similar leaf disc methods have also been used by Oduor et al. (1995). The following procedure was  
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129 used: adult female *T. urticae* were inoculated with conidia of *N. floridana* on bean plants (*Phaseolus vulgaris*  
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130 cv. Masai). After 8–9 days, *N. floridana* infected *T. urticae* had died and dry non-sporulating cadavers were  
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131 collected, wrapped in a cotton cloth and stored in Eppendorf tubes at 5 °C until used in the experiment  
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132 within 30 days.  
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134 *Preparation of leaf discs with fungal spores*

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135 Leaf discs were made from strawberry leaves from the same plant for each observation day. Due to the  
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136 differences in biology between *M. brunneum*, and *N. floridana*, the preparation of leaf discs with fungal  
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137 conidia that was used in the choice experiment was conducted in two different ways.  
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138 For *M. brunneum*, leaf discs were inoculated by dipping them in a conidial suspension. This was done as  
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10 follows: the *M. brunneum* isolate was taken out of the freezer and transferred to sabouraud dextrose agar  
1139 (SDA), cultured for 19-25 days at ambient laboratory conditions (21-25 °C; 20-35% RH) placed in a plastic  
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1340 box (22x16x7 cm) and wrapped with aluminum foil for darkness. Conidia of *M. brunneum* from SDA were  
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1541 then harvested with a sterile spatula, in sterile water with 0.05% Tween 80 to make the hydrophilic fungal  
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1742 conidia suspended in water. The resulting conidia suspension was filtered through a 3-layer cotton cloth and  
18  
1943 conidia suspended in water. The resulting conidia suspension was filtered through a 3-layer cotton cloth and  
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21 adjusted to  $1 \times 10^7$  conidia/mL by the use of a Neubauer Improved hemocytometer. Strawberry leaf discs  
2244 (diameter 15 mm) were then dipped in the *M. brunneum* conidial suspension before air-dried on a tissue  
23  
2445 paper with the abaxial side up. Leaf discs with conidia were placed in Petri dishes with water agar (1.5%) at  
25  
2646 6 °C overnight to be used in choice experiments the next day. Conidial viability of conidia suspensions were  
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2847 established by a standard germination test (Inglis et al. 2012), and only suspensions with > 95% germinating  
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3048 conidia were used in the experiment.  
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3650 Since *N. floridana* is a biotrophic fungus it is difficult to produce conidia from other substrates than the host  
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3851 (*T. urticae*) itself. Hence, a method taking this into account was used. Three *N. floridana*-killed *T. urticae*  
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40 cadavers, dorsal side up, were evenly distributed onto one strawberry leaf disc and placed in darkness for 24  
4152 h at 20 °C and 90% RH for primary conidia to discharge and germinating of primary conidia to form  
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4353 infective capilliconidia (secondary conidia). For each leaf disc, sporulation of all cadavers and an even  
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4554 distribution of conidia were assured by observing each leaf disc under a compound microscope (X80) prior  
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4755 to the observations. Spore producing cadavers were carefully removed, to obtain similar conditions as for  
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4956 leaf discs with *M. brunneum*, i.e. presence of conidia only, before the introduction of healthy *T. urticae* (see  
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5157 below) and the predator species. All leaf discs were dipped in 0.05% Tween 80 as described for *M.*  
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5559 *brunneum* prior to inoculation with *N. floridana* to ensure that a possible Tween 80 effect was similar for  
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160 both fungal treatments. Leaf discs with no fungal conidia (control) were also dipped in sterile water and  
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161 0.05% Tween 80.  
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### 163 *Experimental set-up of choice experiment*

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164 The experimental set-up is shown in Fig. 1. One strawberry leaf disc with fungal conidia and one strawberry  
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165 leaf disc with no fungal conidia were placed with a small gap between them, onto 1.5% water agar in a Petri  
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166 dish (diameter 5 cm). The leaf discs were connected by a bridge of Parafilm (10x10 mm) as described by  
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167 Asalf et al. (2011). Six *T. urticae* adults (for *O. majusculus* choice) or deutonymphs (for *P. persimilis* choice)  
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168 were transferred to each leaf disc approximately one h before predators (one per dish) were introduced.  
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169 During the observation time, *T. urticae* remained on the leaf disc, no webbing was observed, and only on  
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170 rare occasions did they lay eggs. In such cases, eggs were removed before the introduction of the predator.  
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171 The set up of the choice experiments was as follows: *O. majusculus* or *P. persimilis* choosing between a leaf  
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172 disc with 1) *M. brunneum* conidia vs no conidia, or 2) *N. floridana* vs no conidia. Petri dishes with two leaf  
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173 discs without any fungal conidia served as the control. Young adult of *T. urticae* females were used as prey  
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174 for fourth and fifth stage nymphs of *O. majusculus* while smaller *T. urticae* female deutonymphs were used  
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175 as prey for adult females of *P. persimilis*. All predators were starved individually in plastic vials, with moist  
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176 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  
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177 experiment.  
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183 encountering prey (number of events, when prey was within a body length of the predator and reacted to the  
2 presence of the prey), 3) feeding, 4) resting, 5) grooming. If feeding continued after the observation time of  
184 15 min, observation continued until feeding stopped to obtain total feeding time per prey. Furthermore, the  
3 number of prey encounters were used to assess the success rate. Searching, resting and grooming time was  
185 recorded as it affects predation, especially if conidia attach to the body and legs of the predators.  
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1388 Observations were made under an even light source. All treatments were replicated three times a day,  
14 between 9 am and 4 pm, with the sequence of treatments rotated between observation days (n=9). Each  
15 observation was conducted in a new petri dish with new leaf discs and a new predator. The position of the  
16 treated leaf disc (left/right of predator) was randomized. Observations with no feeding events were discarded  
17 and the experiment was continued until at least 20 replicates were achieved for each treatment. An average  
18 of four observations per day were discarded, mainly due to predator inactivity or it was disturbed by the  
19 water barrier surrounding the leaf discs.  
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### 196 *Statistical analysis*

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

206 **Results**

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207 Of the total observation time, both predators spent the majority of the observation time feeding, followed by  
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208 time spent searching (Table 1). Little time were invested in resting (0.1-1.5% of total observation time) and  
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209 grooming (0.1-1.1% of total observation time), and are therefore not considered further as behaviors of  
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10 significance in the present study.

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211 For both predators no significant relations were found between conidia and number of prey encountered,  
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212 success of prey encounters turning into a feeding event, and time spent feeding per prey. However, for *O.*  
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213 *majusculus*, there was a borderline influence of the presence of conidia on the success rate of the predators  
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214 (p=0.10), and time spent feeding per prey (p=0.06). The odds ratio for a successful feeding event was 3.5  
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215 times larger (95% CI: 0.9–13.2) on leaf discs with no conidia relative to leaf discs with *M. brunneum*  
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216 (adjusted p=0.07). The feeding time per prey was 1.8 times longer (95% CI: 1.0–3.2) on leaf discs with no  
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217 conidia compared to leaf discs with *M. brunneum* (adjusted p=0.06). As expected, no differences were found  
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218 between control treatments.

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220 **Discussion**

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321 The presence of entomopathogenic fungal conidia did not affect the behavior of either predator species. As  
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422 described above, the conidia of the generalist fungus *M. brunneum* and the specialist fungus *N. floridana*  
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423 both have the potential to influence predator behavior in different ways due to their very different biological  
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424 characteristics.

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425 The primary conidium of *N. floridana* germinates into an infective sticky capilliconidium on a long capillary  
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5226 that will rise 60–100 µm (Keller, 1997) above the leaf surface (Trandem et al, 2015). Capilliconidia easily  
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527 break off and can attach to the body and legs of host and non-host arthropods (Delalibera et al. 2003).

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528 Specialist fungi cannot infect the predators and do therefore not pose a threat to them as such. This being

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229 said, we considered the physical presence of *N. floridana* conidia as likely to disturb the preying behavior of  
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230 the predator, but this showed not to be the case. A longer observation time would perhaps reveal an  
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231 interference between the specialist fungal conidia and the predators, as found by Wekesa et al. (2007).  
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232 *Metarhizium brunneum* produces smaller conidia (length 5.0-7.0 µm (Bischoff et al. 2009)), and can as other  
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1233 generalist entomopathogens induce avoidance responses, by being perceived as a threat by predators that  
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1234 encounter them (Alma et al. 2010; Meyling and Pell 2006; Ormond et al 2011). Previous studies with  
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1535 generalist entomopathogenic fungi have shown behavioral changes in predators (Pourian et al. 2011; Seiedy  
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1736 et al. 2012), but unlike in the present study, previous studies have been conducted with inoculated prey.  
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2037 Infected prey are likely inducing a stronger volatile profile, ultimately increasing the likelihood of an altered  
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2238 predator response. Both situations are relevant and important for the understanding of the interactions  
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2439 between natural enemies, and both must be considered when developing strategies for pest control.  
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2740 No differences found in behavioral allocation by fungal conidia can also be a response of low conidial  
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3041 concentration. Because of distinct differences in life styles of the two fungal species, it was necessary to  
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3242 utilize two methods of applying fungal inoculum. The presence of fungal inoculum was established by agar  
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3443 imprints and visual observations in the microscope throughout the experiment, while the specific  
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3644 concentrations on the leaf discs was not known and not comparable between species.  
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3945 There was a trend towards an influence of *M. brunneum* conidia, on the behavior of *O. majusculus*. *Orius*  
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4146 *majusculus* spent more time feeding and had more prey encounters turned into feeding events on leaf discs  
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4447 with no conidia than on leaf discs with *M. brunneum*, i.e. where there was no risk for the predator to engage  
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4648 in these behaviors. This trend may be confirmed with a longer observation time. If that is the case, this would  
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4849 support the findings from other studies (Alma et al. 2010; Meyling and Pell 2006; Ormond et al 2011).  
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5150 Three of the four organisms used in the present study (not *N. floridana*) are commonly used individually in  
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5451 augmentative biological control against various pests (Eilenberg et al. 2001; Gacheri et al. 2015; Gerson and  
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5652 Weintraub 2007; van Lenteren 2012). The outcome of such augmentative releases would be affected by the

253 interaction between the released organisms. This study shows that the combined use of these natural  
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254 enemies, of taxonomically remote groups, in augmentative releases will not initially interfere with each  
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255 other. We can hereby not confirm our initial expectations; that the presence of entomopathogenic fungal  
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256 conidia would alter the preying behavior of predators. Considering beyond this point, inoculated prey and  
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1257 risk of infection of predators should be investigated further as it may have long-term negative or positive  
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1258 effects on pest control.  
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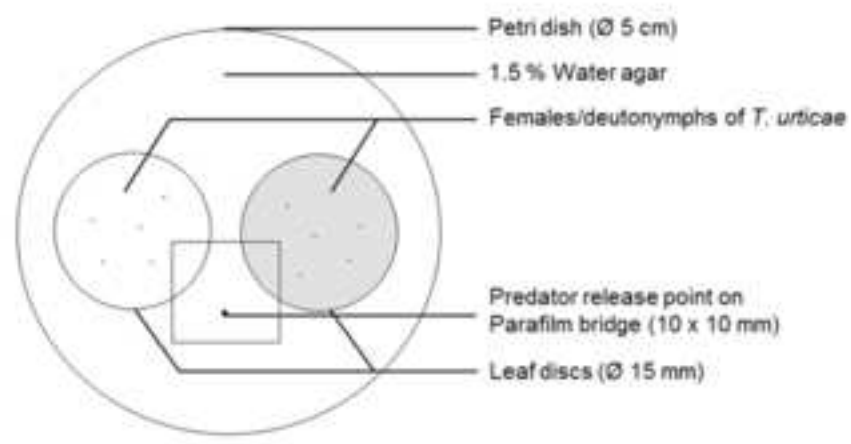


342 **Figure captions:**

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343 **Fig. 1** Set-up of the experimental arena. White leaf disc (left) = no fungal conidia, grey leaf disc (right) =  
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344 with fungal conidia. The Petri dish contained water agar with healthy *Tetranychus urticae* on strawberry leaf  
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345 discs. The leaf discs were connected by a Parafilm bridge where the predator was released.

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## Tables

Table 1. Predator searching time, feeding time and prey encounters by *O. majusculus* and *P. persimilis*. Each treatment consists of one fungal species and one predator species given a choice between a leaf disc with fungal conidia (+) and a leaf disc without fungal conidia (-).

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

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