

# Effects of *Metarhizium anisopliae* on host choice of the bee-parasitic mite *Varroa destructor*

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## ABSTRACT

The ectoparasitic mite *Varroa destructor* poses one of the biggest threats to the western honeybee *Apis mellifera*. Possibilities to control the mites are limited; therefore, it is important to assess the performance of novel alternatives like the use of biological control agents. The goal of our work was to evaluate the effects of the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae* BIPESCO 5, which is a potential biological control agent against the bee parasitic mite *V. destructor*, on the behavior of the parasite. In detail, we investigated whether the presence of fungal spores on the surface of host nurse bees, *A. mellifera*, affects the host choice behavior of adult female mites. We conducted two behavioral assays to monitor the behavior of the mites towards adult bees inoculated with a solution of fungal spores. Both choice and no-choice experiment revealed that *M. anisopliae* has a significant repellent influence on *V. destructor*'s host selection behavior. The mites preferred, and stayed longer on, nurse bees free from fungal spores over bees carrying fungal spores. Our study provides the first evidence of sub-lethal behavioral effects of a fungal biocontrol agent against *Varroa* mites.

**Keywords** *Apis mellifera*, biological control, *Metarhizium anisopliae* var. *anisopliae* BIPESCO 5, *Varroa destructor*, arthropod behavior

## Introduction

*Apis mellifera* is worldwide the most widely distributed honeybee species (van Engelsdorp and Meixner 2010). Recently elevated losses in managed honeybee colonies have been documented across the Northern hemisphere (Neumann and Carreck 2010). The performance of *A. mellifera* may be compromised by a range of diseases and environmental threats. One of the prime organisms challenging *A. mellifera* is the ectoparasitic mite *Varroa destructor*. Its direct damage through sucking hemolymph and its function as vector of viruses have made *V. destructor* the most detrimental honeybee pest (Genersch 2010). Attempts developed so far to control this ectoparasite have not yet been fully satisfactory and entail various disadvantages concerning bee, user and consumer (Genersch 2010; Rosenkranz *et al.* 2010). An alternative approach to established strategies, such as treatment with organic acids or synthetic acaricides, is the use of natural enemies, such as entomo-pathogenic fungi (Kanga *et al.* 2002; Rosenkranz *et al.* 2010; Meikle *et al.* 2012). According to Chandler *et al.* (2001), who evaluated the prospects of biological control of *V. destructor*, entomo-pathogenic fungi appear to be the most promising functional group of natural enemies. Fungi showing promising potential as biological control agents comprise primarily *Beauveria bassiana*, *Hirsutiella thompsoni* and

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*Metarhizium anisopliae* (for review Chandler *et al.* 2001; Meikle *et al.* 2012), with the latter being the focal fungal species in our study. The risk of non-target effects of *M. anisopliae* on bee hives seems low to negligible (for review Meikle *et al.* 2012) but more investigations are needed (Bull *et al.* 2012). The lethal effects of *M. anisopliae* on *V. destructor* were observed both in the laboratory (Shaw *et al.* 2002; Kanga *et al.* 2002, 2007, 2010; García- Fernández *et al.* 2008; Rodríguez *et al.* 2009) and in the field (for review Meikle *et al.* 2012; Ahmed and Abd-Elhady 2013). However, nothing is known about possible sub-lethal and/or behavioral effects of *M. anisopliae* on *V. destructor*.

Here, we investigated behavioral changes of *V. destructor* in its phoretic phase when exposed to adult bees carrying fungal spores of *Metarhizium anisopliae* var. *anisopliae* BIPESCO 5. During the mites' phoretic phase the adult female mites stay and feed on adult bees. Riding on adult bees, the mites can be transported to brood cells or can be distributed by foraging and swarming bees (Kuenen and Calderone 1997; Rosenkranz *et al.* 2010). For having reproductive success it is crucial for *V. destructor* to choose a suitable host, not only for their reproductive phase, but also for their phoretic phase. Therefore, the mites show distinct host preferences in both phases and are able to distinguish the age and task of adult worker bees. Nurse bees, at an age of 3 to 12 days, are the most infested group in the hive and are preferred by *V. destructor* over older forager bees and freshly emerged bees (Kuenen and Calderone 1997). Choosing nurse bees as their hosts ensures the successful transport of *V. destructor* to brood cells.

*Varroa* mites are able to perceive differences in light, vibration and temperature to find their hosts, however, most important are chemical cues (Rosenkranz *et al.* 2010). Several studies have focused on the influence of semiochemicals in *Varroa* host-finding behavior, for example the repellent effect of the forager bees' Nasanov pheromone (Hoppe and Ritter 1988) and various cuticular hydrocarbons of the bees (Del Piccolo *et al.* 2010). Changes in patterns of cuticular hydrocarbons by age have been found in drones, workers and larvae (Rosenkranz *et al.* 2010). Aumeier *et al.* (2002) showed that there are clearly distinct patterns in the relative amounts of cuticular hydrocarbons in the fourth and fifth larval stage and proposed that these patterns could function as signals in the host-parasite interaction between the bees and the mites. Further investigations on the signaling role of these hydrocarbons have been made (Del Piccolo *et al.* 2010) with the conclusion that *V. destructor* is very likely able to distinguish between different cuticular compounds, enhancing its host choice decisions. Here, we conducted two behavioral assays, choice and no-choice, to determine whether host finding and acceptance decisions of *V. destructor* are influenced by the application of fungal spores of *M. anisopliae* on bees.

## Materials and methods

### Mites and bees

*Apis mellifera* nurse bees were collected randomly from 12 Dadant hives established in 2013. Honeybees were considered as nurse bees when observed repeatedly visiting brood cells and tending larvae (Del Piccolo *et al.* 2010). The colonies belonged to the subspecies *Apis mellifera carnica*. All collected bees were killed by deep-freezing and stored at -20°C. No measures to control *V. destructor* were taken in the apiary used for bee and mite collections. Phoretic mites were collected randomly from the hives. To this end, visibly healthy adult bees infested with *V. destructor* were picked from the combs. They were then ice-chilled to facilitate transport and removal of the mites. The mites were picked up with a camel's-hair brush and placed in a petri-dish, containing wet filter paper to prevent desiccation, until they were used in the experiment (Dietemann *et al.* 2013).

## Behavioral assays

Two kinds of behavioral assays, choice and no-choice test, were conducted. Before use in the behavioral assays, all petri-dishes were cleaned with the non-polar solvent pentane. In both experiments, we used dead instead of live bees to avoid any inadvertent influence of different moving activities and cleaning behaviors of the bees on the mite's host choice behavior. For the choice test (experiment 1) two dead adult bees were placed in a petri-dish (60 mm diameter) at opposite sides facing each other. A solution was prepared consisting of conidial *M. anisopliae* var. *anisopliae* BIPESCO 5 spores and the detergent 0.1% TWEEN®20. Using a pipette, one of the bees was inoculated with 30 µl of spore solution at a concentration of  $3.8 \times 10^8$  CFU/ml, the second bee was inoculated with 30 µl of 0.1% TWEEN®20. An adult female mite in its phoretic phase was placed in the middle of the petri-dish, which was then placed in a climate cabinet (35°C, 70% RH). In regular intervals over 3 h, the position of the mite was categorized as follows: (i) climbed on bee (fungus-treated or control bee), (ii) closer to a given bee (shorter distance between mite and fungus-treated or control bee), and (iii) undecided (mite at equal distance to either bee). Further, mite activity (moving or stationary) was recorded. Observations were made every 10 min in the 1<sup>st</sup> h, and every 30 min in the 2<sup>nd</sup> and 3<sup>rd</sup> h. Fungus-treated and control bees were placed alternately on each side, to minimize any inadvertent positional effects of external factors. The experiment was replicated 60 times; no petri-dish, bee or mite was used more than once.

In the no-choice test (experiment 2), an adult female mite in her phoretic phase was presented one adult bee, either inoculated with fungal spores and the detergent 0.1% TWEEN®20 (fungus treatment) or only treated with the detergent (control treatment), in a petri-dish (60 mm diameter). Fungus-treated bees were randomly placed on the right or the left side of the mite to minimize any inadvertent influence of external factors on the behavior of the mites. For each group, control and fungus treatment, 20 replicates were conducted. Each petri-dish, each bee and each mite were used only once. The mites' activity and position were recorded over 3 h, at the same time intervals as in the choice test, with the only exception that positions were only categorized as 'on bee' or 'not on bee'.

## Statistical analyses

All statistical analyses were carried out using IBM SPSS Statistics 22 (IBM Corp., Armonk, NY, USA). In experiment 1, the choice of the mites for control or fungus-treated bees and their activity (moving/stationary) over time (used as inner subject variable) were analyzed by separate generalized estimation equations (GEE; binomial distribution, logit link function). In both analyses, AR-1 was used as working correlation matrix, as the repeated observations of single mites were correlated over time. Choice decisions were scored when mites were either observed directly on bees or when they were clearly closer to a given bee. Accordingly, GEE for choice was run twice, with and without including the score "closer to a given bee". The number of mites deciding to climb on a fungus-treated or control bee within 20 min was compared by a binomial test, assuming equal probability (0.5).

In experiment 2, we used generalized estimating equations (GEE; binomial distribution, counts of events) to analyze whether the number of mites sitting on bees over time differed between mites presented control bees and those presented bees treated with *M. anisopliae*. The activity patterns (on bee, moving or stationary) over time were compared between mites presented control bees and those presented fungus-treated bees by GEE (multinomial distribution, cumulative logit link function). In both analyses, AR-1 was used as working correlation matrix, as the counts and observations of individual mites were correlated over time.

## Results

### Choice test

Mites given a choice between nurse bees with fungal spores (fungus treatment) and nurse bees without fungal spores (control treatment) preferentially orientated towards control bees (GEE: Wald  $\chi^2_1 = 6.064$ ,  $P = 0.014$ ), which did not significantly change over time (Wald  $\chi^2_1 = 0.035$ ,  $P = 0.85$ ) (Figure 1A). This was also true when excluding the option ‘close to bee’ and only scoring mites that had climbed on bees (Wald  $\chi^2_1 = 8.660$ ,  $P = 0.003$ ). However, in the latter analysis also time had an influence (Wald  $\chi^2_1 = 5.863$ ,  $P = 0.015$ ) (Figure 1B). The number of mites sitting on treated bees slightly increased over time, while the number of mites sitting on control bees stayed at the same level over time (Figure 1B). 22 out of 60 mites were observed sitting on a control bee at least once in the course of the experiment, in contrast to 13 mites sitting at least once on a fungus-treated bee.

The mites decided more quickly to climb onto control bees than on fungus-treated bees. Decisions for the control bees were made by 18 of 60 mites (30%) within 20 min while decisions to climb onto fungus-treated bees took longer; only 4 of 60 mites (7%) took such a decision within 20 min (binomial test;  $P = 0.004$ ). 32 of 60 mites (53.33%) were observed at least once on a bee, 28 mites (46.67%) were never observed on a bee. Activity (moving/stationary) of the mites did not change over the course of the experiment (GEE: Wald  $\chi^2_1 = 0.673$ ,  $P = 0.41$ ) (Figure 2).

### No-choice test

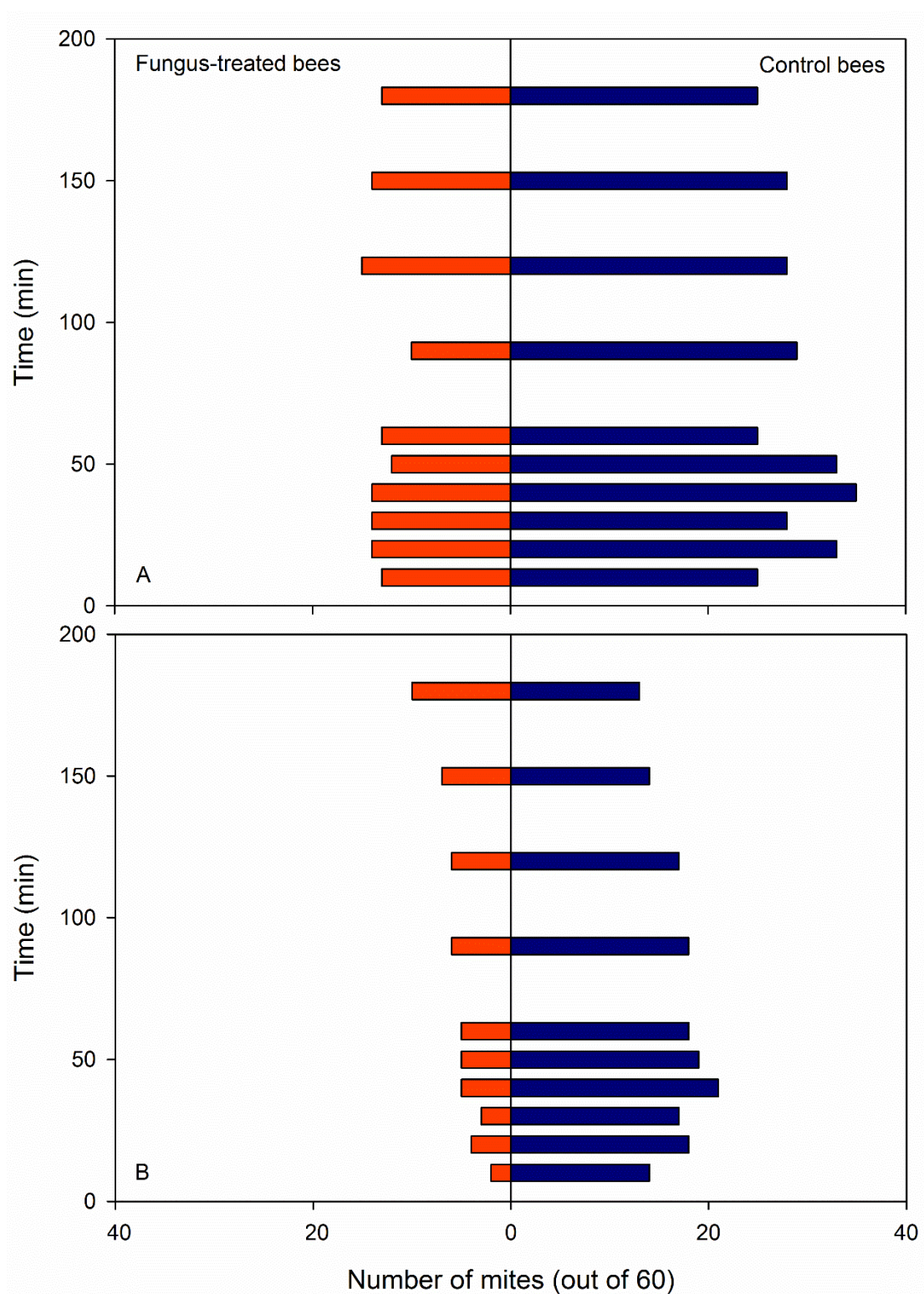
The number of mites sitting on bees differed significantly between mites presented control bees and mites presented *Metarhizium*-treated bees (GEE; Wald  $\chi^2_1 = 8.395$ ,  $P = 0.004$ ) but progressed similarly over time (Wald  $\chi^2_2 = 1.083$ ,  $P = 0.58$ ). Across time, more mites climbed on control than *Metarhizium*-treated bees (Figure 3).

The activity patterns differed between mites presented fungus-treated and control bees (GEE; Wald  $\chi^2_1 = 5.316$ ,  $P = 0.021$ ) but progressed similarly over time (Wald  $\chi^2_2 = 3.060$ ,  $P = 0.22$ ). Mites on control bees showed a higher activity, especially from minute 30 to 60, than those on fungus-treated bees (Figure 4).

## Discussion

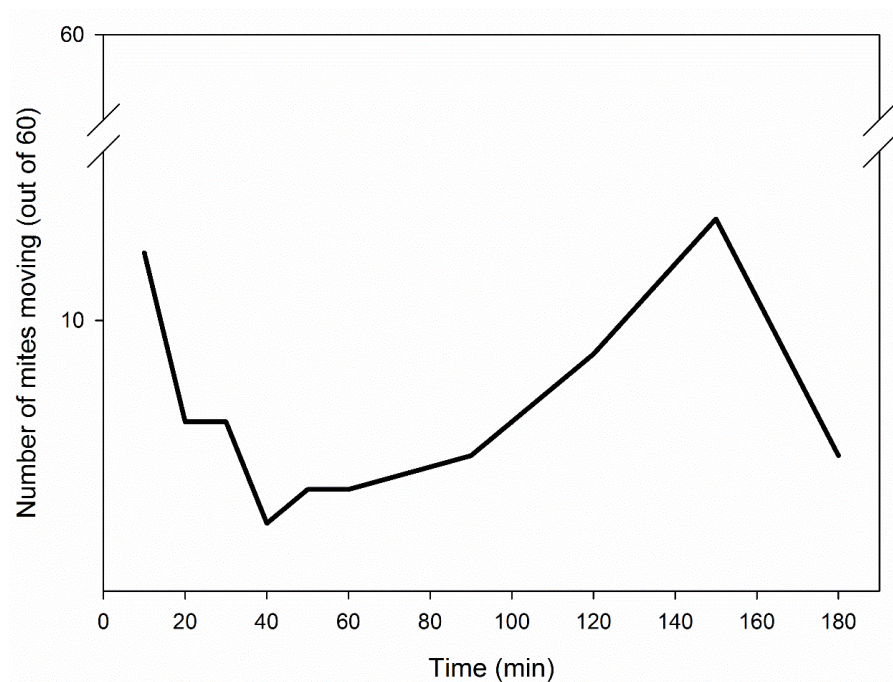
For highly specialized parasites the chance to locate a suitable host of the correct taxon is often low, even when large numbers of offspring are produced (Gunn and Pitt 2012). Living inside honeybee hives, *V. destructor* is perfectly adapted to its host and is perpetually surrounded by bees, making finding the right taxon not a problem. Nevertheless, the mites have to choose suitable individuals among a large number of possible hosts and their reproductive success largely depends on this decision. Disrupting the orientation behavior towards their hosts could reduce reproduction of the mites. Therefore, understanding the mechanisms of host choice decisions and examining which substances and chemical compounds influence these decisions are highly relevant topics regarding the future prospects of *Varroa* control.

Several substances have been tested for their effects on *Varroa* orientation behavior in similar experiments as done in our study. Investigations of compounds that are produced by the honeybee itself like the Nasanov pheromone (Hoppe and Ritter 1988) and cuticular hydrocarbons (Del Piccolo *et al.* 2010) increased the knowledge about how the mites recognize their favored host, the nurse bee. The Nasanov pheromone is produced in larger abundance by older forager bees (Pickett *et al.* 1980). This pheromone exerts a repellent effect on the mites, as does its main component geraniol (Hoppe and Ritter 1988). Del Piccolo *et al.* (2010) found that forager and nurse bees differed in production of (Z)-8-heptadecene, which also acted as repellent. Kraus (1990) discovered the repellent function of 1-octanol, a honeybee alarm pheromone.

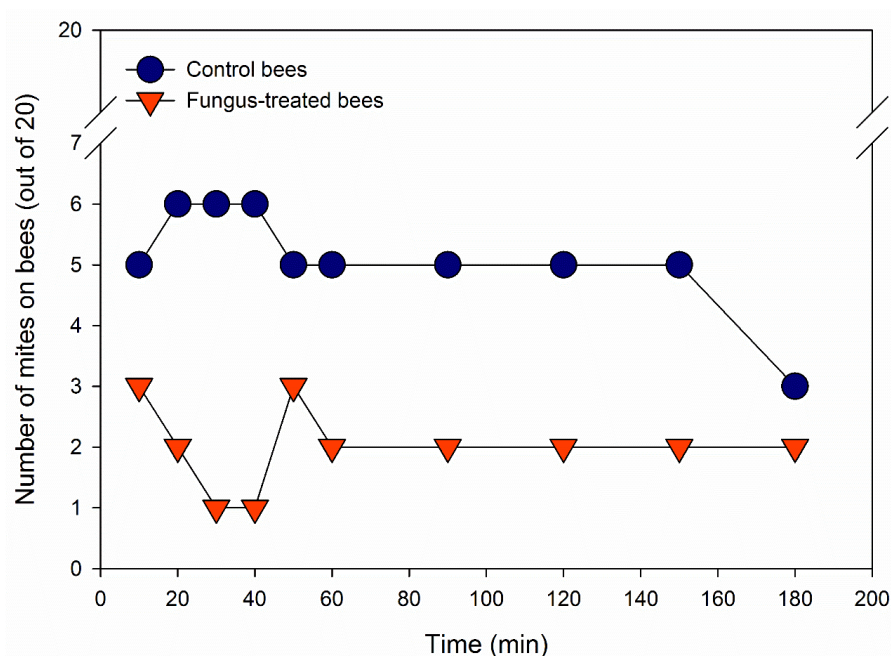


**Figure 1** Mites preferring control or fungus-treated bees in the binary choice test over 3 h (n = 60). Preference for the control or fungus-treated bee was scored when the mite was sitting closer to or on one of the two bees (A) or just when sitting on one of the two bees (B). Individual mites were given a choice between one control and one fungus-treated bee and their behavior observed every 10 min during the 1<sup>st</sup> h and every 30 min during the 2<sup>nd</sup> and 3<sup>rd</sup> h. In both (A) and (B), significantly more mites (GEE;  $P < 0.05$ ) were closer to and/or had climbed onto control bees than onto fungus-treated bees.

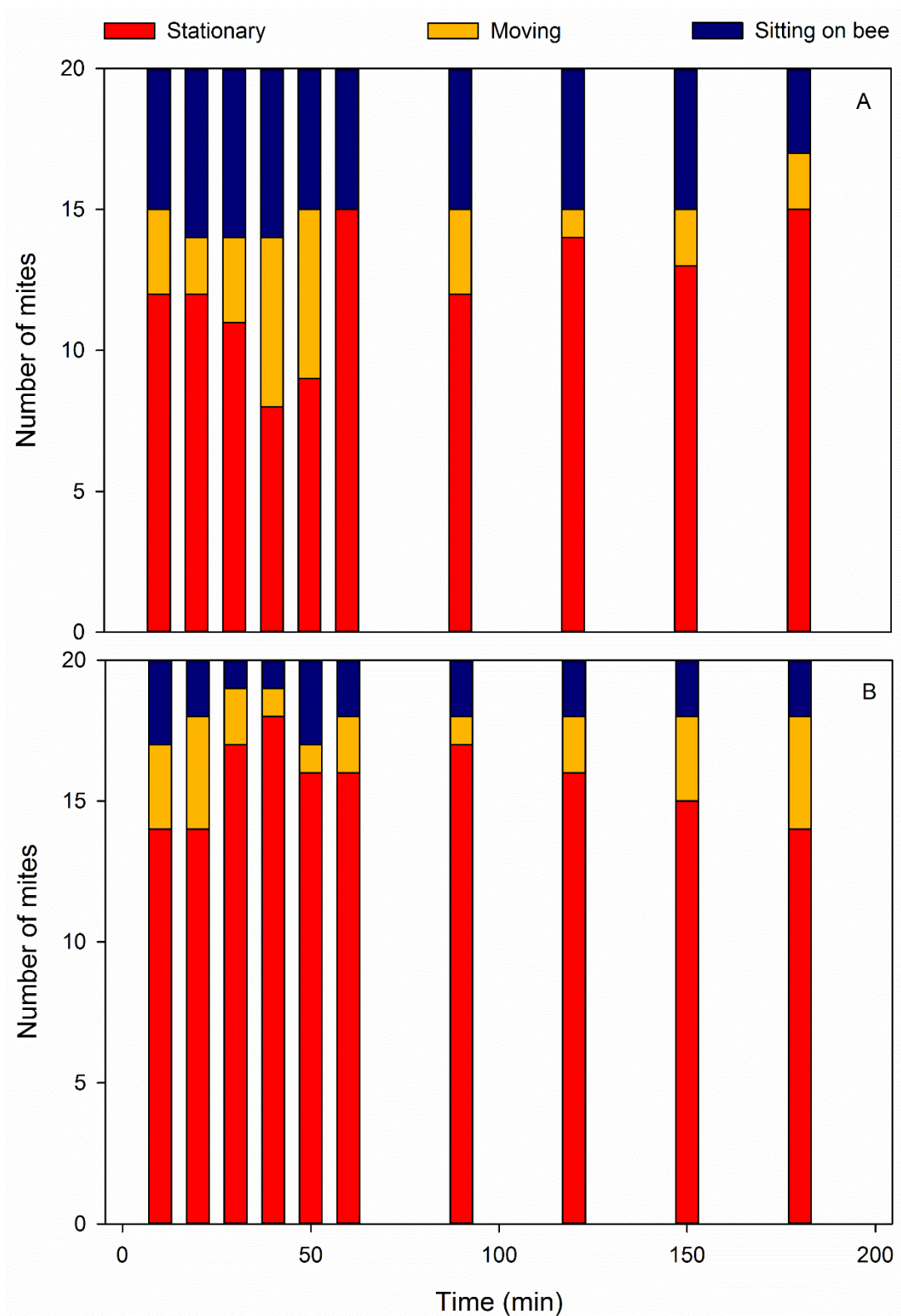




**Figure 2** Activity of mites in the binary choice test (n=60). Individual mites were given a choice between one control and one fungus-treated bee and their activity observed every 10 min during the 1<sup>st</sup> h and every 30 min during the 2<sup>nd</sup> and 3<sup>rd</sup> h. The number of moving and stationary mites did not significantly change over time (GEE;  $P > 0.05$ ).



**Figure 3** Number of mites observed on control and fungus-treated bees (n = 20 for each treatment) in the no-choice test over time. The number of mites climbing onto control bees was significantly higher than the number of mites climbing onto fungus-treated bees (GEE;  $P < 0.01$ ).



**Figure 4** Activity of mites presented either control bees (A) or fungus-treated bees (B) in the no-choice test ( $n = 20$  for each treatment). The activity differed significantly between mites presented fungus-treated bees and those presented control bees (GEE;  $P < 0.05$ ). Mites presented control bees (A) were more likely sitting on bees across time and moved around more at the beginning of the test than mites presented fungus-treated bees (B).

Repellent properties of compounds that do not naturally occur inside the hive have been detected in substances derived from plants like various essential oils (Imdorf *et al.* 1999; Ruffinengo *et al.* 2005), ethanolic extracts (Damiani *et al.* 2011) or the oil extracted from neem seeds (González-Gómez *et al.* 2012). Especially essential oils and their single components have been found to influence the mites' behavior (Imdorf *et al.* 1999). Most of them exhibit repellent properties, for example oil of lavender or citral (Kraus 1990), while only a few are attractive to the mites, for example oil of clover or eugenol (Kraus 1990). To successfully disturb the reproduction of *V. destructor* the repellent properties must be rather strong. Only few substances were efficient in field trials (Ruffinengo *et al.* 2005). Combined with toxicity, however, repellency, as sub-lethal effect, can be a useful asset for *Varroa* control (Damiani *et al.* 2011) and is already exploited by several acaricidal products (Rosenkranz *et al.* 2009).

In our experiments, the application of the entomo-pathogenic fungus *Metarhizium anisopliae* had a significant repellent influence on the host selection behavior of *Varroa destructor*. The choice test revealed a clear preference of the mites for nurse bees that were not in contact with the fungal spores: the number of mites staying on, or in close proximity to, control nurse bees was significantly higher than the number of those staying on or nearby bees treated with fungal spores. At the end of the choice experiment, the number of mites on control bees somewhat decreased because some mites had switched to fungus-treated bees. This was probably due to the fact that dead nurse bees, which do not represent optimal hosts for the mites, were used in the experiment. Thus, after their initial decision, the mites probably dispersed again to determine whether there was an alternative host available. The preference for untreated control bees was also evident in the no-choice experiment. Significantly more mites decided to stay close and/or climb onto control than *Metarhizium*-treated nurse bees. The first host choice decision was made by all mites within 20 min. During the initial phase of the experiment the mites showed a higher moving activity, resembling the initial phases of host searching behavior, which later declined. However, the moving activity differed between mites offered control and *Metarhizium*-treated bees. The activity of mites presented control bees increased up to minute 50 while the initial host search of mites presented *Metarhizium*-treated bees lasted only up to minute 20 and then intensified again during the last hour of the experiment. This activity pattern indicates that the *Metarhizium* treatment may have masked the bee-derived cues used by the mites to recognize nurse bees rather than acting as repellent inducing active avoidance behavior. It seems that, due to the lacking perception of bee-derived cues, the mites terminated earlier searching for a suitable host, but resumed searching in the last part of the experiment when the masking effect of *Metarhizium* started to fade. Actively searching for a host is energy demanding and thus non-random (Gunn and Pitt 2012).

The fungal concentration used in the behavioral assay was  $1.14 \cdot 10^7$  CFU/bee. It could be that the observed effects of the fungus on the mites decrease when also the density of spores on the bee cuticle decreases. In addition, bees remove foreign particles from their body by grooming themselves (auto-grooming) or each other (allo-grooming) (Evans and Spivak 2010). Grooming likely reduces the spore loads of individual bees and could, under field conditions, weaken the effect of the fungus on the mite's host choice observed in our study.

In conclusion, our study suggests that *M. anisopliae* as biocontrol agent may, additional to its lethal effect on *V. destructor*, exert sub-lethal behavioral effects by influencing the mites' orientation behavior towards, and preference for, its hosts. To examine how strong and persistent these effects are under field conditions, further investigations are needed, especially with regard to the concentration of the fungus (dose-response tests) and its persistence under variable abiotic conditions.

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