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Original article

Nosema sp. influences flight behavior of infected honey bee (*Apis mellifera*) foragers*

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Abstract – In this study we investigated whether the microsporidian *Nosema* sp. affects the flight behavior of forager bees. Bees released 6 and 10 m away from the colony took longer times to return. The proportion of bees that did not return was higher in the diseased bees compared to the healthy bees when released 30 m away from the colony. That diseased bees get lost from the colony was also supported by a considerably lower rate of infected bees among the returning foragers compared to departing foragers. In a hive entrance orientation test, diseased bees scored lower than healthy bees, indicating impaired orientation skills. These results are in line with previous results on foragers infested by the parasitic mite *Varroa destructor*. The similar influence of *Nosema* sp. and *V. destructor* on flight behavior, in that foragers might not return to the colony, can be interpreted as a general response of honey bees to diseases to decrease pathogen load within the colonies.

honey bees / pathogens / *Nosema sp.* / foragers / flight

1. INTRODUCTION

Nosemosis in honey bees (*Apis mellifera*) is a disease of the digestive tract caused by *Nosema apis* and *Nosema ceranae.* The species *N. ceranae* was originally reported from the eastern honey bee *Apis cerana* (Fries et al.,1996) and only recently was found to also occur in the western bee *A. mellifera* in many countries (Higes et al. 2006; Fries et al., 2006; Klee et al., 2007). Both species infect epithelial cells of the ventriculum and shed spores in the gut lumen where mature spores infect additional epithelial cells. Eventually spores are released with feces, which are the primary source of infection (Furgala and Mussen, 1990; Fries, 1988). The damaging effects of Nosemosis on physiology are well described for *N. apis* and are likely to be similar in *A. ceranae*. These include impaired protein metabolism, indicated by a lower proteolytic activity of the mid-gut (Malone and Gatehouse, 1998). The lower amount of amino acids in hemolymph cause a reduction of hypopharyngeal size and function (Wang and Moeller, 1969, 1971; Liu, 1990) and lower levels of proteins in the fat bodies (Lotmar, 1939).

The effects of Nosemosis on honey bees also include premature aging, leading to reduced longevity (Furgala and Mussen, 1990). These effects are accompanied by shifts in task division and precocious foraging (Wang and Moeller, 1970), thus affecting the behavior of the bees. Woyciechowski and Kozlowski (1998) also demonstrated increased foraging of infected workers with *N. apis* during adverse weather conditions.

Recently we have demonstrated disease-related changes in honey bee forager behavior

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caused by the parasitic mite *Varroa destructor* (Kralj and Fuchs, 2006). Infested bees took longer flights, took longer times to return to the colony and showed impaired orientation to the nest entrance, resulting in increased losses of infested foragers outside the colony. We interpreted this as a modulation of foraging behavior to the effect of a self sacrifice of diseased bees for the benefit of the colony. Infested workers would not return as a result of an adaptive suicidal mechanism (Smith-Trail, 1990) to increase survival of the colony by removing pathogens. This might pay, as costs of individuals are low and self sacrifice is well known in honey bees in the context of colony defense.

As *Varroa destructor* is a relatively recent parasite of the western honey bee and the demonstrated response thus cannot be a specific adaptation, we hypothesize that non-returning to the colony could likely be a response to diseases in general.

In this study we tested whether *Nosema* sp. causes similar behavioral changes in foragers as observed in *V. destructor*. As in our previous study, we investigated infection of returning foragers in relation to that of departing foragers and then focused on flight behavior. We examined time needed to return and the rate of returning after release of infected and uninfected bees. Further, we investigated orientation of bees toward the nest entrance.

2. METHODS

2.1. General methods: obtaining spores, bee inoculation and marking

For the experiments, bees were infected by fresh spores to assure equal levels of spore infectivity. *Nosema* sp. spores were initially obtained from macerated abdomens of infected dead bees collected from an infested colony. From these, a "nosema" solution was obtained for the production of fresh spores. Bees (20–25) were caged and inoculated by bulk feeding of contaminated 1.2 mL honey solution (approximately 5 million spores per ml from mid-guts) from a gravity feeder. The bees consumed the solutions during the first day and were then fed by inverted sugar (Api-Invert) for an additional 11 days to develop sufficient spore load to infect a first batch of experimental bees. To obtain these fresh spores the digestive tract was pulled out with a forceps and the mid-guts were macerated in water using a mortar. The solution was examined with a light microscope (400× magnifications) and diluted with honey water to a concentration of approximately 5 million spores per ml. The spore concentration was determined by using a counting chamber (Neubauer Improved). Further infections were made with fresh spores from bees used in the ongoing experiments. Every bee was examined for *Nosema* sp. infection after a behavioral test and, if infected, contributed spores to produce fresh infective solutions.

2.1.1. Nosema *identification*

For determining the *Nosema* species, 8 samples derived from experimental bees were examined from the years 2006 and 2007. *N. ceranae* and *N. apis* were determined from the spore solution by using quantitative PCR technique (Cox- Foster et al., 2007) in LLH Kirchain, Germany. To verify the presence of *N. apis* in low quantities, an additional PCR protocol was used. Of the 8 tested samples, six samples contained only *N. ceranea*, and in two samples *N. apis* (one of 5 in 2006 and one of 3 samples in 2007) was additionally present in low quantities.

2.1.2. Inoculation and marking of experimental bees

One day old bees from several healthy colonies were hatched in an incubator and were marked by colors representing the day of emergence. The bees were then introduced into small nucleus colonies containing approximately 2000 bees. Four days later 60 marked bees were retrieved from the colony and distributed equally among two cages. In one cage, they received 1.2 mL honey solution containing *Nosema* spores (approximately 200 000 spores per bee) and in the other cage they received solution without spores. The honey solution was consumed during 7–9 h. Inoculated and non-inoculated bees were caged for an additional 45 min without food and then introduced into the colony separately at a time interval of half an hour to prevent food transfer between the inoculated and non-inoculated bees. Bees had been color marked according to inoculation or non-inoculation or by numbered tags for individual identification.

2.1.3. Inspection for Nosema infection

The behavioral experiments were conduced at least 12 days after infection to enable disease development. As some inoculated bees might not develop the disease and some non-inoculated might, we checked every bee after the behavioral experiment. The mid-gut was macerated in few droplets of water (0.1 mL) using a metal stick and examined with the light microscope (400× magnification). The criterion for *Nosema* sp. infection was the presence of spores in every microscopic observation field (400× magnification). When there were no spores or a single spore in every second or third observation field, bees were considered uninfected.

2.2. Proportion of diseased departing and returning bees from the inoculated colony

Three small nucleus colonies containing 3000–4000 bees were infected by inoculating 100 bees from each of the colonies. These were caged and bulk fed with 4 ml honey solution (ca. 120 000 spores per bee). Fifteen days after inoculation we captured departing and returning bees from the mini-hives between 11 h–15 h. Departing bees were caught in plastic bags attached to the hive entrance for about 5 min, and returning bees were caught by blocking the entrance and sucking the bees through a plastic tube into a bottle. The bees were frozen and later inspected individually for the presence of *Nosema* spores in the mid-gut. Experiments were carried out in July 2005.

2.3. Time needed to return to the hive

The ability of infected and uninfected bees to return to the colony after release from a distance of 10 m and 6 m from the colony was investigated from 12 July to 17 August in 2005 and 21 July to 17 August in 2006, respectively. We released 180 bees from one nucleus colony in the first year and 205 from three nucleus colonies in the second year. For the experiment we selected 1–2 inoculated and non-inoculated marked bees of the same age, covering age ranges between 16–27 days and placed them individually in 5ml glass vials with perforated plastic lids for respiration. These were released simultaneously in pairs in 2005 or in groups of 2–4 bees in 2006.

Bees were caught at the colony entrance for later *Nosema* inspection. To facilitate recognition and retrieving of marked returning bees, the colony entrance was equipped with a 10 cm landing board and was narrowed by a transparent plastic foil to an opening of 2 cm × 1.5 cm, which could be closed by a transparent plastic slider. The flight duration of returning bees was recorded for a maximum duration of 15 min. Bees that returned later than 15 min were collected from the colonies in late evening.

For analysis of returning time, inoculated and non-inoculated bees were regrouped according to their actual *Nosema* health status. Bees were analyzed in pairs consisting of one infected and one uninfected bee of the same age released at the same time. In 2006, when several infected and uninfected bees were released at a time, the average time within each health status was taken for pair analyses.

2.4. Rate of bees not returning to the colony

A larger number of bees were used to determine the proportion of bees which did not return. Bees of the same age were released in groups consisting on average of 18 inoculated and 19 non-inoculated workers. In 7 experiments, a total of 257 workers aged 16–19 d were released from a distance of 30 m from the colony. The experiment was conducted in 4 colonies between 17 July and 8 August, 2007. Workers were marked with an additional color so they could be recognized upon returning, when they were caught at the colony entrance or within the colony 15 min after the release. Colonies were examined again in the evening at approximately 19 h. Colony examinations took about 10–15 min. All recovered bees were checked for *Nosema* infection.

2.5. Orientation of bees toward the nest entrance

The ability of infected and uninfected bees to locate the nest entrance was tested in August 2005 and from July to September 2006. A nucleus colony was placed behind a 2 × 3 m white wall, and was connected to its entrance at the front of the wall by a transparent tunnel (diameter 2 cm). In each year, a different colony was used. The tunnel could be blocked by a slider when a returning bee was expected, and could be opened from the side to catch the bee. The entrance was marked with a blue-colored (10 × 10 cm) wooden square and bees were

allowed to learn the entrance over at least 16 days. During experiments an additional identical dummy entrance marking was presented 10 cm apart from the marked nest entrance. To counter side preferences, the dummy position was altered between the left and right side between experiments. The returning of released bees was observed from a fixed position 1.5 m away from the wall, in front of the nest entrance. We scored whether bees returned to the colony directly or approached the dummy blue square before finding the nest entrance. An approach was scored when a bee was seen to cross the dummy entrance from the observer's position. Additionally, the number of approaches toward the dummy was recorded. A total of 160 (93 inoculated and 67 non-inoculated) marked bees of foraging age (between 16–32 d) were released individually from vials 4 m in the front of the nest entrance. Each release of an inoculated bee followed the release of non-inoculated bee of the same age. Bees were caught at the entrance and were then checked for *Nosema* infection.

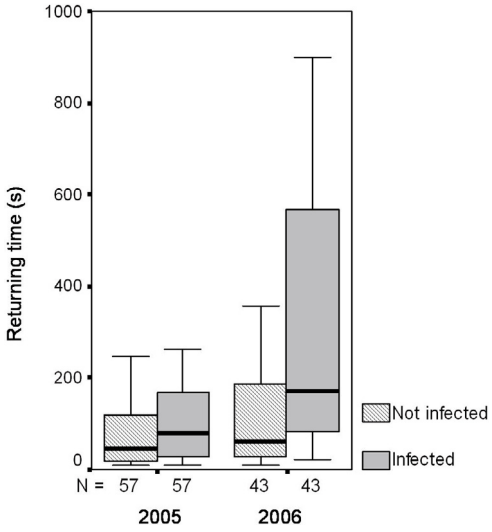

Figure 1. Time needed to return to the colony for 57 and 43 pairs of infected and uninfected bees of the same age released at the same time in the year 2005 and 2006, respectively. The chart indicates medians, interquartile ranges, 10% and 90% percentiles.

3. RESULTS

3.1. Proportion of diseased departing and returning bees from the inoculated colony

The total of twelve samples of departing and returning bees contained 147 and 139 workers, respectively. Samples of departing and returning bees ranged from 3 to 20 bees. The infection of returning bees was 63.0% (88 of 139) while that of departing bees was 82.3% (121 of 147), thus infection of returning bees was 23% lower than that of the departing bees. The difference in infection for paired samples was highly significant (Wilcoxon matched pairs rank test, n = 12, $P < 0.003$).

3.2. Returning time

We released 200 inoculated and 185 non-inoculated foragers in 148 pairs. As actual health status was determined after *Nosema* inspection and some bees did not return during the observation period (25 non-inoculated and 29 inoculated bees), out of the 148 released pairs we could retain only 100 pairs (57 in the year 2006 and 43 in the year 2006). In some cases more than one bee of the same health status was released. In this case the average time for each health status was calculated. Bees that returned later than 15 min were scored as 15 min returning time (900 s).

The median returning time of *Nosema*-positive workers was 116.5 s, that of *Nosema*-negative bees was 54 s. Infected bees thus took 2.1 times longer to return than uninfected bees. The difference was highly significant ($z = -3.521$, $P < 0.0005$, Wilcoxon matched pairs rank test). By year, median returning times of infected bees were 1.4 times longer in 2005 and 2.2 times longer in 2006 (Fig. 1). Differences were significant in each year for the paired samples (2005: $z = -2.30$, $P < 0.022$; 2006: $z = -2.69$, $P < 0.007$, Wilcoxon matched pairs rank test). In 2006, differences were also significant without pairing the samples (n = 159, $z = -4.40$, $P < 0.0005$, Mann Whitney U test).

3.3. Rate of bees not returning to the colony

The rate of bees not returning to the colony until evening was determined from the release of 126 inoculated and 131 non-inoculated bees from a distance of 30 m from the colony. From the inoculated bees 19.0% (24 from 126) and from non-inoculated bees 6.9% (9 from 131) did not return. Thus, inoculated bees failed to return to the colony 2.7 times more frequently than non-inoculated bees ($Chi^2 = 8.51$, df = 1, $P < 0.005$). To ascertain health differences between inoculated and non-inoculated bees we examined the returning bees for *Nosema* infection. In the inoculated group we found 12.7% uninfected bees and in the non-inoculated group we found 12.4% infected bees.

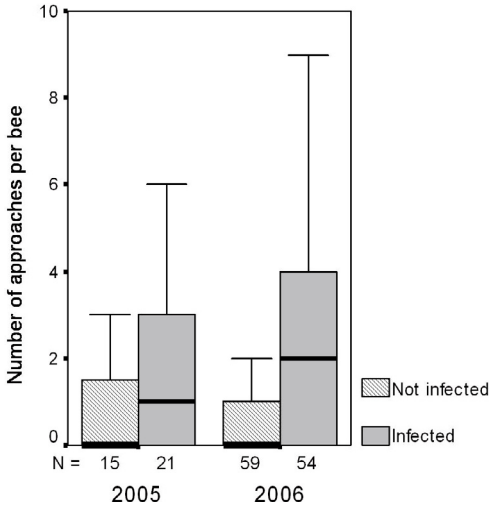

Figure 2. Mean number of approaches per infected and uninfected bees toward the dummy entrance before entering the nest entrance in the year 2005 and 2006. The chart indicates medians, interquartile ranges, and 10% and 90% percentiles.

3.4. Orientation of bees toward the nest entrance

Of the 93 inoculated and 67 non-inoculated bees released in the year 2005 and 2006 to determine the accuracy of locating the nest entrance in the presence of a dummy entrance, 6 and 5, respectively, did not return during the observation time of 15 min. Of the 149 bees that did return, 75 were infected and 74 were uninfected. There were no differences between years. Two thirds of the infected workers (69.3%, 52 from 75) approached the blue square dummy at least once before entering the nest entrance. In contrast, this was the case in only about one third of the uninfected workers (32.4%, 24 from 74), showing that a higher proportion of the diseased bees approached the dummy. This difference was highly significant ($Chi^2 = 20.30$, df = 1, $P < 0.0005$). Also, infected bees approached the dummy entrance significantly more frequently before entering the nest than did uninfected bees (Fig. 2, $z = -4.58$, $P < 0.0005$, Mann Whitney U test).

4. DISCUSSION

Sampling bees at the entrance of colonies infected by *Nosema* sp. showed that infection rates were 23% lower in the returning foragers compared to the departing foragers. This finding strongly indicates that infected individuals get lost from the colony during flights at higher rates compared to uninfected bees. This result corresponds to observations in colonies infested with the parasitic mite *V. destructor,* where infestation of departing workers was almost twice as high as infestation of returning workers (Kralj and Fuchs, 2006). Increased losses of foragers outside the colony caused by *Nosema* sp. were further supported by the considerably lower returning rate of infected workers when released from a distance of 30 m. In these experiments inoculated workers failed to return to the colony about 2.5 times more frequently than non-inoculated workers. A similarly lower return rate was also observed in bees infested with *V. destructor* (Kralj and Fuchs, 2006).

This substantially higher rate of non-returning by infected foragers suggests that *Nosema* sp. influences flight behavior. Indeed, the infected foragers released outside the colony took about twice as long to return to the colony than infected bees. The differences were more pronounced in 2006, although bees were released from a closer distance than in

2005. In both years uninfected bees returned within a 1 min period, while infected bees took approximately 1.5 min in 2005 and almost 3 min in 2006 to return. The longer times to return required by infected bees were not caused by different delays in departing time after release, as takeoff time was recorded as the starting time. The difference in returning time between years could have different causes, including the different locations of release, different colony conditions, the presence of neighboring colonies or differences in the environments that required different navigation skills. However, this does not alter the conclusions related to *Nosema* sp. infection and foraging behavior. As *N. ceranae* was predominately present in analyzed samples from both years, differences were unlikely to be related to different *Nosema* species tested and our data do not allow conclusions as to whether both species would influence the bees differently.

There could be several causes for the prolonged time in returning. One possibility is that bees were not motivated to return and/or suffered from fatigue and landed somewhere or foraged before return. Woyciechowski and Kozlowski (1998) showed that bees infected by *Nosema* sp. foraged more frequently in detrimental weather conditions than did healthy bees. They interpreted increased foraging in infected bees as compensation for foraging yield due to reduced lifespan. However, impaired orientation as a possible cause is clearly supported by the reduced accuracy to find the nest entrance in the orientation experiment and by weaker learning abilities of infected bees (Kralj and Fuchs, 2007). Together these point to prolonged searching due to difficulties in orientation, rather than changes in motivation state, although both may contribute to the effect.

The reduced accuracy to find the nest entrance and weak learning in infected bees corresponds to changes in flight behavior that was also observed in bees infested with *V. destructor*. A deficit in neural processing, as also shown in bees infested by *V.* destructor (Kralj et al., 2007) and deformed wing virus (Iqbal and Mueller, 2007), could be one of the causes of weakened orientation, leading to prolonged flights and reduced returning rates. That similar responses of the bees were found in two different diseases indicate that the underlying mechanisms may be similar and involve neuronal changes.

Though it is difficult to prove whether non-returning is merely a physiological response to *Nosema* sp. and to other diseases (*e.g.* caused by a general weakening of the bees), or whether it is an adaptation, the behavioral change might fulfill some criteria for adaptation (Poulin, 1995). The pronounced loss of diseased bees could have two effects (a) the spread of pathogens if diseased bees enter other colonies and (b) a decrease in colony infection. Erroneous entering of disoriented bees into other colonies can be to the advantage of the pathogen by facilitating horizontal transmission. In contrast, reduced infection of the colony has an advantage for bees and non-returning behavior could be considered as a mechanism to preserve colony health by removing pathogens from the colony. Such losses add to the increased turnover of infected individuals due to premature aging (Schmid-Hempel, 1998), including premature foraging and death of workers (Bailey and Fernando, 1972; Wang and Moeller 1970; Schneider and Drescher, 1987; Furgala and Mussen, 1990).

The similarity of the behavioral response of foragers to the two considerably different pathogens *V. destructor* and *Nosema* sp. strongly suggests that it may be a general response of honey bees to pathogens. Thus, the behavioral modification could be a general defense mechanism toward diseases. The common observation of empty hives devoid of foragers in colonies diseased by *V. destructor* (Martin, 1997) corresponds well to these observed behavioral changes, though other influences such as a shorter life span of diseased bees may also contribute. In addition, empty hives have also been correlated recently with acute paralysis virus and co-infection with both species of *Nosema* (Cox- Foster et al., 2007). An influence on bee behavior to the effect that infected foragers do not return to the colony can serve to enhance colony survival, but pathogens still may eventually overwhelm the colony.

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***Nosema* sp. agit sur le comportement de vol des abeilles (*Apis mellifera*) butineuses infectées.**

***Nosema ceranae* / *Nosema apis* / butineuse/ pathogène / comportement de vol/ orientation**

Zusammenfassung – *Nosema* sp. wirkt sich auf das Flugverhalten infizierter Sammlerinnen von Honigbienen (*Apis mellifera*) aus. Wir untersuchten, wie sich das Mikrosporidium *Nosema* sp. auf das Flugverhalten von Sammelbienen auswirkt. Infizierte in einer Entfernung von 6 oder 10 m von dem Bienenvolk aufgelassene Bienen benötigten längere Zeit für ihre Rückkehr als gleich alte, nicht infizierte Bienen. Die Verlängerung der Flugzeit betrug in 2005 das 1,4-fache und in 2006 das 2,2-fache (Abb. 1). Wenn die Bienen in 30 m Entfernung aufgelassen wurden, war der Anteil der nicht in das Volk zurückkehrenden Bienen bei den infizierten Bienen 2,7 mal höher als bei den nicht infizierten Bienen. Dass kranke Bienen aus dem Volk verloren gehen wurde auch dadurch belegt, dass der Befall von rückkehrenden Sammlerinnen um 23 % geringer war als der der ausfliegenden. In einem Orientierungstest, bei dem neben dem richtigen Stockeingang eine weitere, identische Eingangsmarkierung angebracht wurde, überflogen zwei Drittel der infizierten Bienen die falsche Eingangsmarkierung bevor sie den richtigen Eingang fanden, bei den gesunden Bienen war dies nur etwa ein Drittel. Weiterhin wurde die falsche Eingangsmarkierung von infizierten Bienen häufiger überflogen als von den nicht infizierten Bienen (Abb. 2). Diese Ergebnisse legen nahe, dass die Orientierungsfähigkeit der Bienen beeinträchtigt war. Diese Ergebnisse entsprechen früheren Resultaten von Versuchen mit von der parasitischen Milbe *Varroa destructor* befallenen Sammlerinnen, bei denen verändertes Flugverhalten ebenfalls dazu führte, dass die Bienen oft nicht in die Völker zurückkehrten. Die Ähnlichkeit der Reaktionen der Sammlerinnen auf den Befall mit *Nosema* sp. und *V. destructor* weist darauf hin, dass es sich um eine generalisierte Reaktion der Bienen auf Krankheiten handelt. Wir interpretieren dies als eine adaptive Verhaltensänderung zum Nutzen des Volkes, bei der durch eine Selbstaufopferung von befallenen Bienen Pathogene und Parasiten aus dem Volk entfernt werden.

Honigbienen / Krankheitserreger / *Nosema spec* / Sammlerinnen / Flugverhalten

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