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Pathological effects of the microsporidium *Nosema ceranae* on honey bee queen physiology (*Apis mellifera*)

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Abstract

Nosema ceranae, a microsporidian parasite originally described in the Asian honey bee *Apis cerana*, has recently been found to be cross-infective and to also parasitize the European honey bee *Apis mellifera*. Since this discovery, many studies have attempted to characterize the impact of this parasite in *Apis mellifera* honey bees. *Nosema* species can infect all colony members, workers, drones and queens, but the pathological effects of this microsporidium has been mainly investigated in workers, despite the prime importance of the queen, who monopolizes the reproduction and regulates the cohesion of the society via pheromones. We therefore analyzed the impact of *N. ceranae* on queen physiology. We found that infection by *N. ceranae* did not affect the fat body content (an indicator of energy stores) but did alter the vitellogenin titer (an indicator of fertility and longevity), the total antioxidant capacity and the queen mandibular pheromones, which surprisingly were all significantly increased in *Nosema*-infected queens. Thus, such physiological changes may impact queen health, leading to changes in pheromone production, that could explain *Nosema*-induced supersedure (queen replacement).

Keywords: *Nosema ceranae*; honey bee queen; *Apis mellifera*; vitellogenin; queen mandibular pheromones; antioxidant capacity

1. Introduction

Microsporidia from the genus *Nosema* are intracellular parasites that infect a variety of insect taxonomic orders (Becnel and Andreadis, 1999). In honey bees, nosemosis is a major disease affecting adults and caused by the proliferation of *Nosema* spores in midgut epithelial cells after spores are ingested through contaminated food or comb and cleaning activity (Webster, 1993). After the initial infection, millions of spores can be found within a few days in the midgut (Bailey and Ball, 1991), which causes a reduction in the honey bee life span (Higes et al., 2007a; Malone et al., 1995; Rinderer and Sylvester, 1978; Paxton et al., 2007). Currently, two microsporidian parasites have been described from honey bees: *N. apis* (Zander, 1909) and *N. ceranae* (Fries et al., 1996) isolated from the European (*Apis mellifera*) and the Asian honey bee (*Apis cerana*), respectively. Recently, natural infections of *N. ceranae* in *Apis mellifera* have been found across the world (see Fries (2010) for a review). This emergent pathogen of the European honey bee is believed to be more virulent than *N. apis* (Paxton et al., 2007; Higes et al., 2007a) and to be one of the causes of colony collapse, notably in Spain (Higes et al., 2008). However, a recent study demonstrated similar virulence between both *Nosema* species (Forsgren and Fries, 2010); thus, virulence might actually depend on honey bee race or regional climate (Higes et al., 2010).

To date, most of the studies investigating the pathological effects of *N. ceranae* were performed in workers, but *N. ceranae* can also parasitize the queen and induce similar tissue lesions (Higes et al., 2009). While both workers and the queen can be infected, the consequences for the hive could be very different. Because she monopolizes reproduction and maintains colony homeostasis by continuously producing pheromones (Slessor et al., 2005), a queen weakened by *N. ceranae* infection might compromise the renewal and the stability of worker population. Accordingly, queen quality is of primary importance. In addition, according to beekeepers, 'poor queens' are estimated to be the major cause of the actual colony losses (van Engelsdorp et al., 2008). Data on the pathology of *N. apis* in queens are available. For example, infected queens have decreased ovary development, which can lead to infertility (Fyg, 1964; Liu, 1992), and are more often superseded (process by which an old or failing queen is replaced) (Farrar, 1947; Furgala, 1962 but see Czekonska, 2000). However, the pathological effects of *N. ceranae*, a newly emerging pathogen of European honey bees, remained to be studied in queens.

There are many measures that can serve as parameters for estimating the effects of *N. ceranae* on queen health. Vitellogenin (Vg) is a yolk protein taken up by developing oocytes and associated to egg

production in queens (Engels, 1974; Tanaka and Hartfelder, 2004) but also has antioxidant functions that protect bees from oxidative stress and enhance longevity (Corona et al., 2007; Seehuus et al., 2006). Therefore, the hemolymph titer of Vg, which is synthesized in the fat body, the main site of energy storage (Ricks and Vinson, 1972; Toth and Robinson, 2005), is as an indicator of queen fertility and longevity. The proper functioning of the colony is regulated by different pheromones, notably produced and transmitted by the queen. The main one is the queen mandibular pheromone (QMP), which stimulates queen attendance by workers, inhibits worker ovary development and regulates worker behavioral maturation (Slessor et al., 2005). Because they are both important for the queen health and colony organization, we determined the effects of *N. ceranae* infection on these physiological parameters. In addition, due to the energetic stress induced by *N. ceranae* (Mayack and Naug, 2009), we measured the total antioxidant capacity of queens. Knowing the lethal effects of this parasite on workers, we hypothesized that we would observe a reduced level of most of those physiological parameters in queens infected with *N. ceranae* compared to non-infected queens.

2. Materials and Methods

2.1. Honey bee queen rearing

Experiments were performed in Avignon (France) with local hybrid colonies (*A. m. ligustica*/*A. m. mellifera*). Queens were reared according to standard beekeeping methods (Laidlaw and Page, 1997). However, young larvae used for the queen grafting originated from the same colony to reduce genetic variation and thus potential variation between control and *Nosema* treatments regarding the physiological parameters that we analyzed, especially pheromones (Plettner et al., 1997; Pankiw et al., 1996).

Four days before hatching, queen cells were removed from their hive and placed individually in a cage at 34°C and 60% RH with 30 one day-old bees, which were obtained from honey combs containing last-stage pupae removed from the source colonies used for queen rearing. Bees were fed *ad libitum* with water, candy (30% honey, 70% powdered sugar) and pollen. Since *N. ceranae* can be found in pollen (Higes et al., 2007b) and honey (Chauzat et al., 2007), before feeding the bees, we checked under microscope that both pollen and honey were free of microspores. The queens emerged and were kept in those cages.

Nosema count in ventriculi, lipid stores in fat bodies, vitellogenin titer in hemolymph and QMP levels in heads were all analyzed in the same queens, while total antioxidant capacity in the whole abdomen was determined in another set of queens.

2.2. *N. ceranae* infection

Queens were infected with *N. ceranae* at emergence and collected at day 8 to analyze the pathological effects of the microsporidia. This age was chosen since queens usually start their mating flights one week after emergence. To infect each queen with the same dose of spores when starting the experiments, they were fed individually as in Malone and Gatehouse (1998) with 2 µl of a freshly prepared 50% sucrose solution containing 200, 000 spores of *N. ceranae*. Similar spore number is known to cause an infection in queens (Higes et al., 2009). Control queens were fed with a sucrose solution.

Spores were isolated from a colony infected with *N. ceranae*. The digestive tract of individual bees was dissected and crushed in distilled water, then the suspension was filtered and centrifuged to collect the spores. The identification of *N. ceranae* was confirmed by standard PCR amplification and sequencing of the PCR results as in Alaux et al. (2010). The spore concentration of the feeding solution and the level of *Nosema* infection at the end of the experiment were determined by counting with a haemocytometer.

2.3. Histological analysis of lipid stores in fat bodies

The lipid stores in the fat bodies were determined using the Oil Red O staining protocol. This method has proven to be efficient in detecting differences in lipid content in worker bees (Toth and Robinson, 2005). We followed the same procedure by removing the abdomen of the queens and dissecting out the sternites 3 to 7. This piece of cuticle with the fat bodies attached was rubbed onto a slide, which was then fixed with 10% formaldehyde, and washed with 60% isopropanol. The slide was then stained with Oil Red O for 15 minutes and washed with water. The stained tissue was observed under microscope at x400 and 10 photographic pictures of each slides were taken using a CANON Powershot A650 digital camera. Lipid content was quantified by automatically counting red pixels with Adobe Photoshop version 7.0.

2.4. Quantification of vitellogenin titers

Vitellogenin quantification in queens was determined by SDS-PAGE, following the standard method commonly used for honey bee workers (Lin et al., 1999; Amdam et al., 2006; Nelson et al., 2007). After puncturing the abdomen between the third and fourth tergite, hemolymph was extracted by microcapillary (Hirschmann Ringcaps). Samples that were contaminated with gut content were discarded. Hemolymph (1 µl) was diluted in 50 µl PBS (phosphate buffered saline) and separated by SDS-polyacrylamide gel electrophoresis with a 3% acrylamide stacking gel and 8% separation gel using standard methods. A β-galactosidase standard (Sigma–Aldrich) was also loaded in equal amount on all gels to control for variation between gels and densitometrically quantification of vitellogenin (single band of 180 kDa) (Wheeler and Kawooya, 1990; Lin et al., 1999). Protein molecular weight markers ranging from 70 to 250 kDa (Bio-Rad, France) were used and electophoresis was run at 220 V

at 4°C. After staining the gels with Commassie Brilliant Blue, band intensities were densitometrically measured with the software Image-J 1.36.

2.5. Total antioxidant capacity

The total antioxidant capacity in queens was determined using the antioxidant assay kit (#709001, Cayman chemical, USA) following the kit instruction as in Williams et al. (2008). Whole abdomens were individually homogenized at 4°C, using a TissueLyser (Qiagen, Courtaboeuf, France), in 20% (w/v) ice-cold phosphate buffer (5 mM potassium phosphate, 0.9% osdium chloride, 0.1% glucose, pH 7.4). The homogenates were then centrifuged at 15,000 g for 15 min at 4°C. The supernatant was diluted in the phosphate buffer (1/100) and used for analysis of total antioxidant activity. The capacity of the antioxidants in 10 µl of the solution to inhibit the oxidation of ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{+®}$ was assessed. The amount of $ABTS^{+®}$ produced was determined by reading the absorbance at 750 nm. Then, the total antioxidant capacity was compared to trolox standards and quantified as mM trolox equivalents.

2.6. Pheromone analysis

The analysis of queen pheromone components (9-ODA, HOB, HVA, 9-HDA) was performed following the same procedure published by Maisonnasse et al. (2010). Briefly, queen heads were stored at -20°C before chemical analysis of the QMP components. Pheromone compounds were extracted by crushing individual heads in 200 µl of methanol and 100 µl of decanoic acid (250 ng/µl; internal standard) for 2 minutes on ice. 20 µl of the supernatant was collected and concentrated under nitrogen stream and then derivatized with 5 µl of bistrimethylsilyltrifluoroacetamide. The solution was homogenized and left at room temperature for 40 min. The derivatized sample was then diluted in 100 µl of isohexane and 1 µl of the solution was injected into a fast gas chromatograph (Shimadzu 2014, Japan). The samples were injected in split mode. Hydrogen was used as carrier gas. Oven temperature was set at 100°C, then 100°C to 200°C at 40°C min^{-1} and 200°C to 250°C at 10°C min^{-1} and held at 250°C for 2 min. Identification and quantification of HOB, 9ODA, HVA, 9HDA were based on retention times of synthetic compounds (Sigma-Aldrich, France and PheroTech, Canada). The confirmation of QMP compounds was done by mass spectrometer (Shimadzu CP2010, Japan).

Since the data were not normally distributed, *Nosema* effects on the different parameters of queen physiology were determined using Mann-Whitney U tests. In order to determine whether some of the measured physiological parameters (fat body content, Vg and QMP) were correlated, we performed a correlation analysis including all queens.

3. Results

After 8 days, the level of *Nosema* infection was analyzed in experimentally infected and control queens (n = 8 for both). In infected queens, the mean number of *Nosema* spores per queen was 18.2 millions (± 6 x 10^6). No spore was detected in control queens, except in 2 queens that were infected with 40,000 and 20,000 spores, which is 5 and 10 times lower than the dose use to infect the experimental queens.

Regarding the pathological effects of *N. ceranae*, the microspore infection induced a slight decrease in the fat bodies lipid staining but the difference with control queens was not significant (Fig. 1A). However, the Vg titer increased by around 58% when queens were infected with *N. ceranae*, which was significantly higher than control queens (Fig. 1B). Similarly, the total antioxidant capacity significantly augmented in infected queens (Fig. 1C).

Analysis of queen heads revealed that the QMP components 9-ODA, HOB and 9-HDA were present in both control and parasitized queens (Fig. 2), but HVA was not detected since queens were virgin (Ledoux et al., 2001). However, all QMP components, except HOB, were significantly higher in *N. ceranae* infected queens compared to control (Fig. 2).

The correlation analysis performed on the measured physiological parameters (fat body content, Vg and QMP) revealed a significant negative correlation between the amount of lipid stores in the fat bodies and the Vg titer (Table 1). No significant correlation was found between the others physiological parameters.

4. Discussion

In this study, we experimentally infected queens with *N. ceranae* to determine whether microsporidia affects the queen physiology and health (the minor infection observed in two control queens probably came from spores ingested by chewing the wax capping at emergence (Malone and Gatehouse, 1998)). Based on the lethal effects of *N. ceranae* in workers and the similar lesions induced in queens (Antúnez et al., 2009), we expected a weakening of queen physiology. However, our results clearly demonstrated that *N. ceranae* infection actually boosted the main physiological functions of the queens.

Since recent studies demonstrated that *Nosema* parasitism cause a nutritional stress in workers (Mayack and Naug, 2009; Naug and Gibbs, 2009; Alaux et al., 2010) and a reduction in fat body content in winter bees (Bailey and Ball, 1991), a loss of energy store could be expected in queens. However, although slightly decreased in infected queens after one week, the lipid content in fat bodies (indicator of nutritional status) was not different from control queens, suggesting that queens might be able to compensate for the nutrition stress induced by *Nosema* by increasing their food demand to workers and thus their food consumption. However, we cannot exclude the possibility that in the long-

term the decrease in lipid stores may become more pronounced. Alternatively, the higher Vg titer observed in infected queens could explain the slight decrease in fat body content, since this hormone is synthesized in this tissue (Pan et al., 1969). An increase in Vg synthesis could lead to the impoverishment of its site of production (fat bodies), as indicated by the negative correlation between lipid store and Vg level (Table 1).

Workers and queens develop from the same genome, but opposing responses to *N. ceranae* were observed regarding Vg production. Contrary to the Vg decrease observed in infected workers (Antúnez et al., 2009), *N. ceranae* triggers the increase of Vg synthesis in queens. The lower amount of Vg in parasitized workers might explain their shorter lifespan, taking into account the positive effect of Vg activity on bee longevity (Seehuus et al., 2006). Alternatively, since Vg is present at lower levels in old foragers compared to young nurses (Page and Amdam, 2007), and because *Nosema* causes a precocious onset of foraging (transition from nurse to forager tasks) (Wang and Moeller, 1970), the decrease in Vg might simply reflect the precocious forager profile of infected bees compared to control bees. Thus, this response could be interpreted as a mechanism by which infected workers remove themselves from the hive in order to decrease pathogen load within the colony (Kralj and Fuchs, 2010). However, compared to the loss of workers, queen replacement is more costly and critical to the colony, and thus queens may have evolved to cope with parasite infection. Vitellogenin function is pleiotropic and has been shown to play a role in maintaining the population of functioning hemocytes in honey bee workers (Amdam et al., 2004) and to be involved in the regulation of innate immune response against bacteria and fungi in fishes (Liu et al., 2009; Li et al., 2008; Tong et al., 2010; Li et al., 2009) and mosquitos (Raikhel et al., 2002). This latter function has not been characterized in honey bees but seems unlikely in this case as the observed spore loads seen in queens here were similar to those of workers experimentally infected with the same amount of *Nosema* microspores in a previous study (Alaux et al., 2010) and this study). Similar rates of infection with *N. apis* were also found in workers and queens (Webster et al., 2004). Thus, there is no correlation between the spore loads and the Vg level. Although we cannot exclude the possibility that the increase in Vg level reflects an immune response against an increase in bacterial or viral populations (Bailey et al. 1983) associated with *Nosema* infection, the most direct explanation supports a primary effect of *Nosema* on host physiology. Indeed, *N. ceranae* is strictly dependant on host energy for its development and germination (Keeling and Fast, 2002; Cornman et al., 2009), which would lead to an increase in host metabolism and oxidative stress. In that case, the increase in Vg production observed in infected queens might be a response to the energetic stress caused by the spore population, since Vg is able to reduce oxidative stress by scavenging free radicals and therefore prolong the lifespan of bees (Seehuus et al., 2006). The elevated Vg titer would suggest a higher capacity to resist oxidative stress, which was demonstrated by higher total antioxidant enzyme activity in infected queens (Fig. 1C). Despite this protective response, *Nosema*-infected queens seems to have a shorter lifespan compared

to healthy queens (Higes et al., 2009), suggesting that they are not able to cope with the physiological stress of *Nosema* over the long-term.

Our chemical analysis demonstrated that *Nosema* can significantly modify pheromone production in queens; similar results were found in workers in which *Nosema* parasitism altered the production of the pheromone ethyl oleate (Dussaubat et al., 2010). Based on the observation that *Nosema*-infected queens are more likely to be superseded (Farrar, 1947; Furgala, 1962), Butler (1958) suggested that infected queens produced lower amount of pheromones. In fact, we found that infected queens produced higher quantities of QMP compared to healthy queens. Richard et al. (2007) found that the QMP profile changes according to insemination quantity, with virgin or single-drone inseminated queens producing higher amounts of 9-ODA and 9-HDA compared to mated or multi-drone inseminated queens, respectively. In our study, the QMP compounds, 9-ODA and 9-HAD, were higher in infected queens, suggesting that elevated levels of QMP are a hallmark of poorly fertile or sick queens. It is not known how those pheromone changes would affect the queen-worker relationships, but some data indicate that queens with lower amount of QMP are more attractive and groomed than queens with higher amount of QMP (Richard et al., 2007), suggesting that sick queens would be less attended by workers.

Physiological stress and changes in QMP might affect the ability of queens to mate and/or to be attractive for drones. In the field, the presence of spores in mated queens can lead to a *Nosema*-induced supersedure, in which the infected queen is replaced by a new, presumably healthy queen (Farrar, 1947; Furgala, 1962). The QMP modification induced by *N. ceranae*, coupled with the ability of workers to detect and respond differently to the pheromone changes (Richard et al., 2007) might explain the reports of *Nosema*-induced supersedure. However, those assumptions remain to be tested in the field and represent the next step to further understanding the consequences of the queen pathology on its ability to mate and on the colony fate.

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Figure legends

Figure 1. Pathological effects of *N. ceranae* on different queen physiological parameters (mean ± SE). The levels of lipid store in the fat bodies (A), Vg titer in hemolymph (B) and total antioxidant capacities in the whole abdomen (C) between control and infected queens were compared using Mann-Whitney U tests. Sample size is indicated in each bar.

Figure 2. Pathological effects of *N. ceranae* on QMP levels in queen heads (mean ± SE). The levels of 9-ODA, HVA 9-HDA and HOB (µg/head) between control (n = 9, white bar) and infected queens (n = 10, grey bar) were compared using Mann-Whitney U tests.

Table 1. Correlation analysis between the different queen physiological parameters (lipid store in the fat bodies, Vg titer in hemolymph and QMP levels (9-ODA, HOB and 9-HDA) in heads). r values are shown in the table. Number in bold indicates a significant correlation between lipid store and Vg titer (P = 0.013). The others parameters are not significantly correlated (P > 0.05).

Parameters	Lipid store	Vg titer	9-ODA	HOB
Vg titer	**-0.604**			
9-ODA	0.161	0.089		
HOB	-0.134	-0.290	-0.385	
9-HDA	-0.168	0.265	0.066	-0.215

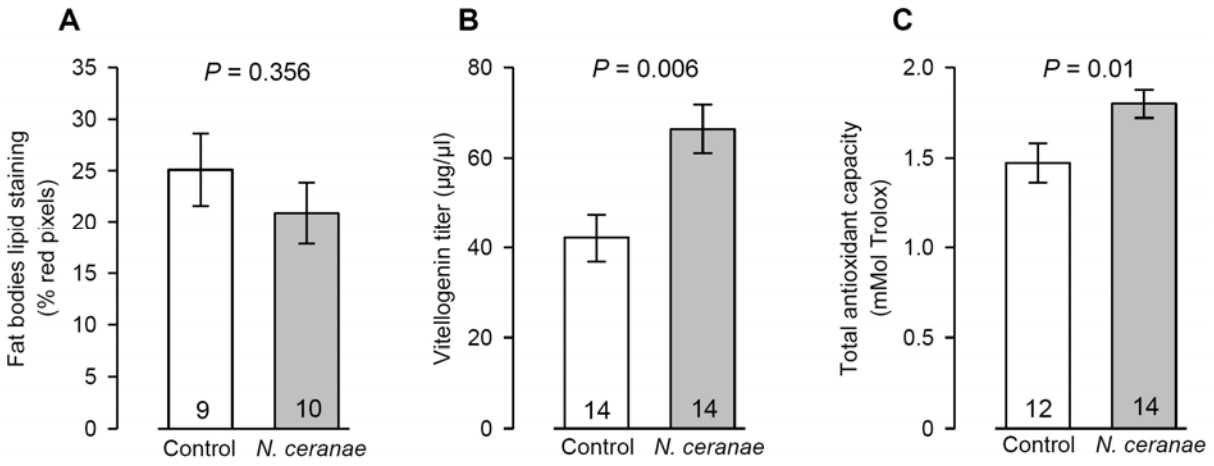

Figure 1

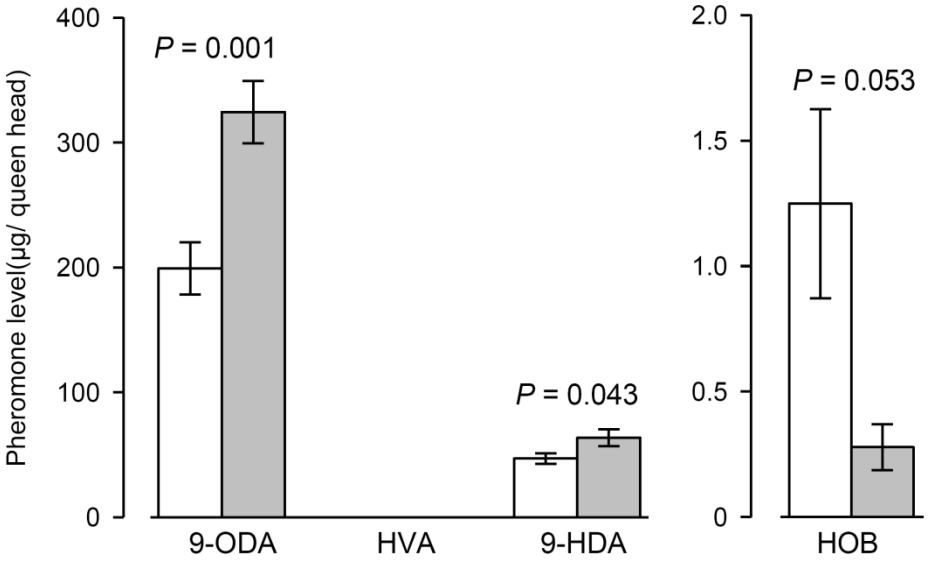

Figure 2