Effets de *Nosema ceranae* sur la maturation comportementale des abeilles le cas de la phéromone oléate d'éthyle et le butinage

Présentation

Dans l'étude des effets de *N. ceranae* sur la maturation comportementale des abeilles, nous nous sommes intéressés particulièrement aux changements induits dans la division des tâches des ouvrières. Ce polyéthisme d'âge est sous le control d'un réseau des phéromones parmi d'autres facteurs endogènes. Une de ces phéromones est l'oléate d'éthyle (EO) émise par les abeilles butineuses qui ralentit la progression naturelle des nourrices vers le statut de butineuses (Leoncini *et al*., 2004) et qui régule ainsi l'équilibre entre la quantité de nourrices et de butineuses dont la colonie a besoin.

Dans ce contexte le présent chapitre est basé sur l'**hypothèse spécifique** du potentiel de *Nosema* spp. à altérer la production de la phéromone EO chez les ouvrières et en conséquence de perturber leur maturation comportementale. Nous avons ainsi développé notre premier objectif qui était la recherche des effets de *N. ceranae* sur l'organisation sociale de la colonie.

La première partie expérimentale (article n°1) fait partie d'un travail plus vaste développé en collaboration avec Alaux *et al.* (2010a) (Annexe 1). Dans cette expérience, des abeilles élevées dans des cagettes au laboratoire ont été exposées à deux facteurs de risque : un pathogène (*Nosema* spp.) et un pesticide (l'imidaclopride). Les résultats de ce travail ont mis en évidence une augmentation de la demande énergétique et de la mortalité des abeilles infectées par *Nosema* spp., et notamment les effets de l'interaction de ces deux facteurs sur l'immunité sociale. En parallèle, nous avons analysé le taux d'EO chez les mêmes abeilles exposées auparavant par Alaux *et al*. (2010a). Cela nous a permis de constater un changement dans la production d'EO chez les abeilles parasitées. Suite à ces résultats, nous avons conduit une deuxième expérience dans des conditions naturelles (article n°2), afin de recréer le contexte social d'une colonie, qui est fondamental pour l'étude du comportement lié à cette phéromone.

Article n°1:

Nosema spp. infection alters pheromone production in honey bees (*Apis mellifera*)

Journal of Chemical Ecology
2010. 36, 522-525.
doi:10.1007/s10886-010-9786-2
Rapid Communication
Version « .txt » du texte publié

Claudia Dussaubat[1*], Alban Maisonnasse[1*], Cédric Alaux[1], Sylvie Tchamitchan[2], Jean-Luc Brunet[2], Erika Plettner[3], Luc P. Belzunces[2], Yves Le Conte[1]

[1] UMR 406 Abeilles et Environnement, Laboratoire Biologie et Protection de l'abeille, INRA, Site Agroparc, 84914 Avignon, France
[2] UMR 406 Abeilles et Environnement, Laboratoire Toxicologie Environnementale, INRA, Site Agroparc, 84914 Avignon, France
[3] Department of Chemistry, Simon Fraser University, Burnaby V5A 1S6, BC, Canada

[*]Claudia Dussaubat and Alban Maisonnasse contributed equally

Résumé

Chez les insectes sociaux les phéromones participent activement à l'homéostasie du groupe. Dans la littérature scientifique il est bien référencé que les parasites peuvent modifier les signaux hormonaux de leur hôte. Contrairement, les connaissances sur l'effet des parasites sur les signaux phéromonaux sont très limitées. Nous avons donc voulu analyser l'effet d'un parasite de l'abeille largement distribué à niveau mondiale, *Nosema* spp., sur la production d'oléate d'éthyle (EO), la seule phéromone modificatrice identifiée chez les ouvrières d'*A*. *mellifera*. Vu que d'autres facteurs environnementaux, tels que les pesticides, peuvent aussi affaiblir les colonies d'abeilles, nous avons également analysé l'effet sur la production d'EO de l'imidaclopride, un pesticide de la famille des néonicotinoïdes largement utilisé en agriculture. Des abeilles ont été exposées en cagettes au pathogène et au pesticide. Après 10 jours, les taux d'EO chez des abeilles traitées et des abeilles contrôles ont été analysés. Contrairement à l'imidaclopride, *Nosema* spp. a modifié la production de l'EO. Le niveau d'infection des abeilles par *Nosema* spp. a été positivement corrélé avec leur niveau de production d'EO. En conséquence, comme l'EO est impliqué dans la régulation de la division du travail des ouvrières, nos résultats suggèrent que des augmentations de production d'EO par des abeilles infestées peuvent perturber la communication chimique de la colonie et donc son homéostasie.

Abstract

Pheromones in social insects play a key role in the regulation of group homoeostasis. It is well-established that parasites can modify hormone signaling of their host, but less is known about the effect of parasites on pheromone signaling in insect societies. We, thus, tested in honey bees (*Apis mellifera*) the effect of the widespread parasite *Nosema* spp. on the production of ethyl oleate (EO), the only identified primer pheromone in honey bee workers. Since environmental stressors like pesticides also can weaken honey bees, we also analyzed the effect of imidacloprid, a neonicotinoid widely used in agriculture, on EO production. We show that, contrary to imidacloprid, *Nosema* spp. significantly altered EO production. In addition, the level of *Nosema* infection was correlated positively with the level of EO production. Since EO is involved in the regulation of division of labor among workers, our result suggests that the changes in EO signaling induced by parasitism have the potential to disturb the colony homoeostasis.

1. Introduction

Analogous to the hormones that control the organism homoeostasis, pheromones in social insects play a key role in the regulation of group homoeostasis. However, homoeostasis of both organisms and insect societies can be threatened by parasite infection. For example, in mammals there is evidence that parasites can modify the endocrine system of the host to favor their development and reproduction (Escobedo *et al*. 2005). In honey bees, the cuticular hydrocarbon profile involved in social recognition can be altered by an activation of the immune system (Richard *et al*. 2008) or parasitization by the mite *Varroa destructor* (Salvy *et al*. 2001). However, it is not known whether, analogous to the modification of hormone signaling in the organism, parasites can affect pheromone signaling in insect societies.

To answer this question, we asked whether the microsporidia *Nosema* spp., potentially involved in worldwide honey bee losses (Higes *et al*. 2008), could affect the production of pheromone in workers. We analyzed the production of the only indentified primer pheromone in workers: ethyl oleate (EO), which regulates worker behavioral maturation (i.e., inhibits the transition from inside-nest tasks performed by young bees (nurse) to foraging tasks performed by old bees (forager)) (Leoncini *et al*. 2004). The focus was on primer pheromones because they are essential to the regulation of social behaviors and colony homeostasis. Therefore, a modification in their production could affect the whole colony organization and endanger its survival. Since survival of honey bees can be threatened by other stressors, such as pesticides, we also tested the

effects of a neonicotinoid (imidacloprid) widely used in agriculture on EO production. Pesticides are known to disrupt pheromone perception, but they also can affect their production (Desneux *et al*. 2007).

2. Methods and Materials

2.1. *Nosema* infection and imidacloprid exposure

This experiment was part of a larger study described in detail by Alaux *et al*. (2010) (see "Annexe 1" for further details on Experimental procedure). Briefly, in order to test the effect of *Nosema* infection and/or imidacloprid exposure, 1-d-old bees were reared in cages and split into four experimental groups: control groups, groups infected with *Nosema*, groups exposed to imidacloprid, and groups both infected with *Nosema* and exposed to imidacloprid. For each experimental group, 3 colonies were used, with 2 cage eplicates for each colony (N = 120 bees per cage). For the *Nosema* infection, bees were fed individually at the beginning of the experiment with a sugar solution containing 200,000 spores (Alaux *et al*. 2010). Spores were isolated previously from infected colonies as in Higes *et al*. (2007), and genetic analysis showed that our bees were infected with both *N. apis* and *N. ceranae* (see Alaux *et al*. 2010). For the pesticide exposure, caged bees were chronically exposed 10 h per day to imidacloprid by ingesting a sugar solution containing 7 µg/kg of imidacloprid (a concentration encountered in the environment) (see Alaux *et al*. 2010). The solution was replaced each day. After 10 d, bees were collected and stored at -20°C in order to measure the level of EO and *Nosema* infection.

2.2. EO Quantification

Pools of 5 bees were analyzed. Wholebody extracts were prepared in 1.9 ml of iso-hexane with the addition of 100 µl of two internal standard solutions at 10 ng/µl (arachidic acid methyl ester, and methyl heptadecanoate, Sigma-Aldrich, France). Samples were crushed with a glass rod for 2 min at 0°C and centrifuged for 20 min at 4°C (2,500 g). The supernatant was collected and applied to a silica column (silica gel 60, particle size 40–63 m, 230–400 mesh). The first fraction was eluted in 3 ml of a solvent mixture (98.5% iso-hexane, 1.5% diethyl ether). The second fraction containing the EO was eluted in 3 ml of a second solvent mixture (94% iso-hexane, 6% diethyl ether). One ml of this fraction was concentrated to 10 µl under a nitrogen stream, and 1 µl was injected into a fast gas chromatograph (2014, Shimadzu, Japan) equipped with a split-splitless inlet, a flame ionization detector, and a capillary column Omegawax 100 (10 m x 0.10 mm, 0.10 µm film thickness). Samples were injected in split mode. Hydrogen was used as carrier gas with a column flow of 0.52 ml/min. Oven temperature was set at 90°C for 1 min, raised to 195°C at 40°C min^{-1}, stabilized for 3 min, then augmented to 210°C at 1°C min^{-1}, stabilized

again for 2 min, then increased to 270°C at 40°C min^{-1} and held at 270°C for 3 min. Identification and quantification of EO was based on retention times of EO synthetic compound (Sigma-Aldrich, France) and by comparison of internal standard area, respectively, using a gas chromatography solution program (Shimadzu, Japan). The EO confirmation was done by a mass spectrometer (CP2010, Shimadzu, Japan) operated in the electron impact mode at 70 eV with continuous scans (every 0.2 sec) from a mass to charge ratio (m/z) of 70–400.

2.3. *Nosema* spore counting

Since *Nosema* is an intestinal parasite, the honey bee intestinal tract was dissected and macerated in distilled water as in Higes *et al*. (2007). The spore concentration from the suspension then was determined by using a haemocytometer.

3. Results and Discussion

Nosema infection caused a significant increase in EO production compared to non-infected groups (Fig. 1a), thus demonstrating that pheromone production can be modified by environmental stressors. However, EO production in imidacloprid-exposed bees did not differ significantly from non-exposed bees (Fig. 1a). Neonicotinoids target the nicotinic acetylcholine receptors and thus can affect neural function (Decourtye *et al*. 2004), but here no effect was found on pheromone production. Studies with different pesticides are needed to determine whether this absence of modification that we observed is a general phenomenon.

One would expect that *Nosema* infection induces a cost to pheromone production. Contrary to this expectation, parasitized bees produced more EO than healthy ones. Since, EO is present at higher levels in foragers compared to nurses (Leoncini *et al*. 2004), and because *Nosema* causes a precocious onset of foraging (Wang and Moeller 1970), the EO increase might reflect a forager profile of infected bees compared to control bees. However, further investigation tended to show that the EO increase is not just a consequence of a forager profile. First, the level of EO in parasitized bees was 6 times higher than healthy bees, which is greater than the difference naturally found between nurses (young bees) and foragers (old bees) (100 ± 19 ng EO/nurse, $N = 60$ and 213 ± 25 ng EO/forager, $N = 120$, unpublished data from $N = 3$ colonies, A. Maisonnasse). Second, there was a positive and significant relationship between EO level and the number of *Nosema* spores per bee (Fig. 1b), showing that the EO increase is not an all-or-nothing response but is linked to the level of *Nosema* infection.

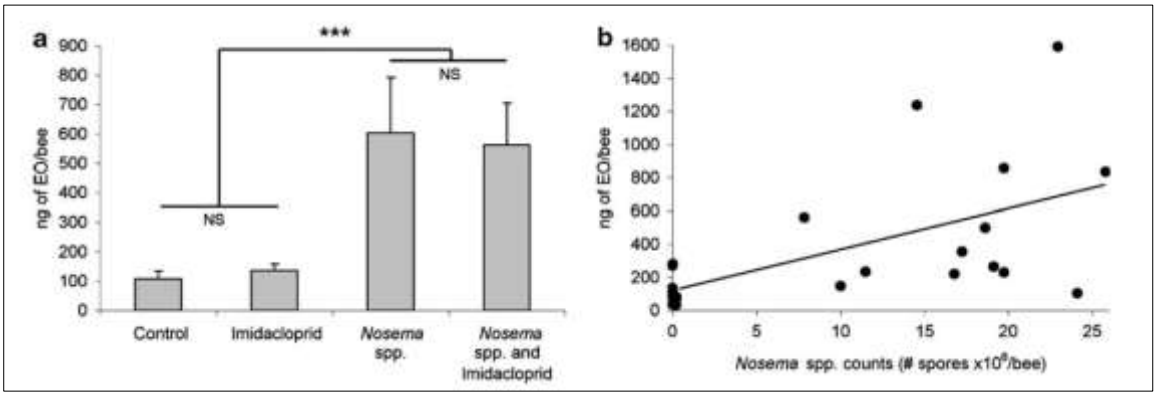

Figure 1. Effect of *Nosema* infection and/or imidacloprid exposure on EO production in honey bee workers. (**a)** EO level for each experimental group. Two pools of 5 bees per cage were analyzed, with 2 cages per treatment. The experiment was replicated on 3 colonies giving a total of *N* = 60 bees per treatment. Treatment and colony effects were determined by using two-way ANOVA on log transformed values followed by Fisher post-hoc tests. There was a significant treatment effect on EO production ($F_{3,47}$ = 17.35, $p < 0.001$). Bees infected with *Nosema*, with or without an exposure to imidacloprid, had a higher level of EO than control and imidacloprid-exposed bees ($p < 0.001$ for each comparison). However, imidacloprid did not affect EO production (control vs. imidacloprid exposed bees: $p = 0.81$; or *Nosema*-infected bees vs. *Nosema*-infected and imidacloprid-exposed bees: $p = 0.14$). There also was a significant effect of colony origin ($F_{2,47}$ = 4.59, $p = 0.017$), but no significant interaction with the treatments was found ($F_{6,95}$ = 0.88, $p = 0.52$) thus demonstrating a consistent effect of the treatments. Data show mean ± SE. *** and NS denote significant ($p < 0.001$) and non-significant differences between treatments, respectively. (**b**) Relation between EO production and the level of *Nosema* infection. There was a significant positive correlation between the quantity of EO produced and the number of *Nosema* spores infecting bees ($r = 0.58$, $p < 0.005$, $N = 24$).

Even if the earlier onset of foraging could be a bee response that decreases the *Nosema* load within the hive, the higher EO level in infected bees has the potential to disturb colony organization. The abnormally high level of EO could mislead the colony on the actual number of foragers and delay the onset of foraging in non-infected nurses. It is not known, however, how infected bees who accelerate their behavioral maturation would react to the high inhibitory effects of EO. On the other hand, since *Nosema* infection decreases worker lifespan (Higes *et al*. 2007), a loss of EO in the colony also can be expected and to accelerate nurse maturation. Field studies are needed to determine the actual response of the colony and whether a failure in pheromone communication induced by parasitism or disease could lead to the colony collapse.

To our knowledge, this is the first demonstration that parasites can modify pheromone production in insect societies. Therefore, our finding indicates that pathogens, besides their effect at the individual level, also can cause damage at the social level.

Acknowledgements

We thank D. Beslay, V. Marteau, C. Gines, and lab members for assistance with bees. Fundings were provided by HFSP (RGPP0042/2007-C101) and FEOGA grants. C. Dussaubat, A. Maisonnasse, and C. Alaux were supported by a CONICYT/French Embassy of Chili grant, a HFSP grant (RGP0042/2007-C101), and an INRA young researcher position (INRA SPE department), respectively.

Article n°2:

Flight behavior and pheromone changes associated to *Nosema ceranae* infection of honey bee workers (*Apis mellifera*) in field conditions

Soumis à Journal of Invertebrated Pathology

Claudia Dussaubat[a], Alban Maisonnasse[a,1], Didier Crauser[a], Dominique Beslay[a], Guy Costagliola[b], Samuel Soubeyrand[c], André Kretzchmar[c], Yves Le Conte[a]

[a] INRA, UR 406 Abeilles et Environnement, Site Agroparc, 84914, Avignon, France

[b] INRA, UR 1115 Plantes et Systèmes de culture Horticoles, Site Agroparc, 84914, Avignon, France

[c] INRA, UR546 Biostatistique et Processus Spatiaux, Site Agroparc, 84914 Avignon, France

[1] Present address : ADAPI (Association pour le développement de l'Apiculture), Maison des Agriculteurs – 22, Avenue Henri Pontier – 13326, Aix en Provence Cedex 1, France.

Résumé

Les parasites des insectes sociaux sont connus pour provoquer la perte des individus. Chez les colonies d'abeilles la disparition d'ouvrières est un facteur commun dans le cas de l'effondrement des colonies observé au niveau mondial. Le parasite émergent *Nosema ceranae* peut réduire la capacité de l'abeille européenne à rentrer à la colonie, diminuer son sens de l'orientation, et altérer son métabolisme. Les abeilles infectées par *N. ceranae* présentent des changements dans leurs taux d'Oléate d'éthyle (EO, Ethyl Oleate), qui est la seule phéromone modificatrice (« primer pheromone ») identifiée à nos jours, impliquée dans le comportement de butinage. Notre hypothèse est donc, que *N. ceranae* (i) modifie l'activité de vol des abeilles, et (ii) induit des modifications dans les niveaux d'EO qui peuvent altérer le comportement de butinage des autres abeilles de la colonie. Nous avons comparé l'activité de vol des abeilles infectées et non infectées placées dans de petites colonies à l'aide d'un compteur électronique-optique d'abeilles pendant 28 jours. Nous avons mesuré les niveaux d'EO (GC/MS) et la charge de spores. Nous avons estimé la mortalité d'abeilles à la fin de l'expérience. Les abeilles infectées ont montré une activité de vol précoce et plus intense que les abeilles saines. Cela est en accord avec des niveaux d'EO plus élevés et une durée de vie raccourcie pour les abeilles infectées. Nos résultats suggèrent que les niveaux plus élevés d'EO chez les abeilles infectées pourraient retarder la maturation comportementale des abeilles saines du même âge, ce qui pourrait expliquer les niveaux d'activité plus bas pour ces dernières. Nous proposons que la maturation comportementale différée constitue une réponse de protection face à l'infection, puisque les abeilles saines exécutent des tâches moins risquées à l'intérieur de la colonie allongeant ainsi leur durée de vie. Nous discutons également sur l'augmentation de l'activité de vol des abeilles infectées qui réduirait la transmission du pathogène au sein de la colonie. Des recherches supplémentaires aideront à la compréhension des conséquences des changements de comportement sur la transmission du pathogène. Cela pourrait contribuer à renforcer les mécanismes naturels de défense de la colonie à travers les pratiques apicoles et réduire ainsi la perte de colonies.

Abstract

Parasites are known to cause the loss of individuals in social insects. In honey bee colonies the disappearance of foragers is a common factor of the wide extended colony losses. The emergent parasite of the European honey bee *Nosema ceranae* has been found to reduce homing and orientation skills and alter metabolism of forager bees. *N. ceranae*-infected bees also show changes in Ethyl Oleate (EO) levels, which is so far the only primer pheromone identified in workers that is involved in foraging behavior. Thus, we hypothesized that *N. ceranae* (i) modify flight activity of honey bees and (ii) induce EO changes that can alter foraging behavior of nestmates. We compared flight activity of infected bees and non-infected bees in small colonies using an electronic optic bee counter during 28 days. We measured EO levels (GC/MS) and spore-counts. Bee mortality was estimated at the end of the experiment. As we predicted, infected bees showed precocious and a higher flight activity than healthy bees, which agreed with the more elevated EO titers of infected bees and reduced lifespan. Our results suggest that the higher EO levels of infected bees might delay the behavioral maturation of same age healthy bees, which might explain their lower level of activity. We propose that delayed behavioral maturation of healthy bees might be a protective response to infection, as healthy bees would be performing less risky tasks inside the hive, thus extending their lifespan. We also discuss the potential of increased flight activity of infected bees to reduce pathogen transmission inside the hive. Further research is needed to understand the consequences of host behavioral changes on pathogen transmission. This knowledge may contribute to enhance natural colony defense behaviors through beekeeping practices to reduce probability of colony losses.

1. Introduction

The worldwide decline of honey bee populations due to multiple environmental stressors (Biesmeijer *et al*., 2006; Paxton, 2010; Ratnieks and Carreck, 2010; vanEngelsdorp *et al*., 2009), may have big consequences for agriculture development and maintenance of natural ecosystems, as insect pollination is essential to reproduction of many plant species (Calderone, 2012; Gallai *et al*., 2009; Klein *et al*., 2007; Lautenbach *et al*., 2012). Attempts to characterize bee loss phenomena have given rise to the definition of "colony collapse disorder", in which the disappearance of honey bee foragers is a common factor (Khoury *et al*., 2011; vanEngelsdorp *et al*., 2009).

Parasites are known to cause the loss of individuals in social insects. Heinze and Walter (2010) showed that when workers from the ant *Temnothorax unifasciatus* are challenged by a fungal infection they leave the nest hours or even days before death and never return. In honey bees, it has been observed that foragers carrying *Varroa destructor* mites (Kralj and Fuchs, 2006), infected with sacbrood virus (Bailey and Fernando, 1972) or deformed wing virus (Iqbal and Mueller, 2007), lose abilities to learn, orient and fly.

Recently, the microsporidian *Nosema ceranae* has also been found to reduce homing and orientation abilities in the honey bee (Kralj and Fuchs, 2010). The presence of this parasite in the European honey bee *A. mellifera* was unknown until 2005 (Higes *et al*., 2006; Huang *et al*., 2007), since then it has been detected worldwide (Chen *et al*., 2008; Fries *et al*., 2006; Klee *et al*., 2007; Rodríguez *et al*., 2012). Before 2005, it was only known to parasitize the Asian honey bee *A. cerana* (Fries *et al*., 1996). *N. ceranae* has the potential to considerably reduce colony strength and productivity (Botías *et al*., 2012) and interact with other environmental stressors weakening colony health (Alaux *et al*., 2010; Pettis *et al*., 2012; vanEgelsdorp, *et al*., 2009). In regions with Mediterranean climate, like in Spain, *N. ceranae* has also been signaled as the main cause of colony losses (Higes *et al*., 2008, 2009), although the asymptomatic presence of this parasite have been reported as well (Fernandez *et al*., 2012).

Nosema spp. produce a resistant spore that transfers its genetic material to the host cell of the midgut epithelium where it multiplies; new spores can re-infect the same individual or be disseminated into the environment through honey bee feces (Wittner and Weiss, 1999). Microsporidia are known to uptake ATP from the host cell environment, as they only have remnant mitochondrial organelles (Williams, 2009), which is related to the energetic stress suffered by infected bees (Alaux *et al*., 2010; Mayach and Naug, 2010; Naug and Gibbs, 2009). *Nosema ceranae* is also able to suppress particular mechanisms of the honey bee individual immune system (Antunez *et al*., 2009; Chaimanee *et al*., 2012) and there is strong evidence that links infection process to oxidative stress, gut tissue degeneration and prevention of epithelium renewal in the honey bee (Dussaubat *et al*., 2012). Mayack and Naug (2009) demonstrated in laboratory conditions that *Nosema*-infected bees were able to counteract infection by increasing feeding to fulfill elevated needs of energy (Mayack and Naug, 2010; Naug and Gibbs, 2009). This behavior could be related to precocious foraging (Hassein, 1953; Lin *et al*., 2009; Wang and Moeller, 1970; Woyciechowski and Moron, 2009), and the increase in foraging activity (Woyciechowski and Kozlowski, 1998). Interestingly, it was observed in laboratory trials that infected bees develop extremely high levels of the primer pheromone "Ethyl Oleate (EO)"

(Dussaubat *et al*., 2010). This pheromone is produced by foragers and its function is to delay the onset of foraging of nurses (Leoncini *et al*., 2004).

Consequently, we hypothesized that first, *N. ceranae*-infected bees have an increased flight activity as an individual response to infection, and second, that the pheromone profile of infected bees can alter social organization. In order to test this we carried out experimental infections of honey bees to record flight activity in field conditions and measured EO levels. We discuss how behavioral changes can influence parasite transmission within the colony and its implications on colony losses.

2. Materials and methods

To study the effect of *N. ceranae* infection on the flight activity of worker bees we carried out honey bee experimental infection in field conditions using four cohort colonies. During the experiment we measured flight activity, EO levels and spore-loads. At the end of the trial we estimated bee mortality. The experiment was finished when almost all infected bees had left the hive (lost or dead) after 28 days.

2.1. Experimental infection

Fresh spores were isolated and purified from naturally-infected foragers from a colony located at the National Institute of Agricultural Research (INRA) from Avignon (France) according to the protocol adapted from Higes *et al*. (2007). Briefly, bees were kept in the cold for 20 min to anesthetize them, abdomens were separated with dissection tweezers, homogenized in distilled water, filtered in Whatman N°4 paper and then centrifuged three times (6 min at 800 g), the supernatant was discarded as the spores remained in the sediment. The pellet was suspended in 10 ml of distilled water and the spore concentration was calculated using a haemocytometer. This suspension was used to prepare a dose of 100 000 spores per bee in a 50% sucrose solution. In laboratory experiments a similar spore dose produced less than 10% of bee mortality in 10 days and 50% in 20 days (Vidau *et al*., 2011). Newly emerged honey bees were fed individually with 2 µl of this solution using a micropipette. After infection honey bees were colored marked and introduced into a small hive. *Nosema* species was confirmed by PCR as described in Alaux *et al*. (2010).

To obtain new born honey bees for infection, frames of capped brood were collected one day before emergence from 3 different colonies and kept in incubators (33°C). New born bees were mixed in order to reduce a potential colony effect. Samples of new born bees used to build

cohorts were analyzed to verify absence of *N. ceranae* spores. Bees were divided in two cohorts, one non-infected bees (control) and one to be infected with *N. ceranae* spores as described before.

2.2. Experimental colonies

The effect of *N. ceranae* was tested in colonies made of four different cohorts of worker bees introduced in a small hive or nucleus ("nuc") (Fig. 1). A total of three identical nucs were used in parallel. Each nuc contained cohorts of both non-infected bees and *N. ceranae* infected bees which allowed comparing the behavior and pheromone profile between both groups sharing the same environment. The nucs were built as follow:

Day 0- introduction of the first two cohorts:

On day 0 of the experiment, each nuc was built with two cohorts composed of new born honey bees. One cohort named "Control 1" was composed of 4000 *N. ceranae*-free bees and a second cohort named "*Nosema* 1" corresponded to 300 *N. ceranae* infected bees. "Control 1" cohort played the role of "background bees" of the nuc representing the main population in the colony.

Day 7- introduction of the second two cohorts:

Seven days after nucs were built, two other identically treated cohorts of new born honey bees were introduced in the same nuc. One cohort named "Control 2" of 300 *N. ceranae*-free bees and a second cohort named "*Nosema* 2" of 300 *N. ceranae* infected bees.

To populate the nucs, frames of sealed brood were kept in an incubator at 34ºC ± 1ºC, when new worker bees started to emerge they were carefully removed, marked with a paint dot on the thorax, with each cohort a separate color and placed in the nuc. In the case of *Nosema* treated-bees, they were infected, marked and then introduced in the nuc. All nucs had the same number of bees and contained two honeycomb frames and two empty frames. To simulate the presence of a queen, each nuc was given a commercially available plastic strip (Bee Boost, PheroTech, Delta, BC, Canada) containing the five components of the Queen Mandibular Pheromone (QMP) blend that releases one queen equivalent per day. Because no brood was produced, colonies had no exposure to brood pheromone. Controlling both QMP and brood pheromone is important as they can affect the age of onset of foraging (Leoncini *et al*., 2004). The last day of the experiment the entrance of the nucs were closed during the evening and live remaining bees were froze at -20°C to be counted and calculate bee mortality.

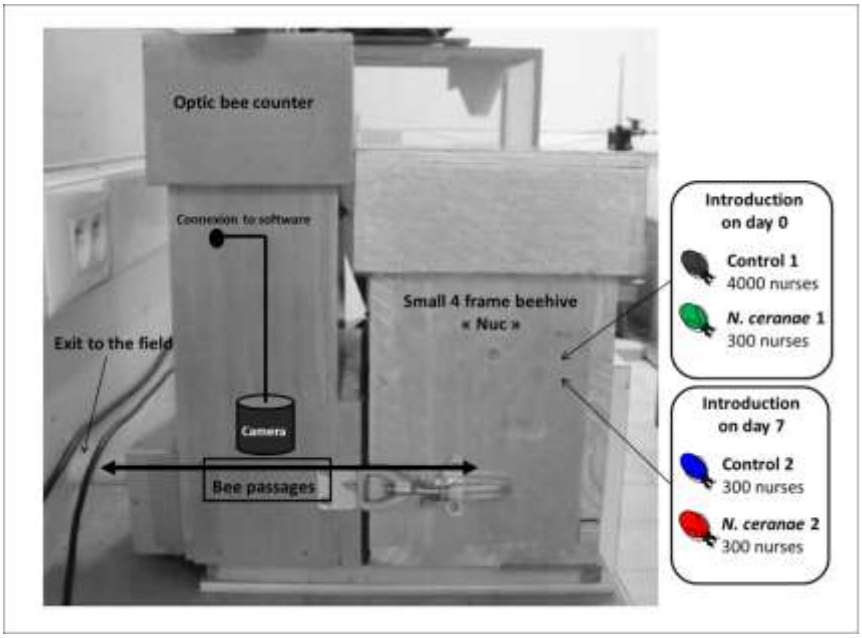

Figure 1. Schematic representation of the optic bee counter and nuc (small hive). The counter is connected to a software that control counting conditions and record data. Treated honey bees cohorts are represented at the side.

2.3. Optic bee counter and flight activity

The three nucs were equipped with an optic-electronic bee counter at the entrance that allows for the recording of outgoing (exits) and incoming (entrances) bees between the nest and the field. The count was recorded every 5 min (i.e. cumulated activity during 5 min) for 24 h a day, yielding 288 measurements per day of both "in" and "out" with no limits on days of registration. Date, time and cumulative values were automatically saved in an Excel file. In this way it is possible to introduce 4 cohorts in a nuc to be counted independently, 3 cohorts marked with 3 different colors and one cohort non-marked.

Nonstop recording is highly important when studying forage behavior in bees since flight patterns are the result of the interaction of weather and other environmental factors. Indeed in some natural landscapes foraging patterns can suffer daily changes as new and better food sources are discovered (Winston, 1987), so a limited time of observations per day can give only a partial idea of flight activity.

The optic counter consists of a camera placed inside of a modified entrance of a nuc. At the bottom of the entrance there are 8 passages with the form of a tunnel with the size of one bee that can be crossed only while exposing the back of the thorax to the camera. The width of the 8

passages corresponds to the camera lens angle. An original software (IDDN. FR.001.130013.000.R.P.2010.000.31235) developed in the laboratory at INRA - Avignon, which runs in LabView environment for graphic programming, allows to calibrate the sensibility to different colors to achieve the highest performance when counting bees with a minimal error (3 - 4 %). It also adjusts the frequency of images captured per second in order to reduce the chance of missing some bees passing in front of the camera. The software controls the three cameras (one in each of the 3 nucs) simultaneously, processes the signal, analyzes the images in real time and acquires the data.

2.4. Ethyl oleate chemical analysis

Twelve incoming honey bees were sampled from each cohort of infected and control bees at the entrance of the nucs. They were picked by the thorax with a pair of soft tweezers and immediately froze and stored at -20°C until analysis. Sampling was carried out when bees were 14 and 21-days-old. Pools of 4 bees were analyzed for EO quantification as described in Dussaubat *et al*. (2010). Briefly, whole-body extracts were prepared in iso-hexane with the addition of two internal standard solutions (methyl eicosanoate and methyl heptadecanoate, Sigma-Aldrich, France). After samples were crushed and centrifuged, supernatant was applied to a silica column and two fractions were obtained, the second fraction containing fatty acid methyl esters including EO was concentrated under a nitrogen stream, and 1 µl injected into a gas chromatograph (GC, 2014, Shimadzu, Japan) equipped with a flame ionization detector (FID), and a capillary column Omegawax 100 (10 m x 0.10 mm, 0.10 µm film thickness). Identification and quantification of EO was based on retention times of EO synthetic compound (Sigma-Aldrich, France) and by comparison of internal standard area, respectively. The EO confirmation was done by a GC-MS (Trace-ISQ, Thermo Scientific, USA), operated in the electron impact mode at 70 eV, continuous scans (m/z 50 – 500), equipped with a capillary column TR5-MS (20 m x 0.10 mm, 0.10 µm film thickness).

2.5. N. ceranae spore-load

Simultaneously to OE sampling (see above), six incoming honey bees were sampled from each cohort of infected and control bees to perform spore count. Honey bee midguts were extracted with dissection tweezers and individually homogenized in an Eppendorf tube with 1 ml of distilled water for 10 sec at a frequency of 30/s in a TissueLyser® (Qiagen). Spore-load per bee was directly determined from the homogenized using a haemocytometer.

2.6. Statistical analysis

Flight activity

Flight activity analysis was based on the number of exits (outgoing bees) from the nucs since the number of entrances and exits per day followed the same pattern and were similar in quantity. The difference between daily entrances and exits fell in the order of magnitude of the counter error (3-4 %). To analyze flight activity we performed both a graphical test and built a model based on the daily number of exits as follow.

Graphical test for the daily number of exits. To test the null hypothesis H_0 of no difference between control and infected bees based on the number of exits, we built a graphical test which can be applied to two time series of numbers of exits. Let $N(i,t)$ denote the number of exits for cohort i (i =1 for control bees and i =2 for infected bees) at day t (t in {0,…,T}). Let n_i denote the initial number of bees in cohort i. Under H_0, at day t, the total number of exits in cohorts 1 and 2 is $N(1,t)+N(2,t)$, a fraction $n_1/(n_1+n_2)$ of exits is expected to be from control bees and a fraction $n_2/(n_1+n_2)$ of exits is expected to be from infected bees. Moreover if we assume that a random multinomial noise is applied to these fractions and leads to the realized numbers of exits, then, for each day and each cohort, we can build a confidence interval for the corresponding number of exits. Thus, for each cohort, we can plot the observed numbers of exits and 95% confidence interval ("confidence envelopes") in which the observed curve should be included under H_0. To get the 95% level for confidence envelopes, we used the Bonferroni's correction (Miller, 1981): since for a given cohort there are T+1 data (one number of exits per day), the confidence envelope is obtained by building T +1 confidence intervals (one for each number of exits) and the confidence level of the intervals has to be fixed at (100 - 5/(T+1))%. The conclusion of the graphical test is drawn following this statement: if the observed time series goes out from the 95% confidence envelope, then the null hypothesis H_0 can be rejected at the risk level 5%.

Model for the daily number of exits. Let $N(i,t)$ denote the number of exits in day t for the cohort of bees i; t starts at zero and ends at T. We built a model for the daily number of exits and fitted it to data by following a quasi-likelihood approach (McCullagh and Nelder, 1989). This approach consists in specifying a model for the expectation and the variance of $N(i,t)$ without

specifying a probabilistic distribution. Thus, we did not make strong assumptions on the mechanisms governing the variability of the daily number of exits.

The expectation of the daily number of exits was modeled as follows:

$$E\left[N(i,t)\right] = \alpha_i n_i d_t P \text{ [bee of cohort } i \text{ is active at day } t\text{]}$$

where α_i is an unknown positive parameter interpreted as the mean daily number of exits per active bee, n_i is the initial number of bees in cohort i and d_t is an unknown day effect which applies to all the cohorts of bees at day t; the mean of the day effects over the period 0, …, T is fixed at one so that the parameters α_i are identifiable. The probability that a bee of cohort i is active at day t (last term appearing in the previous equation) is modeled as the probability that a bee of cohort i is no more "juvenile" ("juvenile state" corresponds to an inhive bee) but not yet "retired" ("retired state" is when bees are non-active because any reason) at day t, these events being assumed to be independent:

P [bee of cohort i is active at day t]
= P [bee of cohort i is no more juvenile but not yet retired at day t]
= P [bee of cohort i is no more juvenile at day t] x P [bee of cohort i not yet retired at day t]

Consider a given bee, let X denote the number of days required for the bee to exit the "juvenile state" and Y denote the number of days to enter the "retirement state". Assume that X and Y are independent and follow Poisson distributions with unknown mean parameters $\beta_i > 0$ and $\gamma_i > 0$ depending on the cohort to which the bee belongs. Under this model, Y can be smaller than X; in this case, the bee is "retired" before the end of the "juvenile state" and, consequently, never becomes active outside the hive. Under these assumptions about X and Y,

$$P \text{ [bee of cohort } i \text{ is no more retired at day } t\text{]} = P\left[t \geq X\right] = \sum_{s=0}^{t} e^{-s/\beta_i} \frac{\beta_i^s}{s!}$$

$$P \text{ [bee of cohort } i \text{ is no retired at day } t\text{]} = P\left[t \leq Y\right] = 1 - P\left[Y < t\right] = 1 - \sum_{s=0}^{t-1} e^{-s/\gamma_i} \frac{\gamma_i^s}{s!}$$

And, therefore,

$$E\left[N(i,t)\right] = \alpha_i n_i d_t \left(\sum_{s=0}^{t} e^{-s/\beta_i} \frac{\beta_i^s}{s!}\right)\left(1 - \sum_{s=0}^{t-1} e^{-s/\gamma_i} \frac{\gamma_i^s}{s!}\right).$$

The variance of $N(i,t)$ is assumed to be proportional to its expectation: $V\left[N(i,t)\right] = \sigma^2 E\left[N(i,t)\right]$, where σ^2 is an unknown positive parameter.

Parameter estimation. The estimation of parameters is carried out in two stages because of the day effect d_t. First, we built estimates of the day effects by using data collected for the three control cohorts of 4 000 bees. These control cohorts correspond to the index i=1, 2 and 3. The model without day effect (i.e. d_t=1 for all t) is fitted to each of the three data sets mentioned above by following the procedure provided in McCullagh and Nelder (1989). Let $\tilde{\alpha}_i$, $\tilde{\beta}_i$ and $\tilde{\gamma}_i$ denote the estimates of the parameters which are obtained. Then, the day effects which satisfy

$$d_t = E\left[N(i,t)\right] / \left\{\alpha_i n_i \left(\sum_{s=0}^{t} e^{-s/\beta_i} \frac{\beta_i^s}{s!}\right)\left(1 - \sum_{s=0}^{t-1} e^{-s/\gamma_i} \frac{\gamma_i^s}{s!}\right)\right\}$$

are estimated by computing, instead of the expectation above, the following average depending on the estimates $\tilde{\alpha}_i$, $\tilde{\beta}_i$ and $\tilde{\gamma}_i$:

$$\delta_t = \frac{1}{3}\sum_{i=1}^{3} N(i,t) / \left\{\tilde{\alpha}_i n_i \left(\sum_{s=0}^{t} e^{-s/\tilde{\beta}_i} \frac{\tilde{\beta}_i^s}{s!}\right)\left(1 - \sum_{s=0}^{t-1} e^{-s/\tilde{\gamma}_i} \frac{\tilde{\gamma}_i^s}{s!}\right)\right\}$$

and recalibrating the δ_t to obtain the estimates $\hat{d}_t$:

$$\hat{d}_t = \delta_t / \left(\frac{1}{T}\sum_{s=0}^{T} \delta_s\right).$$

The recalibration is required to satisfy the constraint over the day effects (their mean is equal to one; see model construction).

Second, the model with day effects is fitted to each of the 12 data sets (for cohorts per nuc: Control 1 and 2, *Nosema* 1 and 2; three nucs) by plugging-in the expression of $E\left[N(i,t)\right]$ the estimates $\hat{d}_t$ and following the procedure provided in McCullagh and Nelder (1989). This procedure yields the final estimates $\hat{\alpha}_i$, $\hat{\beta}_i$ and $\hat{\gamma}_i$ of α_i, β_i and γ_i as well as the estimates of the variance parameter σ^2 and the standard deviations of all the parameters.

EO levels

Differences between Control and *Nosema* cohorts were analyzed using nested t-test on log10 values to attain normal distribution. Differences between nucs were tested by ANOVA and Bonferroni post-hoc test. The correlation between EO titers and spore-loads was also calculated.

Spore-loads and mortality at the end of the experiment

To search for differences of spore-loads between nucs, cohorts and day of sampling of experimentally infected bees, we performed a factorial ANOVA with interactions. The dependence of the proportion of remaining bees at the end of the experiment to *N. ceranae* infection was calculated by *Chi-test* for each nuc.

3. Results and discussion

3.1. Nosema ceranae spore-loads

Experimentally-infected bees developed a mean of $8.49 \times 10^6 \pm 6.07 \times 10^6$ spores per bee, similar to parasitism levels in natural infected bees of same age (Smart and Sheppard, 2012). The spore-loads (Table 1) should have reached a plateau since it didn't show significant differences between 14 and 21-day-old bees ($p = 0.9367$; $N = 36$ and $N = 27$ respectively). We obtained a good repeatability on infection development between the 3 nucs ($p = 0.3205$; $N = 21$, $N = 19$, $N = 24$ for nucs A, B, C respectively) and between infected cohorts ($p = 0.5101$; $N = 24$, $N = 27$ for *Nosema* 1 and *Nosema* 2 respectively). All interactions were statistically similar as well ($p > 0.05$).

Two from 6 newborn bees, used to build the nucs (Control 1 and *Nosema* 1), were found to have very low quantity of spores per bee (20 000 - 40 000) probably acquired by chewing the wax capping, that can contain some spores, at emergence (Malone and Gatehouse, 1998). In newborn bees used for Control 2 and *Nosema* 2 cohorts, no spores were found. Later in the experiment

spores were observed in one-third of the samples of Control cohorts at low counts of 0.77 ± 2.13 million spores/bee. At the experimental dose of 100 000 spores of *N. ceranae* per bee, still no spores were detected in 3% of the sampled bees. Probably, in field conditions bees are able to go for cleansing flights reducing the number of new spores in the midgut.

Table 1. Spore-count in infected bees of 14 and 21-day-old. Mean ± SD expressed in millions of spores per bee.

Cohorts	14-day-old bees	21-day-old bees
Nosema 1	6.956 ± 4.272	8.708 ± 3.379
Nosema 2	10.007 ± 4.943	7.637 ± 2.360

3.2. Flight activity

The graphical test allowed comparing the number of exits per bee through time between Control and *Nosema* cohorts (Fig. 2 a and b). While the model of bee flight activity we built (Fig. 3) was used to estimate the number of exits/bee/day (*Tau*), the lifespan of a bee (α) and the age to exit "juvenile state" (ß) equivalent to the first exit from the hive (Tab 2).

In general, Control 1 showed a higher number of exits/bee/day than *Nosema* 1, by the first 5 days of the experiment (Fig. 2a). From day 5 to 15, the activity was similar between both cohorts. After that, *Nosema* 1 bees increasingly decreased their activity compared to Control 1. Differently, flight activity was similar between Control 2 with *Nosema* 2 at the beginning (Fig. 2b). Then, between days 15 – 20 bees from *Nosema* 2 were significantly more active than bees from Control 2. By the end of the experiment, *Nosema* 2 bees significantly decreased their activity compared to Control 2, similar to *Nosema* 1 bees that also decreased activity by the end.

Differences between cohorts 1 and 2 can be explained by the lack of organization of the nucs at the beginning of the experiment, along with an unbalanced number of bees per cohort. Thus, bees from Control 1, in a greater number (n = 4 000) than *Nosema* 1 (n = 300), may have had a strong response to the need of foragers as single cohorts do (Leoncini *et al*., 2004). Indeed, single cohort colonies are known to rapidly initiate foraging after having been established compared to double cohorts (Leoncini *et al*., 2004). Moreover, bees from large colonies start foraging earlier than bees from small ones (Rueppell *et al*., 2009). Instead, the second two cohorts, Control 2 and *Nosema* 2, were introduced at day 7 in an already organized nuc, and so differences in activity became evident once the infection was developed. A fully developed infection in the midgut epithelium is achieved within 8 to 12 days (Forsgren and Fries, 2010; Higes *et al*, 2007).

In general, the number of exits/bee/day estimated by our model was smaller for Control than *Nosema* cohorts (Table 2) which confirms that infected bees were more active than healthy bees.

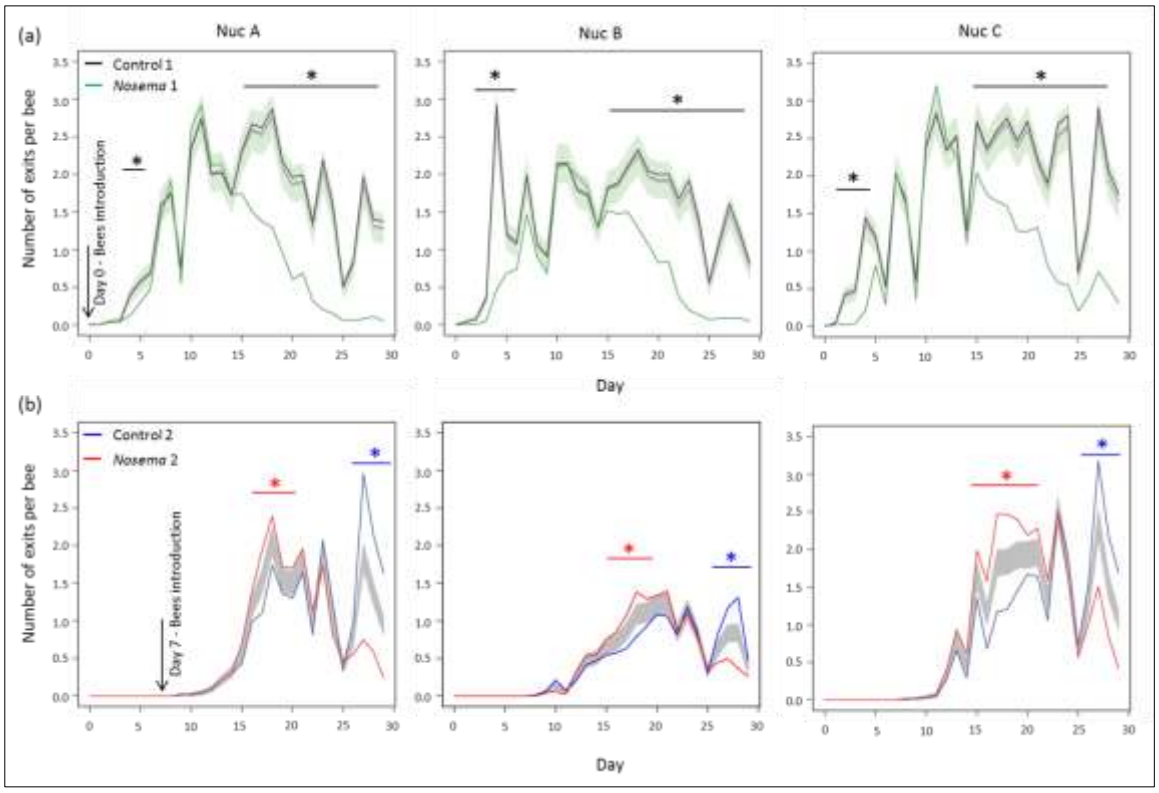

Figure 2. Graphical test for the daily number of exits per bee, applied to two time series of numbers of exits (Control and *Nosema* cohorts). 95% confidence envelopes were calculated using Bonferroni's correction (Miller, 1981). When the observed time series goes out from the 95% confidence envelope, differences between Control and *Nosema* cohorts based on the number of exits are expected. (a) First two cohorts introduced at day 0 in Nucs A, B and C, with 95% confidence envelops in grey for Control 1 (n = 4000) and green for *Nosema* 1 (N = 300), asterisk and line in black means significant higher activity for Control 1. (b) Second two cohorts introduced at day 7 in the same three Nucs, with one single grey envelop that corresponds to both Control 2 (N = 300) and *Nosema* 2 (n = 300), asterisk and bar in red means significant higher activity for *Nosema* 2, asterisk and line in blue means significant higher activity for Control 2.

Increase in flight activity can be interpreted as infected bees performing more but shorter trips to fulfill their own food requirements. Mayack and Naug (2010) estimated that a bee's flight capacity should decrease about one-third due to elevated energy demands because of infection. It is known that *Nosema* spp. develop in close association with the host-mitochondria to uptake host energy (Weidner *et al*., 1999), then to compensate energetic stress infected bees feed on more sucrose (Mayack and Naug, 2009). It seems that infected bees forage for their own extra needs, a behavior which might be modulated by the low carbohydrate levels independent of social cues such as colony demand for nectar (Mayack and Naug, 2010). This is supported by the fact that

infected colonies don't increase nectar storage (Botías *et al*., 2012). Increase in flight activity would be also the result of an inefficient foraging of infected bees because of the reduction of flight skills (Kralj and Fuchs, 2010). It could also be the consequence of increased cleansing flights that eliminate spores cumulated in the rectum, although cleansing flights seems not influence *N. ceranae* intensity in colonies (Williams *et al*., 2010).

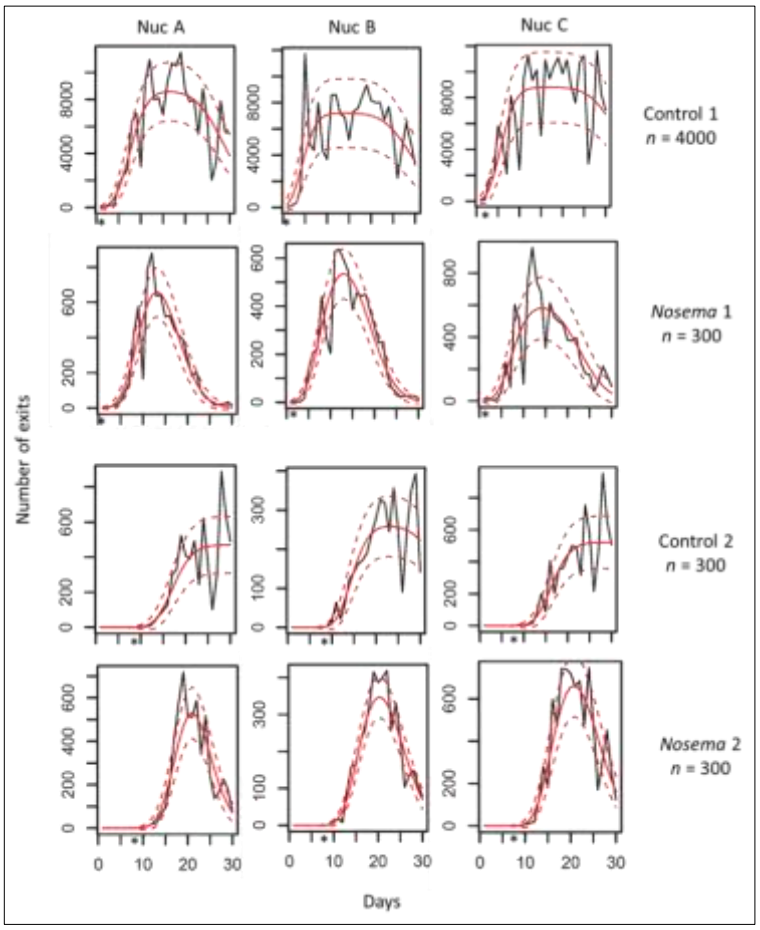

Figure 3. Model for the daily number of exits of honeybees from the hive following a quasi-likelihood approach (McCullagh and Nelder, 1989). Here is shown the fixed model to each treatment and nuc. Solid red lines represent the predicted number of exits per bee for each case; dashed red lines represent 95% confidence interval for predicted values; solid black lines are original data. * day of introduction of the each cohort in the nuc (day 0 of the experiment for Control 1 and *Nosema* 1 and day 7 for control 2 and *Nosema* 2 cohorts).

The increased flight activity may favor dissemination of spores between colonies by drifting (bees returning to other colony different from its colony of origin) especially if bees suffer of disorientation and flight impairment (Kralj and Fuchs, 2010). Trophallaxis (the transfer of food among members of a community through mouth to mouth feeding) is another way of *N.*

ceranae spore transmission (Smith, 2012). However, infected bees are more likely to beg for food because of hunger but the same hunger also makes them averse to share any food that they have obtained, thus decreasing the connectivity of the contact network within the colony (Naug and Gibbs, 2009).

Table 2. Predictions based on the model elaborated for the daily number of exits of bees from the hive following a quasi-likelihood approach (McCullagh and Nelder, 1989). Estimations of honeybee lifespan, age to exit "juvenile state" and the number of exits per day per bee were done for each nuc and treatment.

Nuc	Treatment	Lifespan in days (α)	Age to exit "juvenile state" * in days (ß)	N° exit per day per bee (*Tau*)	SD Lifespan in days (α)	SD Age to exit "juvenile state" in days (ß)	SD N° exit per day per bee (*Tau*)
A	Control 1	28.92	8.38	2.16	0.26	0.56	1.14
	Nosema 1	17.54	9.68	2.78	0.01	0.50	1.24
	Control 2	54.02	11.00	1.56	1391594.98**	0.90	1.28
	Nosema 2	16.72	11.75	2.86	0.05	0.62	1.48
B	Control 1	29.49	5.25	1.80	0.42	0.72	1.21
	Nosema 1	19.05	8.45	2.00	0.01	0.44	0.80
	Control 2	28.05	8.30	0.87	2.43	0.70	0.56
	Nosema 2	18.41	9.39	1.39	0.06	0.40	0.49
C	Control 1	33.75	6.50	2.20	1.16	0.63	1.31
	Nosema 1	22.66	8.21	2.02	0.07	0.74	1.39
	Control 2	40.82	10.21	1.74	669.96**	0.80	1.28
	Nosema 2	18.98	9.68	2.63	0.10	0.56	1.30

α, ß and *Tau*: represent de corresponding parameter in our model.

* A "juvenile" bee corresponds to an inhive bee not yet "retired"; a "retired" bee is a non-active bee because any reason.

** The standard deviations of the lifespans assessed in these cases are extremely large because the duration of the observation of the corresponding groups are too short to show a decrease in the bee activity (see Fig. 3, 3rd line, left and right panels that correspond to Control 2: Nuc A and C).

A higher flight activity can also be part of the strategy to reduce the rate of disease transmission within the colony. Bees simply can get lost into the field because of orientation impairment, where they would probably die, reducing the inoculum in the colony (Kralj and Fuchs, 2010). Moreover, altruistic-self-removal behavior would cause infected bees to abandon their social functions and remove themselves from the colony to prevent disease transmission (Rueppell *et al*., 2010).

Assuming that a honeybee can perform a limited number of flights per day (Winston, 1997), the increase in the number of exits/bee/day should also reflect an increase in the number of bees performing flights and vice versa. Then, the progressive augmentation in the number of exits/bee/day observed in *Nosema* 2 between days 15 – 20 (Fig. 2b), also represents a progressive and higher number of infected bees performing flights compared to healthy bees. A similar accelerated start of foraging was observed previously in *N. apis* infected bees (Wang and Möeller, 1970). However, the estimated age to exit "juvenile state" in our model was, in general, lower in bees from Control than bees from *Nosema* cohorts (Table 2). Probably, the age to exit "juvenile state", equivalent to the first exit from the hive, represents mainly orientation flights after which foraging started.

The age at first foraging seems to be a key determinant of worker longevity and colony survival. Recently, it was proposed that if the number of bees performing precocious foraging exceeds a critical threshold, a rapid population decline can be expected and colony failure is inevitable (Xu, 2012).

3.3. EO levels on honeybees

Comparison between the EO levels of the 3 nucs revealed a good repeatability of the experiment as no significant differences were found between Control cohorts (p = 0.6041 and 0.3738, for bees of 14 and 21-day-old respectively). While *Nosema* cohorts of the 3 nucs had almost similar EO levels, the exceptions were nucs A and B in bees of 14-day-old, but differences disappeared when bees reached 21-day-old (p = 0.0076 and 0.4541 for nuc A vs. nuc B in 14 and 21-day-old bees respectively).

In general, infected bees showed higher levels of EO than healthy bees (Fig. 4a, b). Increases in EO levels of infected bees could affect the colony social organization, as EO regulates behavioral maturation of nurses delaying onset of foraging (Leoncini *et al*., 2004). The EO levels in infected bees were close to natural titers in foragers (Castillo *et al*., 2012), while healthy bees had almost one-third less EO than infected bees. In a previous laboratory study (Dussaubat *et al*., 2010) an extremely high level of EO, along with higher spore-loads compared to this experiment, were found. The lower levels observed in this experiment could be the result of field conditions, since cleansing flights allow bees to eliminate spores outside the hive and reduce probability of re-infection (Fries, 1988).

When comparing EO titers between Control 1 and *Nosema* 1, we found that differences were not statistically significant on 14-day-old bees (Fig. 4a) which corresponds with no

differences in flight activity (Fig. 2a). However, in 21-day-old bees, Control 1 showed significant lower levels of EO than *Nosema* 1, despite Control 1 activity being higher than *Nosema* 1 which was clearly declining. The reduction in activity of *Nosema* 1 should be the result of a decreasing number of infected bees performing flights at the end of the experiment due to mortality (Table 3).

When comparing Control 2 and *Nosema* 2 (Fig. 4b), lower EO levels were observed in 14-day-old bees from Control 2 (day 21 of the experiment), which was consistent with a lower activity of this cohort (Fig. 2b). The EO levels of 21-day-old bees from Control 2 (day 28 of the experiment) were still lower than *Nosema* 2, but differences were not significant. This result also supports the idea that reduction in activity of infected bees by the end of the experiment is not related to EO levels but to mortality.

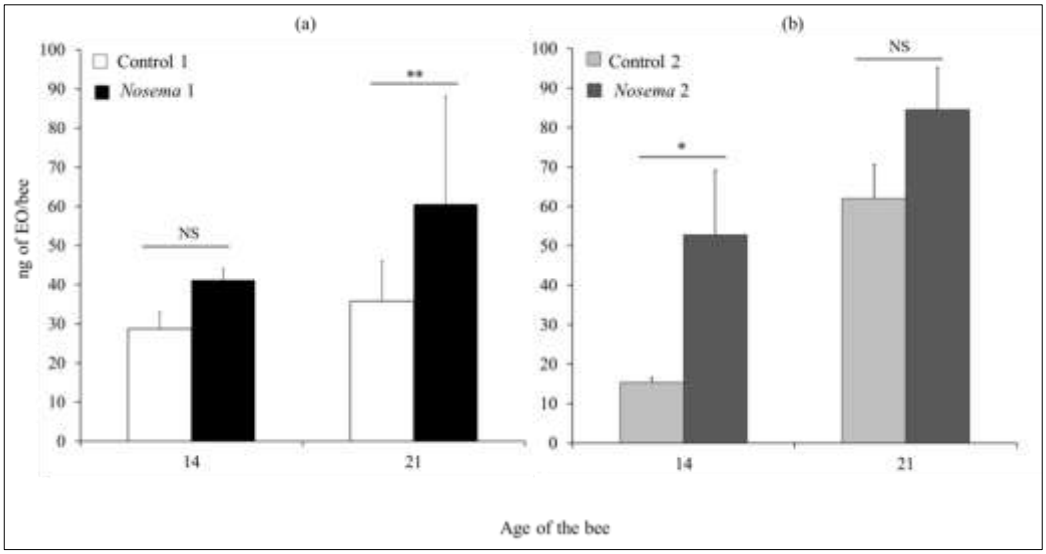

Figure 4. EO levels on honey bees. Infected bees showed higher mean levels of EO than non-infected bees, these differences were statistically significant in bees from (a) Control 1 and *Nosema* 1 of 21-day-old, and in bees from (b) Control 2 and *Nosema* 2 of 14-day-old. Control 1 and *Nosema* 1 were introduced at the beginning of the experiment and Control 2 and *Nosema* 2 were introduced in the same nuc 7 days later. Bars represent mean ± SD of 3 nucs, from each nuc 12 bees per cohort were GC/FID analyzed in 3 pools of 4 bees (n = 9). Differences were compared using t-test for nested values, *, ** denote significant differences at $p < 0.05$ and $p < 0.01$ respectively, and NS non-significant differences between treatments.

Based on our results, we hypothesized that elevated EO levels of infected bees might restrain flight activity of healthy bees. The benefits for the colony would be that a belated transition from nurse to forager could extend healthy bees lifespan (Rueppell el al., 2009). Infected bees would also serve as a spore reservoir since they are less willing to exchange food reducing spore transmission by trophallaxis (Naug and Gibbs, 2009). Finally, as infected bees die

outside the hive, the inoculum that they potentially can transmit inside the hive decreases. In this case infected-foragers might represent a "barrier" to pathogen transmission as, with these behaviors, they would be protecting their nestmates from disease, as described by Evans and Spivak (2010) and Naug and Camazine (2002).

A positive correlation between EO levels and spore-loads was found statistically significant but moderately (Fig. 5). Our results support the idea of a previous study (Dussaubat *et al*., 2010), that the increase on EO is not an all-or-nothing response but is linked to the level of *Nosema* infection. Honey bees biosynthesize EO in the esophagus upon ingestion of ethanol present in fermented nectar collected by foragers, what *N. ceranae* does to interfere with this mechanism merits further investigation. We speculate that since infected bees suffer nutritional and energetic stress (Aliferis *et al*., 2012) that stimulates nectar foraging (Mayack and Naug, 2010), the excess of ethanol from fermented nectar collected during foraging is available to be transformed into EO. This could explain the positive correlation between the number of *N. ceranae* spores and EO levels. Other chemical signals are indirectly modified by *N. apis and N. ceranae. N. apis* indirectly increases the level of juvenile hormone III which is also involved in the regulation of age-related tasks (Huang and Lin, 2004). *N. ceranae* also increases the levels of this hormone (Ares *et al*., 2012) and down-regulates the expression of the vitellogenin gene that participates in the transition nurse to forager as well (Antúnez *et al*., 2009).

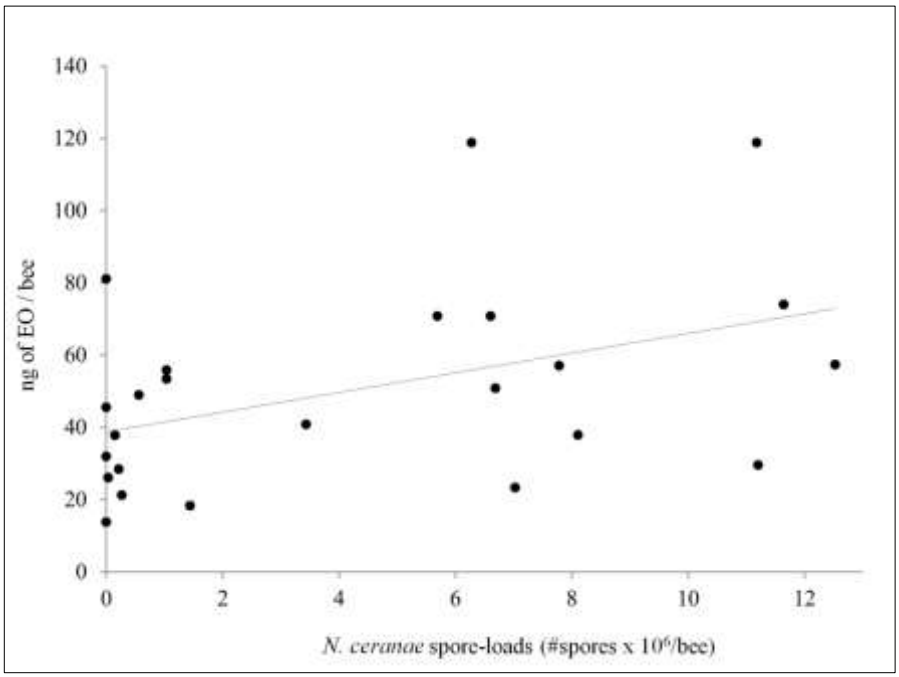

Figure 5. Correlation between EO production and the level of *N. ceranae* infection. There was a significant positive but moderated correlation between the quantity of EO produced and the number of *N. ceranae* spores infecting bees (r = 0.432, p = 0.0349, N = 24).

3.4. Bee mortality

As expected, by the end of the experiment, bee mortality (calculated from the number of remaining bees on the nuc) was dependent on *N. ceranae* infection (Table 3). This agrees with the estimation of bee lifespan based on the model that was greater for Control bees (28.05 - 54.02 days) than for *Nosema* bees (16.72 - 22.66 days) (Table 2). The effect of *N. ceranae*-induced mortality can clearly be observed on the activity of Control 1 compared to *Nosema* 1 bees, which dramatically decreased from day 15 of the experiment (Fig. 2a).

The nucs used in our experiment did not have brood, making evident the loss of infected foragers as there were no bees for replacement. In a colony if brood production and emerging rate are too low to support a sustained level of forager losses the colony will fail (Khoury *et al*., 2011). If survival of both brood and adults bees are compromised, then colonies will be particularly vulnerable to collapse. This could be the case of double parasitism by *V. destructor*, which affects both brood and forager survival, and *Nosema* infection (Khoury *et al*., 2011). In this context, queen performance seems to be extremely important and the reason why commercial beekeepers identify poor queens among leading causes of their losses (vanEngelsdorp *et al*., 2011).

Table 3. Percentage of remaining bees in the nuclei at the end of the experiment. A significant smaller proportion of infected bees compared to control bees was found in the nucs at the end of the experiment (*Chi-test* between cohorts Control and *Nosema* per nuc, $p < 0.0001$ for each analysis; Control 1: $n = 4000$; Control 2, *Nosema* 1 and *Nosema* 2: $n = 300$).

Nuc	% of remaining bees in the nuc			
	Control 1	***Nosema* 1**	**Control 2**	***Nosema* 2**
A	43	2	73	6
B	28	3	76	7
C	42	9	70	8

4. Conclusion

This study compares flight behavior associated to pheromone (EO) changes on bees challenged by the microsporidia *N. ceranae* in field conditions. The observed increment of flight activity in honey bees along with high levels of EO and mortality rate compared to non-infected bees, suggest that infected bees avoid pathogen transmission in the colony. However, colony

homeostasis becomes fragile as the chemical mechanisms that regulate behavioral maturation, especially the balance between nurses and foragers, are disturbed making colonies more susceptible to other environmental stressors. Our model allowed simplifying complex social interactions that characterized a colony making it possible to study specific factors involved in the response to a disease at colony level in field conditions. Nevertheless, we acknowledge that the response to infection might also be influenced by other interactions in hive and environmental factors as well. Finally, this study provides flight data recorded in real-time of a 5 000 honey bee population distributed in 4 cohorts, carried out simultaneously on 3 nucs, representing a powerful tool to study honey bee flight behavior.

Acknowlegments

We thank Mathieu Landrin and Louise Clochey for technical assistance during their professional training at INRA of Avignon Laboratory. We also thank Cynthia McDonnell for English revision and comments. This work was co-funded by a BEEDOC grant (FP7, RTD REG/E.4(2009)D/561221) and Human Frontiers Science Project grant (RGP0042/2007-C101). C. Dussaubat was supported by a CONICYT/French Embassy of Chile grant.

Synthèse

Ces expérimentations nous ont permis de tester notre première hypothèse spécifique sur le potentiel de *Nosema* spp. à altérer la production de la phéromone EO chez les ouvrières et en conséquence à perturber leur maturation comportementale. Nos résultats ont montré un effet très marqué sur la production d'EO chez les abeilles élevées au laboratoire (article n°1) et un effet plus subtil mais consistant, chez les abeilles élevées dans des conditions naturelles (article n°2). Les effets observés chez les abeilles infectées ont été : un butinage précoce, l'augmentation de la fréquence des vols et l'accroissement de la mortalité, alors que chez les abeilles saines, l'activité de vol a été moins élevée et la durée de vie allongée. Ces effets pourraient être liés à une demande énergétique plus élevée des abeilles infectées et à une stratégie visant la diminution de la transmission du parasitaire au sein de la colonie. Ainsi, la survie de la colonie dépendrait d'une part de sa capacité à maintenir la population d'abeilles malgré la perte constante de butineuses infectées et d'autre part de la richesse et disponibilité des ressources mellifères.

Dans le chapitre suivant nous allons incorporer à cette étude, des aspects fondamentaux visant à la compréhension des mécanismes infectieux de *N. ceranae* qui sont à la base des effets observés. D'une part, les changements transcriptomique et d'autre part les effets physiologiques, induits pas *N. ceranae* dans l'intestin de l'abeille.