

# THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ D'AVIGNON

Ecole doctorale Sciences et Agrosciences  
Discipline : Sciences Agronomiques

Présentée par

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Le 13 décembre 2012

## Effets de *Nosema ceranae* (Microsporidia) sur la santé de l'abeille domestique *Apis mellifera* L.

*Changements physiologiques et comportementaux*

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UR 406 Abeilles et  
Environnement



## **Résumé Effets de *Nosema ceranae* (Microsporidia) sur la santé de l'abeille domestique *Apis mellifera* L. Changements physiologiques et comportementaux**

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*Nosema ceranae* est un parasite émergent d'*Apis mellifera* décrit dans certaines régions comme la cause majeure de la mortalité des abeilles. Dans d'autres cas, il est soupçonné d'affaiblir les colonies par l'interaction avec d'autres facteurs de pression de l'environnement. Dans le cadre du phénomène global de la mortalité des abeilles, nous avons orienté nos recherches vers l'étude des effets *N. ceranae*, en faisant l'hypothèse que ce parasite est capable d'induire des changements comportementaux chez *A. mellifera* dus à des altérations physiologiques, ce qui pourrait éventuellement perturber l'organisation sociale et aboutir à la mort de la colonie. Etant donné cette hypothèse, trois domaines d'étude ont été inclus dans notre recherche, (i) les effets de *N. ceranae* sur l'organisation sociale de la colonie, (ii) les mécanismes moléculaires à la base des effets chez les abeilles parasitées, et (iii) les différences en virulence d'isolats de *N. ceranae* ce qui pourrait expliquer la variation des effets du parasite chez l'abeille. Nous avons obtenu trois résultats majeurs. D'abord, nous avons constaté des modifications dans la structure sociale des abeilles après l'infection. Ces changements sembleraient contribuer à la survie de la colonie constituant probablement un mécanisme d'immunité sociale. Ce mécanisme géré par un signal phéromonal, permettrait de diminuer la transmission du parasite au sein de la colonie et prolonger la survie des abeilles saines. Ensuite, nous avons mis en évidence des effets sur la physiologie de l'intestin de l'abeille qui pourraient causer sa mort : l'induction du stress oxydatif et l'inhibition du renouvellement cellulaire de l'épithélium. Finalement, nos résultats suggèrent que certaines caractéristiques de l'hôte et conditions environnementales augmenteraient la probabilité de *N. ceranae* d'induire la mort. En conclusion, *N. ceranae* a le potentiel de causer la mort des abeilles, cependant, la colonie pourrait contrer l'infection, par exemple, par de mécanismes d'immunité sociale. Or la réponse générale à l'infection dépendrait des caractéristiques de l'hôte en combinaison avec les conditions de l'environnement. Le phénomène d'effondrement de colonies à l'échelle mondiale a mis en évidence la fragilité du système colonie d'abeilles – environnement. L'étude de chaque facteur participant au système, entre autres, parasites, pesticides, changements dans l'environnement, pratiques apicoles, est essentielle pour une meilleure compréhension de toutes les interactions qui maintiennent l'équilibre écologique des colonies.

**Mots clés :** *Nosema ceranae*, parasite, microsporidie, *Apis mellifera*, abeille, comportement, phéromone, oléate d'éthyle, pathologie, physiologie, transcriptome, stress oxydatif, virulence.

## **Abstract *Nosema ceranae* (Microsporidia) effects on honey bee (*Apis mellifera* L.) health Physiological and behavioral changes**

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*Nosema ceranae* is an emergent parasite of the honey bee *Apis mellifera*. In some regions it has been found to be the main reason for bee mortality, while in others it is suspected of weakening honey bee colonies by interacting with other environmental stressors. In the context of worldwide colony losses, we focus our research on the study of *N. ceranae*, with the hypothesis that this parasite is able to induce behavioral changes in bees through physiological modifications, which could alter social organization and cause colony death. Given this hypothesis, the program of study falls into three areas; (i) *N. ceranae* effects on colony social organization, (ii) molecular mechanisms of *N. ceranae* infection underlying observed effects, and (iii) differences in virulence of *N. ceranae* strains which could explain the diversity of parasite effects. We obtained three main results. First, we observed modifications in honey bee social structure after infection. This mechanism under pheromone control, would reduce parasite transmission within the colony and increase the lifespan of healthy bees. These changes may contribute to colony survival as part of a mechanism of social immunity. Second, we found two mechanisms whereby the pathogen affects the physiology of bee midgut epithelium that could lead to host mortality: oxidative stress and the inhibition of cellular renewal. Finally, our results suggest that certain host and environmental conditions increase the probability that *N. ceranae* will cause bee mortality. In conclusion, *N. ceranae* has the potential to cause bee death, however at colony level bees might counteract infection through, for example, social immunity mechanisms; although, overall honey bee response to infection would depend on characteristics of the host in combination with environmental conditions. Worldwide colony losses phenomenon have highlighted the fragility of the "honey bee colony – environment" system. The study of each factor involve in this system, including parasites, pesticides, environmental changes and beekeeping practices, is essential to better understand all of the interactions that maintain the ecological balance of honey bee colonies.

**Key words :** *Nosema ceranae*, parasite, microsporidia, *Apis mellifera*, honey bee, behavior, pheromone, ethyl oleate, pathology, physiology, transcriptom, oxydatif stress, virulence.

## **Resumen Efectos de *Nosema ceranae* (Microsporidia) sobre la salud de la abeja doméstica *Apis mellifera* L. Cambios fisiológicos y comportamentales**

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*Nosema ceranae* es un parásito emergente de *Apis mellifera*. En algunas regiones ha sido señalado como la causa principal de la mortalidad de abejas, mientras que en otras, su presencia debilitaría las colonias a través de la interacción con otros factores de presión del medioambiente. En el marco de la pérdida de colonias a nivel mundial, enfocamos nuestra investigación al estudio de los efectos de *N. ceranae* basándonos en la hipótesis que dicho parásito es capaz de inducir cambios en el comportamiento de las abejas a través de modificaciones fisiológicas, dichos cambios podrían alterar la organización social y causar la muerte de la colonia. Dada esta hipótesis, el programa de estudio comprendió tres áreas, (i) efectos de *N. ceranae* en la organización social de la colonia, (ii) mecanismos moleculares que originan los efectos observados en las abejas infectadas, y (iii) diferencias en la virulencia de cepas de *N. ceranae* que podrían explicar la diversidad de efectos del parásito. Distinguimos tres resultados principales. Primero, la modificación de la estructura social de las abejas debido a la infección. Dichos cambios podrían contribuir a la sobrevivencia de la colonia como parte de un mecanismo de inmunidad social. Este mecanismo, bajo control feromonal, permitiría reducir la transmisión del parásito dentro de la colonia y aumentar la duración de la vida de las abejas sanas. Segundo, la puesta en evidencia de dos mecanismos patológicos que afectan la fisiología del epitelio intestinal y que podrían causar la muerte de la abeja: estrés oxidativo y la inhibición de la renovación celular. Tercero, nuestros resultados sugieren que ciertas características del hospedero y condiciones del medio ambiente aumentarían la probabilidad de que *N. ceranae* cause la muerte. En conclusión, *N. ceranae* tiene el potencial de causar la muerte de las abejas, sin embargo, a nivel de colonia estas podrían contrarrestar la infección, por ejemplo, por medio de mecanismos de inmunidad social, no obstante, la respuesta general a la infección dependería de las características del hospedero en combinación con las condiciones del medio ambiente. El fenómeno mundial de pérdida de colonias ha sacado a la luz la fragilidad del sistema “colonia – medio ambiente”. El estudio de cada uno de los factores que forman parte de este sistema, tales como: parásitos, pesticidas, cambios ambientales y prácticas apícolas, es esencial para la comprensión de todas las interacciones que mantienen el equilibrio ecológico de las colonias de abejas.

**Palabras clave:** *Nosema ceranae*, parásito, microsporidio, *Apis mellifera*, abeja, comportamiento, feromona, oleato de etilo, patología, fisiología, transcriptom, estrés oxidativo, virulencia.

## Publications

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## **Bourse d'étude**

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2008/2012 – Bourse Master 2 et Doctorat de la Commission Nationale de la Recherche Scientifique et Technologique (CONICYT) du Chili et du Ministère des Affaires Etrangères de France à travers l'Ambassade de France au Chili.

October 2010 - Short Term Scientific Mission (STSM): “Standardizing laboratory procedures for collaborative research on *Nosema* spp. and pesticide interactions on honey bees”. COST Action FA0803 - Prevention of honeybee Colony LOSSes (COLOSS). Host: Dr. Mariano Higes, Research Director from “Centro Apícola Regional”, Laboratory of Bee Pathology, Marchamalo-Guadalajara, Spain.

## Remerciements

---

Je souhaite remercier toutes les personnes avec lesquelles j'ai pu partager d'inoubliables moments pendant les années de ma thèse...

Je suis particulièrement heureuse d'avoir eu comme directeur de thèses Yves Le Conte. Yves, je vous remercie de m'avoir ouvert les portes de votre équipe scientifique et de m'avoir accompagnée pendant ces années vers la découverte de ce qu'est la recherche en apiculture. Merci d'avoir partagé avec moi votre passion pour les abeilles, vos connaissances, vos idées et surtout votre esprit critique essentiel pour progresser dans la recherche. Je vais garder précieusement votre exemple de sérieux au travail mais aussi votre exemple de bonne humeur. De temps en temps vous veniez me demander si « la vie était belle » pour moi, je vous dis encore que oui et que vous avez contribué largement à ça.

Je tenais à remercier les membres du jury qui m'ont fait l'honneur de lire et de commenter cette thèse : M. Frédéric Delbac, M. Peter Neumann, M. Mariano Higes et M. Mohamerd El Maâtaoui.

Je tiens à remercier les équipes du laboratoire biologie et protection de l'abeille et du laboratoire de toxicologie environnementale. Reprendre le travail de laboratoire et de terrain quelques années après avoir fini la fac, ce n'est pas évident, et j'ai pu le faire grâce à votre aide, patience et précieuses connaissances.

Je remercie Cédric Alaux, pour m'avoir incluse dans son travail, pour avoir été toujours bien disposé à corriger mes manuscrits et pour son sympathie. Egalement merci à Cynthia McDonnell pour les corrections d'anglais si nécessaires et les suggestions sur les articles et les abstracts, et aussi pour les bons moments partagés au bureau.

Je remercie très profondément à Didier Crauser, pour m'avoir appris un peu de la meilleure apiculture pour la science, pour ses conseils sur les manips et son aide très précieuse. Merci à Dominique Beslay pour toute son aide au labo spécialement lors des analyses GC et pour son agréable compagnie. Merci à Jean Marc Becard qui a toujours réussi à faire marcher la petite machine informatique sans laquelle nous ne pouvons pas vivre aujourd'hui.

Un grand merci également à Alban Maisonnasse pour son énergie et bon humeur, pour avoir toujours trouvé le temps de me donner un coup de main, pour m'avoir introduite dans sa sphère phéromone et pour son investissement pour démarrer la manip des reines, pour ses leçons de français-apicole pour débutants sur le terrain et pour avoir partagé son apiculture d'excellence avec moi.

Je remercie également Luc Belzunces et Jean-Luc Brunet pour m'avoir initiée au domaine de la toxicologie et des enzymes, pour leur collaboration indispensable et leur sympathie. Merci également à Marc Bonnet pour son aide très efficace avec les analyses des activités enzymatiques et à Jacques Senechal pour s'être impliqué gentiment dans notre manip de nucléis. Grand merci à Sylvie Tchamitchian pour avoir aimé comme moi les manips que l'on a faites et avoir surveillé tous les petits détails, merci pour son implication et engagement.

Je tiens à remercier à tous les étudiants qui se sont investis avec leurs esprits dans les expériences de labo et de terrain. Merci beaucoup pour les bons moments passés ensemble, pour les discussions très enrichissantes et pour m'avoir fait connaître un petit morceau de vos régions d'origine. Aurelie Baldy, Stan Charbert d'Hiery, Anaïs Appelgren, Mathieu Lindrin, Louise Clochey et Ilyas Boudia. Merci également à d'autres étudiants et CDD qui lors de leurs stages ont créé une ambiance conviviale, dynamique et

délicieusement gourmande : Horiya, Morgane, Jean-François, Emma, Fanny, Claire, Martin, Vincent, Athena, Ghania, et tous les autres personnes que le labo m'a permis de rencontrer. Pour l'ambiance conviviale, la solidarité quand il y avait besoin d'aide pour les manips, et pour m'avoir fait découvrir à travers eux un peu de la France, l'Italie, la Côte d'Ivoire et les Etats Unis, je tiens à remercier les thésards, Guillaume Kairo, Garance Di Pasquale, Fanny Mondet, Teresa Renzi, Koffi Ahadji-Dabla et Kirsten Traynor. Je remercie particulièrement Faten Ben Abdelkader, pour son aide et son amitié et Aklesso Kadala pour sa compagnie très agréable pendant le temps que l'on partageait le bureau.

Merci également à Mercedes Charreton pour sa convivialité et sympathie jour à jour. Un grand merci à Marianne Cousin pour son aide pendant les différentes manips, merci pour avoir partagé avec moi sa passion première pour les fils et les mailles et surtout merci pour son soutien précieux.

Je tiens à remercier également les autres membres de l'unité pour les moments agréables passé de temps en temps à la cafétéria, Claude Collet, Jean Aptel, Laurent Guilbaud, Marie-Josée Buffière, Nicolas Morison, Guy Rodet, Oriane Rollin, Bernard Vaissière et Mikael Henry. Merci également aux gens de l'UMT pour leur sympathie, Julien Vallon, Fabrice Allier et Axel Décourtye. Je remercie Colette Pélissier notre documentaliste et aussi à Corinne Chêne notre secrétaire, pour leur aide avec tout ce qui semble simple mais qui est compliqué.

Merci beaucoup également aux collaborateurs extérieurs à notre unité, Guy Costagliola pour la GC/MS, André Kretzschmar et Samuel Soubeyrand pour s'être impliqués avec passion dans les stats au service de la compréhension des phénomènes qui gèrent la vie des abeilles.

Je tiens à remercier très spécialement l'équipe de recherche espagnole du Centro Apícola Regional Marchamalo, qui m'a accueillie chaleureusement plusieurs fois et avec lesquels c'était toujours un plaisir de collaborer, particulièrement Mariano Higes, Raquel Martí n-Hernández, Cristina Botí as, Almudena Cepero, Soledad Sagastume et Carmen Abascal. Merci également à Teresa Corrales, Mari Carmen Rogerio, Virginia Albendea, Javier Almagro, Germán Sánchez et Tamara Gómez-Moracho. Je remercie également Pilar García Palencia et son équipe de l'Université Complutense à Madrid.

Merci à mon équipe du Volleyball INRA-ADAS d'Avignon pour les super bons moments vécu ensemble. Isabelle, Fred, Yannick, Sarah, Claude, Julien, Naïma, Eric, Caroline, Servane, Savelina, Nico, Norbert, Stéphane, David, Gêrôme, Gisèle et tous les autres.

Merci à mes amis de route Lucia Andreini, Sivasathivel Kandasamy, Tonya Lander, Elmer Ccopa, Alfredo Alessandrini, Hadrien Lalagüe, Sophie Derveau et Hanna Ben Tamarzizt, je garderai dans mon cœur toutes les petites et grandes aventures vécues avec vous!

Je n'aurai pas été ici sans l'encouragement de mon ancienne équipe de travail à l'Université Australe du Chili, merci du fond de mon cœur à Don Miguel, Sra. Nimia, Glorita, Patty, Carito, Alejandro, Patty H., Sra. Irene, Caro, Luis, Germán, vous êtes toujours dans mes pensées malgré le temps et la distance.

Merci également à Daniela, Ximena, María Luisa, Andrea, Patty R. et Marcelo pour votre amitié, vous êtes les meilleurs.

Merci à ma famille, merci à Eric.



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## Abréviations

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### Phéromones :

BEP :	phéromone du couvain	/	brood ester pheromone
EO :	oléate d'éthyle	/	ethyl oleate
(E)- $\beta$ -ocimène :	phéromone du couvain jeune	/	young brood pheromone
QMP :	pheromone mandibulaire de la reine	/	queen mandibular pheromone

### Hormones :

JH :	hormone juvénile III	/	juvenile hormone III
Vg :	vitéllogénine	/	vitellogenin

### Enzymes :

AP :	phosphatase alcaline	/	alkaline phosphatase
GP:	glutathion peroxydase	/	glutathione peroxidase
Gtpx2:	glutathion peroxydase type 2	/	glutathione peroxidase like 2
GR :	glutathion réductase	/	glutathione reductase
GsT :	glutathion-S-transférase	/	glutathione-S-transferase
SOD :	superoxyde dismutase	/	superoxide dismutase

### Autres:

AMP :	protéines antimicrobiennes	/	antimicrobial peptides
CCD :	syndrome d'effondrement des colonies	/	colony collapse disorder
DWV :	virus des ailes déformées	/	deformed wing virus
GB:	GenBank		
GO:	Gene Ontology Term		
IAPV :	virus israélien de la paralysie aiguë	/	Israeli acute paralysis virus of bees
ROS:	espèces réactives de l'oxygène	/	reactive oxygen species



## Présentation générale

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Actuellement l'apiculture et les systèmes agricoles et écologiques qui dépendent de l'action des pollinisateurs, vivent un moment critique vis-à-vis de la diminution à grande échelle des populations d'abeille, ce qui a fait l'objet de nombreuses publications scientifiques durant ces dernières années. Les causes de ce phénomène sont encore mal connues et plusieurs hypothèses sont en train d'être testées. La mortalité des abeilles serait due, dans certains cas, à l'action d'un seul facteur biotique (e.g. pathogène) ou abiotique (e.g. pesticide). Dans d'autres cas l'interaction de plusieurs de ces facteurs présents dans l'environnement en combinaison avec des pratiques apicoles intensives pourrait entraîner la perte des colonies.

L'étude de l'épidémiologie des agents infectieux chez l'abeille européenne (*Apis mellifera*) présente plusieurs défis dus aux caractéristiques sociales et sauvages de cet insecte. Le comportement d'essaimage confère à l'abeille sa caractéristique sauvage, à travers laquelle de nouvelles colonies se déplacent librement pour s'établir ailleurs. Ce comportement, associé à la dérive des abeilles entre colonies et la grande densité de population, sont des facteurs qui favorisent la transmission des pathogènes. De plus, la transhumance et les échanges commerciaux ont donné une dimension globale et ont accéléré la dynamique de dissémination d'agents infectieux. Auparavant, les principaux agents étaient restreints à certaines régions et sous espèces, à présent, ils ont une distribution mondiale. C'est le cas du parasite *Nosema ceranae* (Microsporidia) dont l'hôte original est l'abeille asiatique *Apis cerana*, et qui a été récemment détecté chez *A. mellifera* dans le reste du monde.

Ce parasite émergent de l'abeille européenne, a été décrit dans certaines régions comme la cause majeure de la mortalité des abeilles. Dans d'autres cas, sans être la cause directe de ces mortalités, il est soupçonné d'interagir avec d'autres facteurs pour finalement entraîner l'effondrement des colonies. Dans le cadre des efforts internationaux pour comprendre le phénomène global de la mortalité des abeilles, nous avons orienté nos recherches vers l'étude du parasite *N. ceranae* et son implication dans les pertes des colonies.

Dans la première partie de ce document, nos travaux de recherche sont introduits par une synthèse bibliographique qui permet (i) de placer cette étude dans le contexte global de la mortalité des abeilles, (ii) de décrire les principales connaissances scientifiques sur de *N. ceranae*, et (iii) de présenter certains aspects d'organisation d'une colonie d'abeilles en relation au développement de

l'infection, à partir desquelles est née notre hypothèse générale de travail et les objectifs fixés au cours de cette thèse.

Ensuite, trois chapitres basés sur 4 articles en anglais dont 3 publiés, et un soumis, développent progressivement nos objectifs. Deux publications complémentaires réalisées en co-auteur sont également citées dans le texte et inclus dans les annexes.

Pour finir, la discussion générale donne une vision intégrative et synthétique de l'ensemble des résultats et leur contribution à répondre à notre hypothèse de recherche. Suite à nos travaux, des recherches complémentaires sont en cours, développant ainsi nos perspectives incluses également dans le dernier chapitre.



## **Chapitre 1**

### **Introduction générale**

---

#### **I) La perte de colonies d'abeilles (*Apis mellifera*) à grande échelle**

L'augmentation de la pression environnementale et anthropique à laquelle sont exposées les populations d'abeilles domestiques (*Apis mellifera*) et sauvages est soupçonnée d'être à la base de leur déclin à l'échelle mondiale (Beismeyer *et al.*, 2006 ; Gixti *et al.*, 2009 ; National Research Council, 2007 ; Oldroyd, 2007 ; Paxton, 2010 ; Ratnieks et Carreck, 2010 ; Stokstad, 2007 ; vanEngelsdorp *et al.*, 2009 ; Whitehorn *et al.*, 2012). Des recensements récents ont estimé les pertes d'abeilles domestiques à environ 30 % aux Etats Unis et entre 1,8 à 53 % en Europe (Neumann et Carreck, 2010), alors que le niveau acceptable de mortalité aux Etats Unis est estimé à 15 % (vanEngelsdorp *et al.*, 2012).

Cependant, la perte importante d'abeilles domestiques n'est pas un phénomène récent comme des données historiques du début du XX<sup>ème</sup> siècle le montrent (Neumann et Carreck, 2010). Le développement de l'apiculture au niveau mondial associé à leur rôle clé dans l'agriculture par la pollinisation, ont fait de l'abeille un animal d'importance stratégique et donc d'intérêt public. En effet, 35 % de la production globale des aliments provient de cultures qui ont besoin de pollinisation entomophile et 70 % des 124 cultures majeures utilisées directement dans l'alimentation humaine au monde, dépendent de la pollinisation (Klein *et al.*, 2007). En Europe la production de 84 % des espèces cultivées dépend aussi de la pollinisation des abeilles (Williams, 1994). En 2005, l'activité des abeilles domestiques représentait 9,5 % de la valeur de la production agricole mondiale destinée à la nourriture humaine, ce qui était estimé à 153 milliards d'euros (Gallai *et al.*, 2009).

#### **Mono et multiples causes**

Pendant la première décennie des années 2000, le déclin des abeilles était déjà évident dans quelques régions du monde (Klein *et al.*, 2007). Plusieurs causes étaient soupçonnées d'être à la base du déclin comme la propagation mondiale (i) de l'acarien ectoparasite *Varroa destructor* débutée il y a environ 40 ans (Anderson et Trueman, 2000), (ii) de l'acarien des trachées, *Acarapis woodi* (Downey et Winston, 2001 ; Chen *et al.*, 2004), (iii) du coléoptère *Aethina tumida* (Evans *et al.*, 2003) et (iv)

celle de la microsporidie *N. ceranae* (Higes *et al.*, 2006). L'utilisation abusive de pesticides et d'herbicides était également suspectée d'influencer la diminution des abeilles (James et Xu, 2012). D'autres facteurs ont été considérés comme contributeurs au déclin des abeilles comme le changement climatique (Le Conte et Navajas, 2008) et les altérations du paysage. Ce dernier peut avoir comme conséquences la fragmentation des écosystèmes, la réduction de la biodiversité et la diminution de la quantité et qualité des ressources mellifères, entre autres (Lautenbach *et al.*, 2012 ; Vandame et Palacio, 2010). On peut aussi mentionner des causes éloignées du système écologique des abeilles comme la baisse des prix des produits et services apicoles (Klein *et al.*, 2007).

En 2009 vanEngelsdorp *et al.* ont défini les caractéristiques du syndrome d'effondrement des colonies (CCD, colony collapse disorder) aux USA comme : (i) l'absence d'abeilles mortes dans la ruche et dans le rucher, (ii) la diminution rapide de la population d'abeilles adultes qui laisse le couvain sans soin, (iii) l'absence de signes de pillage, (iv) le fait qu'au moment du collapse les niveaux de populations de *V. destructor* ou *Nosema* ne sont pas suffisants pour provoquer un impact économique ou le déclin de la population. Il a été proposé que le CCD possède un caractère contagieux ou bien qu'il soit le résultat de l'exposition à un facteur de risque commun. Dans ce scénario les pathogènes sont suspectés d'avoir un rôle indirect en interagissant avec d'autres facteurs. Les pathogènes seraient à la base des symptômes du CCD lorsque le syndrome est installé dans les colonies déjà affaiblies par d'autres facteurs ou par la combinaison de plusieurs stress qui réduisent la capacité des abeilles à lutter contre l'infection.

D'autres études ont montré l'implication d'un pathogène dans des cas de colonies ayant succombé au CCD, comme le virus israélien de la paralysie aiguë (IAPV, Israeli acute paralysis virus of bees) (Cox-Foster *et al.*, 2007) et *N. ceranae* (Borneck *et al.*, 2010 ; Higes *et al.*, 2008a). Mais ces parasites peuvent être également présents de façon asymptomatique dans les colonies (Blanchard *et al.*, 2008 ; Fernández *et al.*, 2012 ; Garrido-Bailón *et al.*, 2010 ; Gisder *et al.*, 2010 ; Reynaldi *et al.*, 2011). D'une façon similaire, les pesticides peuvent provoquer directement la mortalité des abeilles (Henry *et al.*, 2012), ou malgré leur présence dans l'environnement ne pas jouer un rôle prépondérant dans les mortalités d'abeilles (Creswell *et al.*, 2011).

Le rôle des interactions multiples est devenu une des hypothèses majeures pour expliquer la perte de colonies. Les différents facteurs de risque peuvent avoir des effets additifs ou synergiques, augmentant ainsi les effets individuels de chaque facteur (Le Conte *et al.*, 2012). Parmi les interactions pathogène - pathogène il a été mis en évidence l'effet conjoint de *V. destructor* et du virus des ailes déformées (DWV, deformed wing virus) (Dainat *et al.*, 2012a) et encore de *N. ceranae* et d'un

Iridovirus (Bromenshenk *et al.*, 2010). Des interactions pesticide - pathogène ont été également décrites entre *N. ceranae* et des pesticides neocotinoïdes (Alaux *et al.*, 2010a ; Aufauvre *et al.*, 2012 ; Pettis *et al.*, 2011 ; Vidau *et al.*, 2011). Des effets de l'interaction entre la nutrition (pollen) et *N. ceranae* ont été aussi montrées sur la survie des abeilles (Porrini *et al.*, 2011).

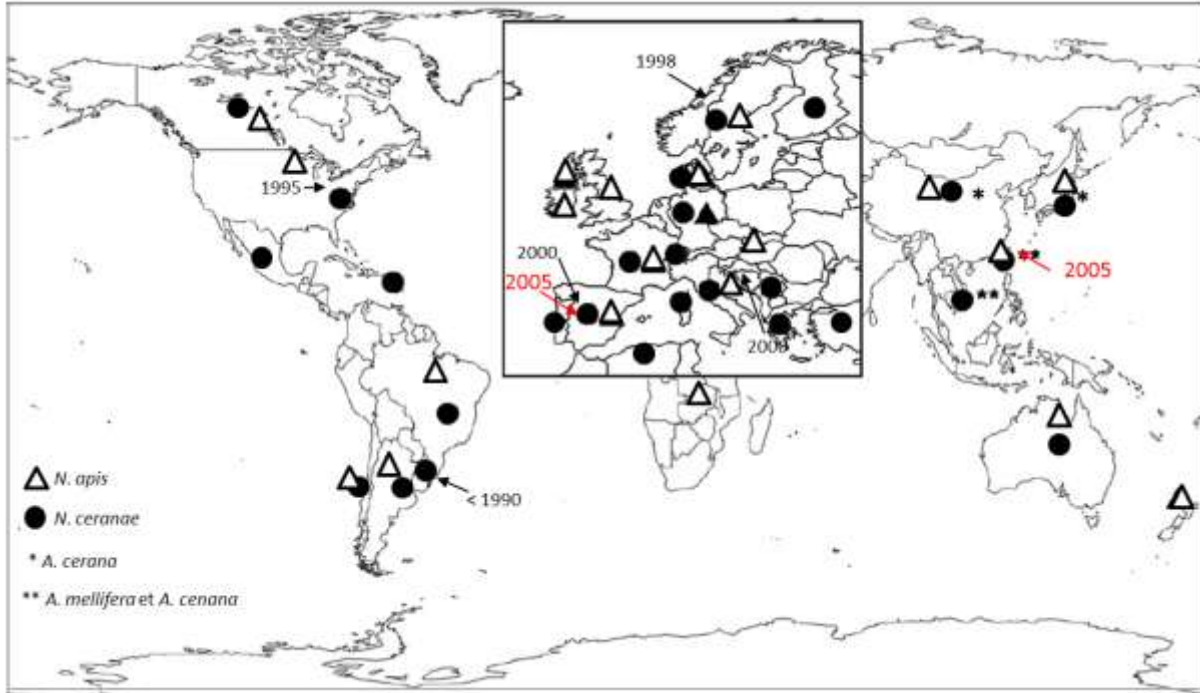
## II) Le rôle de *Nosema ceranae* dans les pertes de colonies

Dans le scénario si complexe des pertes de colonies d'abeilles, la compréhension des mécanismes d'interaction de plusieurs facteurs, pathogènes – pesticides – environnement, ne peut pas être atteinte sans d'abord connaître les mécanismes d'action des facteurs individuels. C'est pourquoi nous avons voulu approfondir et nous concentrer sur l'étude de l'interaction hôte - parasite : *A. mellifera* et *N. ceranae*. A présent ce parasite émergent est largement distribué dans le monde. Il exprime des niveaux de pathogénie différents qui vont d'une présence asymptomatique à la mort de la colonie.

### Distribution géographique

En 1996, une nouvelle espèce de *Nosema* a été découverte chez l'abeille asiatique *Apis cerana* (Fries *et al.*, 1996). La présence de *N. ceranae* chez *A. mellifera* était inconnue jusqu'en 2005, année au cours de laquelle elle a été isolée chez les abeilles européennes (Higes *et al.*, 2006 ; Huang *et al.*, 2007). Cependant, le transfert de *N. ceranae* d'*A. cerana* vers *A. mellifera* remonte à plus de 15 ans avant la première détection chez *A. mellifera*, comme le démontre l'analyse d'échantillons des années 1990 en Uruguay et 1995 aux Etats-Unis (Chen *et al.*, 2008 ; Invernizzi *et al.* 2009). Actuellement *N. ceranae* présente une distribution géographique mondiale (Fig. 1). La présence d'échantillons positifs à *N. ceranae* dans des régions aussi éloignées que l'Uruguay et la Finlande suggère un transfert par différentes voies probablement suivant les échanges commerciaux.

Dans de nombreuses régions *N. ceranae* semble avoir remplacé ou être en train de remplacer *N. apis*, une autre espèce de *Nosema*. Cela soutient l'idée de l'origine récente de *N. ceranae* chez *A. mellifera* (Carreck 2012). Or, il y a des régions, comme en Allemagne, où les deux espèces coexistent sans la prédominance de l'une sur l'autre (Gisder *et al.*, 2010).



**Figure 1.** Distribution géographique de *N. ceranae* et *N. apis*. Les flèches rouges indiquent l'année et l'endroit de la première détection de *N. ceranae* chez *A. mellifera*; les flèches noires indiquent l'année et l'endroit des détections datées avant la première détection de *N. ceranae*. D'autres pays non indiqués sur la carte où *N. ceranae* est présent sont : Israël, Indonésie, Vietnam, les Iles Salomon (adapté de : Botías *et al.*, 2012a ; Chen *et al.*, 2008, 2009 ; Giersch *et al* 2009 ; Invernizzi *et al.*, 2009 ; Klee *et al.*, 2007 ; Martínez *et al.*, 2012 ; Paxton *et al.*, 2007 ; Soroker *et al.*, 2011 ; Williams *et al.*, 2008).

### Classement taxonomique

*Nosema ceranae* appartient à la classe des Microsporidia (phylum Microspora) qui est un groupe très diversifié de parasites obligatoires intracellulaires eucaryotes. Environ 1 200 espèces ont été décrites dans 160 genres, ce qui certainement représente une petite fraction de la diversité réelle (Wittner and Weiss, 1999). Presque toutes les microsporidies sont parasites des animaux, une partie notamment sont responsables de maladies humaines associées à la suppression de l'immunité. Chez l'homme ces pathogènes émergents opportunistes (tel que *Encephalitozoon cuniculi*) sont devenus un problème de santé publique lors de la pandémie de l'AIDS (Acquired Immunodeficiency Syndrome : Syndrome de l'immunodéficience acquise) (Texier *et al.*, 2010). Egalement, ces pathogènes infectent des nombreuses espèces d'importance commerciale comme l'abeille, le ver à soie (*Nosema bombycis*), le saumon (*Loma salmonae*), et des mammifères domestiques (Keeling, 2009 ; Williams, 2009).

L'histoire taxonomique des microsporidies semble avoir atteint une stabilité avec l'arrivée de la biologie moléculaire. Lors de leur découverte en 1857, les microsporidies étaient considérées comme des champignons (schizomycète) puis ils ont été reclassés deux fois (en 1882 Sporozoa et en

1983 Archezoa) avant que la phylogénie moléculaire démontre leur origine fongique en 1996 (Keeling, 2009). Le séquençage de leur génome et la découverte de la présence de « mitosomes » (une forme simplifiée de mitochondrie) ont soutenu leur classement dans le règne Mycota, ce qui continue à être vérifié par de nouvelles recherches moléculaires (Capella-Gutiérrez *et al.*, 2012).

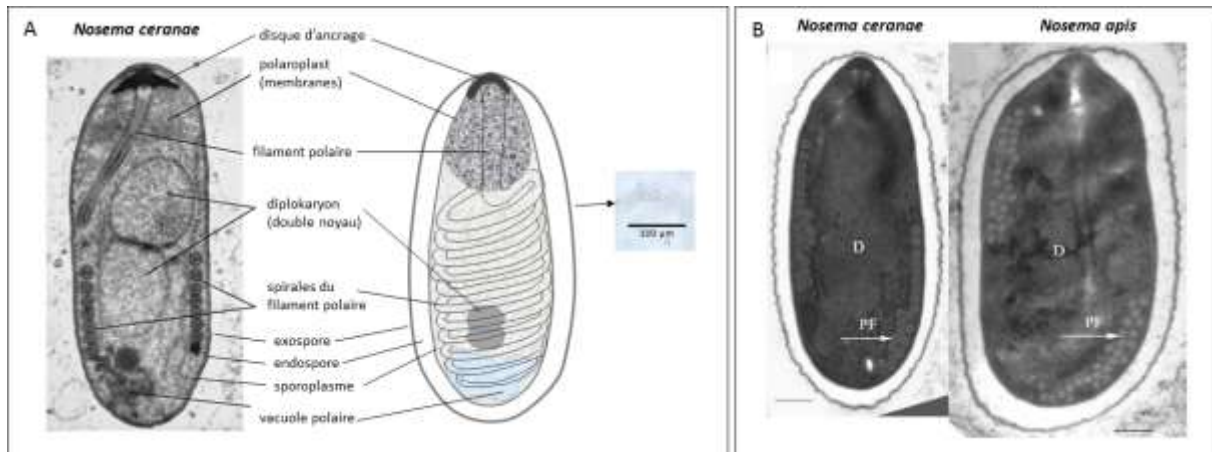
L'absence de mitochondrie a été à la base de l'hypothèse d'une origine primitive (Archezoa) des microporidia, aujourd'hui nous savons que l'absence de cette organite fait partie d'une réduction adaptative au niveau moléculaire, biochimique et cellulaire de ce groupe de parasites (Burri *et al.*, 2006). La présence du mitosome sans la capacité de production d'ATP via la phosphorylation oxydative, rend les microsporidies fortement dépendant es de leurs hôtes pour l'obtention d'énergie, cela correspond à un des effets les plus connus de *N. ceranae* chez l'abeille (Aliferis *et al.*, 2012).

*Nosema apis* fut isolé à partir d'*A. mellifera* en 1909 (Zander) mais le diagnostic moléculaire a été mis au point récemment (Webster *et al.*, 2004). Il est probable que dans le passé les diagnostics des spores *N. apis* basés sur microscopie optique ne différenciaient pas les spores de *N. ceranae* (Fries *et al.*, 2006). *N. apis* a une demande énergétique plus basse que *N. ceranae* (Martín-Hernández *et al.*, 2011) et en général ne cause pas de mortalités élevées (Forsgren et Fries, *et al.*, 2010 ; Martín-Hernández *et al.*, 2011). La maladie causée par *N. apis* se dénomme « nosemosis de type A ». Dans les cas aigües, cette maladie se caractérise par des abeilles qui tremblent, des abdomens dilatés, des tâches de fèces sur les cadres et la façade des ruches, une baisse de la quantité du couvain et de la taille de la population d'abeilles, spécialement au printemps (Higes *et al.*, 2010). La maladie due à *N. ceranae* « nosémoses de type C » ne présente pas les symptômes évidents rencontrés par l'infection par *N. apis*. La nosémosis de type C se caractérise par une période d'incubation asymptomatique longue pendant laquelle la colonie présente une production intensive de couvain, des réserves de miel et de pollen, et l'effondrement de la colonie se déclenche lorsque le taux de ponte de la reine n'est pas suffisant pour remplacer la mort d'abeilles adultes (Higes *et al.*, 2008a).

## **Morphologie**

Le stade infectieux de *N. ceranae* correspond à une spore de paroi épaisse, seul stade à survivre en dehors les cellules hôtes (Keeling, 2009) (Fig. 2a). Sa forme extérieure ovo-cylindrique mesure 4,7 x 2,7 µm (fixée et colorée 3,6 x 1,7 µm) (Fries *et al.*, 1996). La spore possède une paroi fine avec une première couche « exospore » protéinique qui mesure entre 14 – 17 nm qui la protège des facteurs environnementaux et une deuxième couche chitineuse interne l'« endospore » (Fries *et al.*, 1996). La structure interne de la spore qui sert à l'infection est constituée essentiellement de trois parties :

- un filament polaire qui contient des protéines dans sa structure et qui est caractéristique du phylum Microspora,
- des membranes lamellaires et tubulaires « polaroplast »,
- et une vacuole postérieure (Fries *et al.*, 1996 ; Vavra et Larsson, 1999 ; Williams, 2009).



**Figure 2.** (A) Structure d'une spore de *N. ceranae* (adapté de Soroker *et al.*, 2011) et (B) comparaison avec une spore de *N. apis*, D : diplokarya ; PF : spiraux du filament polaire ; bar = 0,5 µm (Fries *et al.*, 2006).

L'extrémité antérieure de la spore contient le disque d'ancrage qui joint le filament polaire à la cellule pendant la germination; le filament est arrangé dans la partie centre-postérieure de la spore en formant des spirales dans la périphérie et entourant le sporoplasme. Le nombre des spires est caractéristique de l'espèce qui dans le cas de *N. ceranae* correspond à 18 – 23 spires (Chen *et al.*, 2009 ; Fries *et al.*, 1996; Higes *et al.*, 2010). Au centre de la spore, entourés par les spirales du filament polaire et le sporoplasme, se trouvent deux noyaux apposés (diplokaryon) structurellement identiques qui sont également visibles dans les stades végétatifs (Fries *et al.*, 1996 ; Vavra et Larsson 1999).

Le diagnostic différentiel de *N. ceranae* se base sur la PCR car les différences structurelles avec *N. apis* ne sont pas évidentes par microscopie optique. Les spores de *N. ceranae* sont en moyenne légèrement plus petites en taille et en nombre de spires du filament polaire que celles de *N. apis* (Fig. 2b). Les cycles de vie suivent des étapes similaires dans les deux espèces (Wittner et Weiss, 1999).

### Le cycle de vie

Le cycle de vie de *N. ceranae* commence lorsque l'abeille adulte ingère la spore. Nous distinguons deux étapes essentielles : la germination de la spore et la multiplication de la microsporidie (Fig. 3).

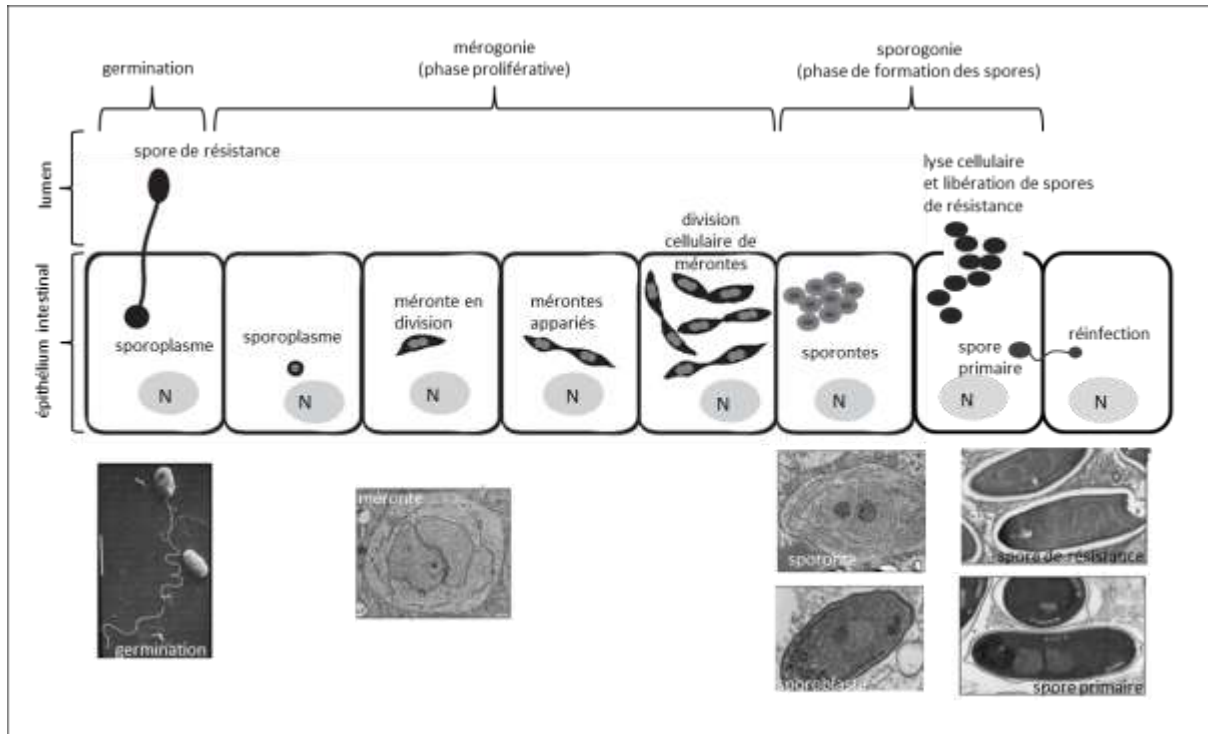
(i) *Germination:*

Dans le lumen, la spore extrude son filament polaire qui pénètre les cellules épithéliales de l'intestin. Une fois dans le cytoplasme de la cellule hôte, le parasite injecte son sporoplasme qui contient le matériel génétique (Wittner et Weiss, 1999). Les voies de signalisation qui gèrent la germination de la spore restent à présent inconnues. La vision actuelle considère que la germination est le résultat de l'augmentation de la pression osmotique à l'intérieur de la spore suivi par un influx rapide d'eau à travers la membrane cellulaire qui provoque l'éjection du filament polaire et le passage du sporoplasme vers l'extérieur de la spore à travers le filament. La recherche aujourd'hui vise à comprendre (i) l'interaction de protéines et glycoprotéines présentes dans le filament polaire et la paroi de la spore qui sont importants pour la fonctionnalité du filament, et (ii) la présence des autres composants impliqués dans la reconnaissance des tissus cibles et l'initiation de l'invasion de la cellule hôte (Texier *et al.*, 2010).

(ii) *Multiplication intracellulaire :*

- Mérogonie (phase proliférative). Dans cette étape le microorganisme se multiplie en contact direct avec le cytoplasme de la cellule hôte. Le sporoplasme se développe en méronte lorsque la multiplication commence à avoir lieu par fission cytoplasmique ayant comme résultat la formation de mérontes appariés qui vont continuer à se diviser. Le nombre de cycles de division n'a pas été encore déterminé (Fries *et al.*, 1996 ; Higes *et al.* 2010 ; Vavra et Larsson, 1999). Dans cette étape, la multiplication du parasite élargit les cellules hôtes et déplace les noyaux en position apicale; de nombreuses mitochondries sont placées autour des mérontes, ce qui facilite probablement l'obtention d'ATP dont le parasite a besoin (Higes *et al.* 2010).
- Sporogonie (phase de formation des spores). Une fois que la multiplication a eu lieu, les mérontes appariés vont se séparer et transformer en sporontes et sporoblastes par condensation et la formation d'une paroi épaisse (Gisder *et al.* 2010). Par la suite, deux types de spores peuvent se former, des spores « primaires » caractérisées par une forme ronde et les spores de « résistance » de forme ovale. La spore primaire a une paroi cellulaire plus fine que la spore de résistance et est capable de réinfecter des cellules hôtes voisines (Higes *et al.* 2010). La spore de résistance, de paroi plus épaisse, est libérée dans le lumen de l'intestin lorsque la cellule hôte se lyse, et est finalement dispersée dans l'environnement portée par les fèces des abeilles (Gisder *et al.*, 2010 ; Wittner et Weiss, 1999).

Après trois jours d'infection, des états végétatifs sont visibles dans quelques cellules épithéliales. A 6 jours la plupart des cellules contiennent des états végétatifs et des spores mûres (Higes *et al.*, 2007). L'infection se développe en totalité dans l'intestin au bout de 10 – 12 jours après l'inoculation (Forsgren et Fries, 2010).



**Figure 3.** Cycle de vie de *N. ceranae* dans les cellules épithéliales de l'intestin de l'abeille (adapté de Gisder *et al.*, 2010 ; Solter, 2010).

*Nosema ceranae* est considéré comme un organisme tissu – spécifique qui se développe et se multiplie uniquement dans les cellules épithéliales de l'intestin (Fries *et al.*, 1996 ; Higes *et al.*, 2007). Or, la présence d'un signal de PCR dans d'autres tissus, comme les glandes hypopharyngiennes, salivaires et mandibulaires, les tubes de Malpighi, le corps gras et le sac à venin, la spermathèque et les ovaires des reines (Chen *et al.*, 2009 ; Copley et Jabaji, 2011 ; Traver et Fell, 2012), remet en question la spécificité et la forme de transmission du parasite (voir plus bas les voies de transmission). Ces observations restent à être confirmées par microscopie électronique ce qui permettra de mieux comprendre la présence inattendue du parasite dans ces tissus et la relation avec son cycle de vie.

### Caractéristiques du génome

Avant 2009 la connaissance du génome de *N. ceranae* était limitée à certaines séquences de l'ARN ribosomal. La plupart des recherches sur les microsporidies correspondait à des séquences d'espèces



d'intérêt pour la santé humaine comme l'*Encephalitozoon cuniculi* et l'*Enterocytozoon bieneusi*. L'arrivée du pyroséquençage a permis la caractérisation rapide du génome de *N. ceranae* à un coût moins élevé que celui des techniques antérieures (Cornman *et al.* 2009).

En général, les microsporidies possèdent des petits génomes dont la taille ressemble plus à celle des procaryotes qu'à celle des eucaryotes (Cornman *et al.* 2009). La condensation et la réduction du génome sont liées à la dépendance qui caractérise les microsporidies de leurs hôtes étant parasites obligatoires intracellulaires (Texier *et al.*, 2010).

*N. ceranae* possède un génome biaisé AT (74%) extrêmement réduit (7.86 MB, 2614 gènes). Le génome contient des gènes conservés qui n'ont pas été détectés en dehors de ce phylum et qui pourraient être éventuellement associés à des facteurs de virulence, ce qui mérite des études complémentaires (Cornman *et al.*, 2009). Une partie importante du génome est dédiée aux différents niveaux de développement alors qu'une partie minime sert au transport et aux réponses aux stimuli chimiques. Cette caractéristique les éloignent des autres champignons et levures et reflète des adaptations suivies pendant leur évolution vers un cycle de vie à l'écart des variations environnementales et qui demandent une reproduction rapide. Une autre caractéristique liée à la forme de vie parasitaire est l'existence d'un groupe de gènes qui codifient des signaux peptidiques associés à des protéines sécrétoires qui peuvent interagir avec le tissu hôte. Actuellement, des recherches sont en cours de réalisation pour la description du génome de *N. apis*, ce qui révélera des caractéristiques corrélées au transfert d'hôte de *N. ceranae* et sa distribution rapide entre les colonies d'*A. mellifera* (Cornman *et al.*, 2009). Les microsporidies sont un modèle unique pour l'étude des effets hôte-parasite, comme pour la manipulation cellulaire, les interactions au niveau moléculaire et l'adaptation extrême à l'environnement intracellulaire. Cependant, une haute capacité de transformations génétiques d'origine inconnue (la transfection ou la reproduction sexuelle ont été proposées) c'est une contrainte pour leur utilisation comme modèles biologiques (Sagastume *et al.*, 2009 ; Williams, 2009).

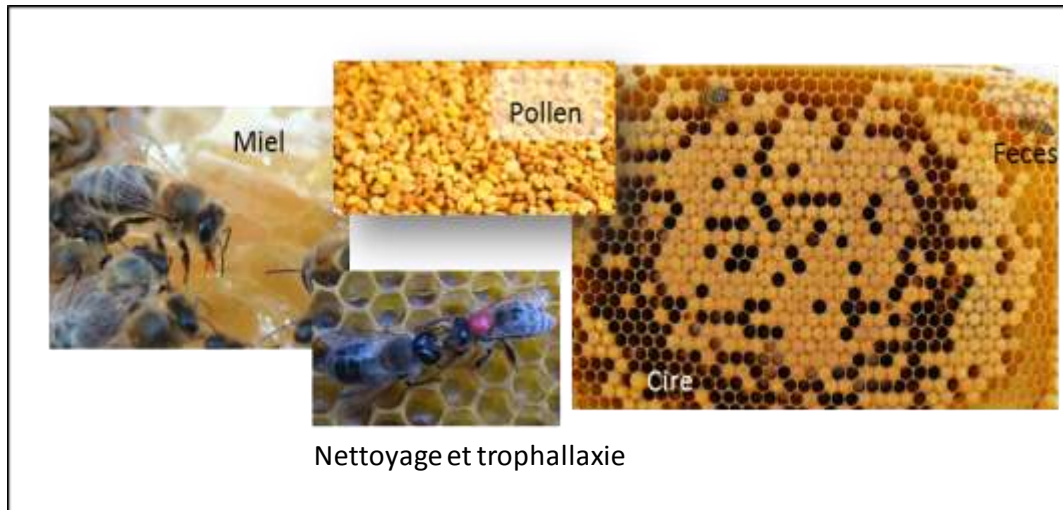
### **Voies de transmission de *N. ceranae* chez *A. mellifera***

Des infections naturelles de *N. ceranae* ont été détectées dans les trois castes d'*A. mellifera*, les reines (Traver et Fell, 2012), les ouvrières (Higes *et al.*, 2006; Huang *et al.*, 2007) et les mâles (Traver et Fell, 2011).

Il existe deux grands modes de transmission des pathogènes : la transmission horizontale et la transmission verticale. La transmission horizontale a lieu entre les individus d'une même génération, alors que la transmission verticale passe vers la descendance. Comparée avec la transmission

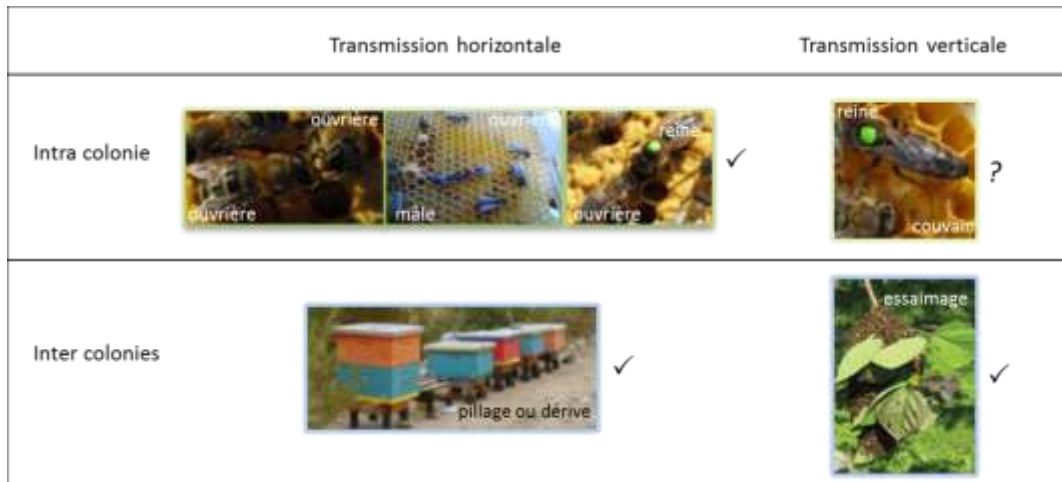
horizontale, la transmission verticale est sensée produire une sélection vers la diminution de la virulence du pathogène (Fries et Camazine, 2001). Dans ce cas, pour assurer sa transmission le parasite a besoin que son hôte se reproduise sans contrainte, donc le parasite a intérêt à ne pas affecter fortement la santé de son hôte ni sa capacité reproductive (Fries et Camazine, 2001). *Nosema ceranae* a une forme de transmission horizontale qui favoriserait le développement de la virulence du parasite. Cependant, des recherches récentes ont suggéré également la possibilité d'une transmission verticale (Traver et Fell, 2012).

L'infection se transmet lors des activités de nettoyage des fèces qui contiennent les spores de résistance (Higes *et al.*, 2010). Ce comportement est caractéristique des abeilles jeunes dédiées à maintenir un environnement propre en utilisant leurs structures buccales pour nettoyer le matériel à l'intérieur de la colonie (Winston, 1987) et qui ingèrent ainsi les spores. La trophallaxie est également un autre vecteur de transmission (Smith, 2012). Ce comportement correspond à l'échange de nourriture entre les abeilles et est probablement la voie d'infection des reines (Higes *et al.*, 2009) et des mâles qui sont essentiellement nourris par les ouvrières (Winston, 1987). Le miel, le pollen et la cire deviennent probablement contaminés pendant leur manipulation par les ouvrières lors de la récolte ou du stockage, ils constituent un réservoir de spores qui peut contribuer à leur transmission (Botías *et al.*, 2012b ; Higes *et al.*, 2008b) (Fig. 4).



**Figure 4.** Sources et vois de transmission de spores de *N. ceranae* à l'intérieur de la colonie.

Dans le cas des insectes sociaux, les modes de transmission horizontale et verticale peuvent être divisés dans deux sous-groupes : « intra colonie » entre les individus d'une colonie, et « inter colonie » entre les individus des différentes colonies (Fig. 5). La transmission des spores entre colonies peut avoir lieu à travers la dérive, pillage ou essaimage.



**Figure 5.** Différents modes de transmissions horizontales et verticales de *N. ceranae* dans des colonies d'*Apis mellifera* (basé sur Fries et Camazine, 2001).

La dérive est un comportement typique des ouvrières butineuses ou des mâles qui rentrent dans une colonie différente de celle d'origine, probablement dû à des problèmes d'orientation lorsque la densité des colonies dans un rucher est très élevée. Les mâles ont la tendance naturelle à dériver plus que les ouvrières pendant la recherche des endroits d'accouplement (Traver et Fell, 2011 ; Winston, 1987). Le pillage est déclenché par le manque de ressources mellifères qui stimule les ouvrières à voler le miel, qui peut contenir des spores (Botías *et al.*, 2012b), stocké dans des colonies étrangères (Winston, 1987). Les essaims sont formés par des abeilles ouvrières qui accompagnent une nouvelle reine lorsque la colonie d'origine est trop peuplée (Winston, 1987), avant de partir les ouvrières ont le réflexe de remplir leurs estomacs avec du miel, apportant ainsi les spores de *Nosema* avec elles.

### Les mécanismes de défense de l'abeille à l'infection par *N. ceranae* au niveau individuel

Les mécanismes de défense des abeilles aux pathogènes, comme en général chez les animaux, comprennent deux stratégies principales : la résistance et la tolérance. La résistance s'exprime par la construction de barrières qui empêchent l'infection ou par l'activation de réponses de défense lorsque l'infection a eu lieu, alors que la tolérance vise à compenser le coût énergétique ou le dommage tissulaire provoqués par le pathogène ou par l'activation de la réponse immunitaire de l'hôte (Evans et Spivak, 2010). Chez les insectes, les barrières sont la cuticule et les membranes épithéliales qui dans de nombreux cas évitent l'adhérence et l'entrée des microbes dans le corps. L'invasion par les microorganismes peut être également inhibée par des changements physiologiques de pH ou d'autres conditions chimiques de l'intestin de l'insecte (Evans et Spivak, 2010).

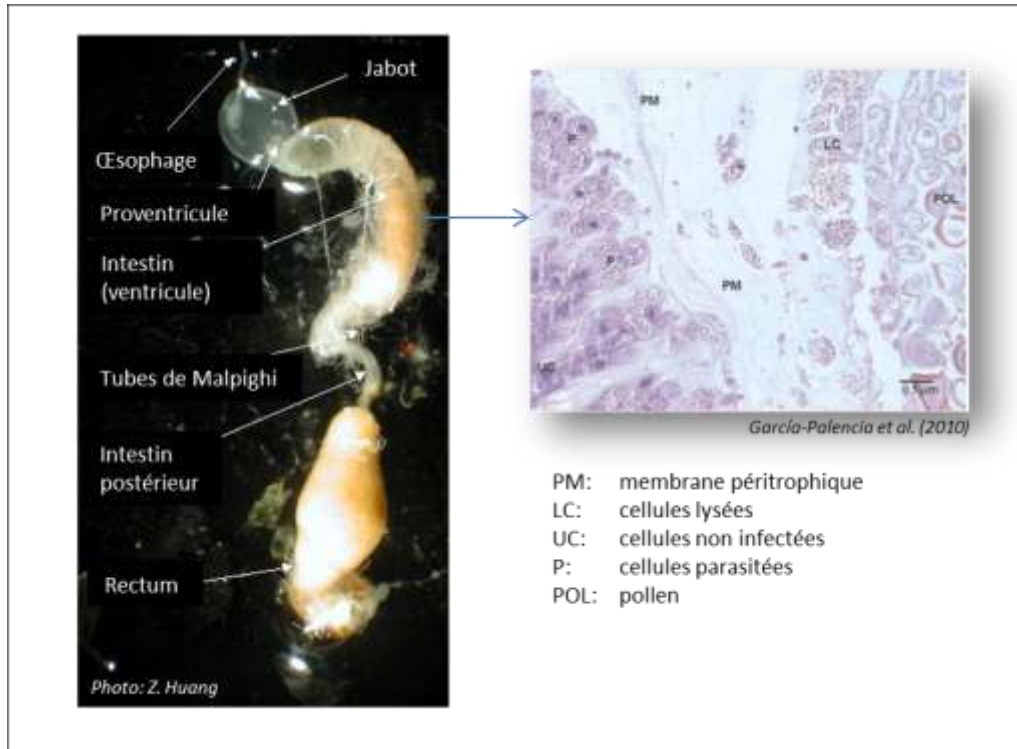


Figure 6. Système digestif de l'abeille et détails sur l'épithélium de l'intestin parasité par *N. ceranae*.

(i) Effets sur l'épithélium de l'intestin

Après la germination de la spore et l'introduction de son sporoplasme dans le cytoplasme de la cellule hôte, une série d'altérations morphologiques et physiologiques sont visibles dans les cellules de l'épithélium de l'intestin (Fig. 6). García-Palencia *et al* (2010) décrivent les lésions les plus importantes durant le développement et la multiplication du parasite :

- L'altération des membranes cellulaires, observée occasionnellement en dessous de la couche superficielle de l'épithélium de l'intestin.
- La lyse de cellules épithéliales, caractérisée par la présence de vacuoles dans le cytoplasme et par des agrégats de ribosomes et lysosomes.
- Le déplacement apical du noyau des cellules épithéliales, avec la présence de stades végétatifs ou de spores matures de *N. ceranae* dans des invaginations de la membrane nucléaire.
- La condensation ou réduction du noyau cellulaire, souvent associée à l'hyperchromatosis (la condensation irréversible de la chromatine dans le noyau des cellules qui suivent un processus de nécrose ou apoptose).

- L'absence de la membrane péritrophique, ou bien d'une structure fragmentée de celle-ci. Cette membrane est considérée analogue au mucus qui recouvre le lumen de l'estomac des vertébrés. Son rôle principal est de protéger les cellules épithéliales des lésions mécaniques dues à l'action abrasive des particules de nourriture. Son origine serait le délaminage des couches de cellules épithéliales de la partie apicale de l'intestin (Da Cruz Landim, 1985).

La préservation de la fonctionnalité de l'intestin semble être un facteur clé dans la capacité de l'abeille à contrebalancer l'augmentation des demandes énergétiques induites par le parasite (Aliferis *et al.*, 2012 ; Mayack et Naug, 2009), d'où l'intérêt d'approfondir nos connaissances sur les mécanismes pathologiques de *N. ceranae* notamment les effets sur l'intestin.

#### *(ii) La réponse du système immunitaire de l'abeille*

Le système immunitaire des abeilles est activé lors de l'exposition à des blessures ou à des pathogènes. Evans *et al.* (2006) ont proposé que l'abeille, comme d'autres insectes, possède quatre voies moléculaires majeures interconnectées entre elles qui peuvent s'activer face à l'exposition à un parasite : Toll, Imd, Jak/STAT et Jnk. En général, ces voies consistent à (i) des protéines qui reconnaissent des signaux des parasites envahisseurs, (ii) des protéines qui modulent et amplifient le signal de reconnaissance, et (iii) des protéines ou métabolites qui sont impliqués directement dans l'inhibition du parasite. Ces voies moléculaires sont à la base des réponses immunitaires de type cellulaire et humorale (James et Xu, 2012). L'immunité humorale commence avec la reconnaissance d'un antigène tel qu'un parasite, ce qui déclenche la synthèse des différentes protéines antimicrobiennes (AMPs, antimicrobial peptides). Une réponse immunitaire localisée peut avoir lieu dans l'intestin des insectes par la production d'AMPs ou également par la production d'espèces réactives de l'oxygène (ROS, reactive oxygen species), molécules très réactives ayant une action antimicrobienne. La production de ROS ( $H_2O_2$ ,  $O_2^-$ , ou  $OH^\bullet$  entre d'autres molécules) a lieu dans les cellules par différentes voies comme par exemple la respiration cellulaire. Ces molécules assez réactives peuvent aussi endommager les cellules, c'est pourquoi l'organisme possède des mécanismes antioxydant (Cadet *et al.*, 2005). La production de ROS dans l'intestin est extrêmement rapide ce qui lui confère une fonction de première barrière contre l'invasion microbienne. Au contraire, pour la plus parts des insectes les AMPs ne sont pas efficaces dans le contrôle des champignons entomopathogènes. L'immunité cellulaire implique la reconnaissance du pathogène suivie par la phagocytose (virus et bactéries), la nodulation (champignons et quelques bactéries) et l'encapsulation (parasites multicellulaires). La phagocytose est accompagnée par la production de mélanine et la mélanisation de nodules ou capsules. La voie de mélanisation est activée par les hémocytes et

déclenche la cascade de phénoloxydase (enzyme terminal du processus de mélanisation); la mélanisation peut induire également la production de ROS (James et Xu, 2012).

Il a été observé au niveau moléculaire (Antúnez *et al.*, 2009) et biochimique (Alaux *et al.*, 2010a) que l'infection par *N. ceranae* n'active pas la voie de mélanisation, et inhibe l'expression des gènes d'AMPs (hymenoptaecin et abaecin) (Antúnez *et al.*, 2009, Chaimanee *et al.*, 2012), au contraire de *N. apis* (Antúnez *et al.*, 2009). En revanche, d'autres études ont montré l'activation du système immunitaire par la surexpression des gènes des voies moléculaires Toll, IMD et Jak/STAT chez des abeilles sélectionnées pendant longtemps pour leur tolérance à *N. apis* (Huang *et al.*, 2012).

L'abeille possède un nombre réduit de gènes d'immunité individuelle comparé à d'autres insectes non-sociaux. Une des hypothèses qui pourrait expliquer une telle diminution est liée à l'efficacité des barrières sociales que possèdent les abeilles domestiques pour se défendre des pathogènes, alors que les insectes non sociaux comptent uniquement sur l'immunité individuelle (Evans *et al.* (2006).

La connaissance des mécanismes qui sont à la base du système de défense de l'abeille est actuellement très limitée. Cette carence est particulièrement évidente lorsque nous voulons expliquer les effets de *N. ceranae* sur la survie et les comportements des abeilles.

### **Les mécanismes de défense de l'abeille à l'infection par *N. ceranae* au niveau social**

En plus des mécanismes de défense individuels, les abeilles possèdent également des mécanismes collectifs pour limiter les impacts des microorganismes pathogènes. Les réponses d'immunité individuelle sont renforcées lorsque les comportements de la communauté sont coordonnés, ce qui a comme résultat une réponse immune au niveau colonial. Le mécanisme de défense collective contre les parasites qui résulte de la coopération entre les individus se dénomme « immunité sociale » (Evans et Spivak, 2010).

Parmi les comportements d'immunité sociale le transport des abeilles adultes mortes hors de la colonie contribue à réduire le contact des autres individus avec de potentiels pathogènes (Evans et Spivak, 2010). Egalement l'auto-expulsion altruiste des individus malades est sensée être un comportement des abeilles face aux infections comme celle de *N. ceranae* qui permettrait la diminution de l'inoculum dans la colonie (Rueppell *et al.*, 2010). Le polyéthisme d'âge (division des tâches selon l'âge), peut rendre plus vulnérable à une maladie des groupes spécialisés dans certaines

tâches plus risquées comme le butinage. Cependant la concentration de la maladie dans ce groupe servirait de barrière à la transmission du parasite lorsque ce groupe meurt à l'extérieur de la colonie (Evans et Spivak, 2010). Par exemple, les butineuses infectées par *N. apis* butinent plus précocement que les abeilles saines, ce qui aiderait à réduire la transmission de l'infection au sein de la colonie puisque les butineuses meurent à l'extérieur du nid (Wang and Möeller, 1970).

Cependant, certains mécanismes d'immunité sociale qui aident à la diminution de parasites comme *Varroa destructor* (acararien macro parasite), *Acarapis woodi* (acararien des trachées) et de maladies du couvain (Evans et Spivak, 2010), ont été signalés comme des comportements qui peuvent augmenter la transmission d'autres parasites (Schmid-Hempel, 1998). Par exemple, le comportement de toilettage, par lequel les abeilles éliminent des particules et parasites en se nettoyant leur surfaces individuellement ou les unes aux autres, est efficace pour l'élimination de *V. destructor* et *A. woodi*, mais lorsque le toilettage implique l'utilisation des parties buccales (Evans et Spivak, 2010), il pourrait entraîner l'échange de sécrétions entre les abeilles avec un effet similaire à la trophallaxie. Smith (2012) récemment a démontré le potentiel de la trophallaxie comme voie de transmission de *N. ceranae*. Un autre comportement social dénommé «fièvre comportementale» observé chez des abeilles infectées par *N. ceranae* semble également favoriser le parasite (Campbell *et al.*, 2010). La fièvre comportementale est une réponse commune des animaux face à une infection qui dans presque tous les cas sert à la défense de l'hôte et qui consiste à élever la température corporelle de l'individu. Cependant, l'augmentation de la température à l'intérieur de la colonie dans le cas de *N. ceranae* favorise son développement au lieu de le restreindre (Martín-Hernández *et al.*, 2009).

Les connaissances actuelles sur l'immunité sociale des abeilles face à l'infection par *N. ceranae* laissent plusieurs questions ouvertes. Notamment, les mécanismes physiologiques à la base de l'immunité sociale, probablement manipulés par le parasite, sont très peu connus. De même, les interactions entre les individus des différents castes et âges, qui dans des conditions normales régulent l'organisation de la colonie, sont probablement modifiées par le parasite. L'influence de l'environnement est également très importante dans le scénario d'une colonie malade, car c'est la source de nutriments dont la qualité est fondamentale pour renforcer le système immunitaire (Alaux *et al.*, 2010b) et pour contrebalancer le stress énergétique dû par exemple à *N. ceranae* (Mayack and Naug, 2009). Le pollen représente aussi une source de microbes avec des effets protecteurs bénéfiques pour l'abeille (Mattila *et al.*, 2012). Dans le même sens, Porrini *et al* (2011) ont démontré que la réponse à l'infection dépend de l'état nutritionnel et physiologique de l'hôte, qui dépend à la fois non pas de la quantité mais de la qualité des nutriments.

Dans le chapitre 2 (article n°1 et 2) nous revenons sur la compréhension du rôle de l'immunité sociale dans la prévention de la transmission de *N. ceranae* et décrivons les mécanismes phéromonaux qui sont à la base de ces comportements, dans le but d'expliquer la relation entre le parasitisme de *N. ceranae* et la disparition des abeilles lors du phénomène d'effondrement des colonies (vanEngelsdorp *et al.*, 2009).

### **Effets de *N. ceranae* sur le comportement de l'abeille**

La dépendance énergétique de *N. ceranae* à son hôte est associée à des changements de comportements observés chez l'abeille. Une consommation élevée de ressources sucrées a été mise en évidence par Alaux *et al.* (2010a) et Vidau *et al.* (2011) chez les abeilles parasitées par *N. ceranae*. Mayack et Naug (2009) ont montré que les abeilles infectées avec un accès *ad libitum* au sucrose avaient une survie similaire aux abeilles non infectées, ce qui révélait la capacité des abeilles de contrebalancer le stress énergétique par la surconsommation des carbohydrates. Naug et Gibbs (2009) ont observé que les abeilles parasitées présentent une altération du comportement de trophallaxie car elles reçoivent de la nourriture mais, par contre, elles sont moins disposées à en donner aux autres, ce qui serait dû à l'état de faim des abeilles infectées. D'autres comportements liés également au stress énergétique ont été décrits par Karlj et Fusch (2010) tels que des problèmes d'orientation et de réduction de la capacité de vol.

Un butinage précoce et plus intense a été décrit chez les abeilles infectés par *N. apis* (Hasein, 1953 ; Lin *et al.*, 2009 ; Wang and Möeller, 1970 ; Woyciechowski et Kozlowski, 1998 ; Woyciechowski et Moron, 2009), à l'exception de l'étude de Mattila et Ottis (2006) mais ces auteurs n'ont pas vérifié le succès de l'infection des abeilles. D'autres effets sur les comportements des abeilles, notamment la perte des capacités d'orientation, de vol, et d'apprentissage, ont été observés lors du macro-parasitisme de l'acarien *V. destructor* (Kralj et Fuchs, 2006), du virus du couvain en sac «SBV, sacbrood virus», (Bailey et Fernando, 1972) ou du virus des ailes déformées «DWV, deformed wing virus» (Iqbal et Mueller, 2007).

Le comportement de butinage, et en général tous les comportements qui font partie du polyéthisme d'âge (division temporelle de tâches selon l'âge) qui caractérise les abeilles, sont sous le contrôle de facteurs génétiques et de l'environnement. Ainsi, les abeilles adaptent la répartition de tâches en réponse aux conditions de la colonie et de l'environnement (Winston, 1987). Le passage d'une tâche à l'autre comprend un changement physiologique, qui est lié au processus de maturation de l'abeille. Cependant il existe une plasticité importante qui permet aux abeilles de reprendre une tâche malgré son âge (Winston, 1987).

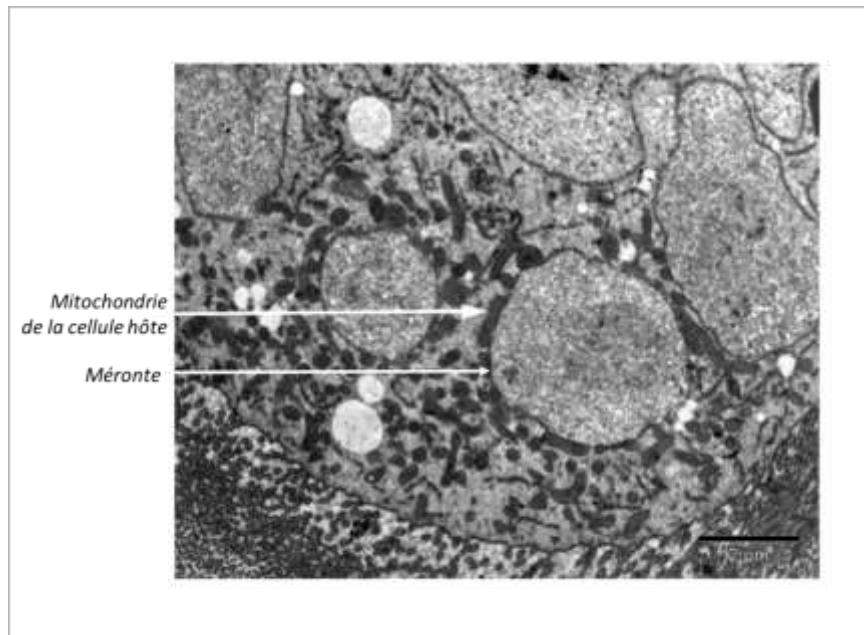


Les changements physiologiques à la base du polyéthisme d'âge sont gérés au niveau individuel par des hormones et au niveau social par des phéromones. C'est le cas de la répartition des tâches réalisées à l'intérieur de la ruche par les abeilles jeunes « nourrices » et les activités développées à l'extérieur de la ruche par des abeilles âgées « butineuses » (Winston, 1987). Parmi les hormones, l'hormone juvénile III (JH: juvenile hormone) et la vitéllogénine (Vg) sont impliquées dans la transition de nourrice à butineuse (Winston, 1987). La JH est produite par les *corpora allata*, une paire de glandes sécrétrices localisées à proximité du cerveau (Sullivan *et al.*, 2000). Le taux de JH augmente avec l'âge des abeilles, mais aussi au fur et à mesure qu'elles mûrissent (Robinson, 1987). La Vg est une hormone produite dans le *corps gras* des abeilles, qui correspond à un tissu lâche localisé le long du corps principalement en dessous du tégument des abeilles. Le corps gras possède une fonction endocrine en plus d'autres fonctions, comme le stockage et l'utilisation de nutriments (Arrese et Soulages, 2010). La Vg participe à l'inhibition de la maturation des abeilles ouvrières, et est sensée interagir avec la JH en s'inhibant réciproquement (Page et Adam, 2007). Ainsi, au fur et à mesure que les ouvrières mûrissent, les taux de Vg diminuent et ceux de la JH augmentent. Également l'octopamine, une neuro-hormone qui se trouve en plus forte quantité dans les lobes antennaires des butineuses que dans ceux des nourrices, participe aux mécanismes de maturation comportementale (Schulz et Robinson, 2001). La perturbation des taux d'hormones pourrait être à la base des changements de comportements observés lors de l'infection avec *N. ceranae*. En effet, chez les abeilles parasitées Antúnez *et al.* (2009) ont observé la sous-expression du gène de la Vg et Ares *et al.* (2012) ont constaté un niveau plus bas de JH. De façon similaire, Huang et Lin (2004) ont montré que des abeilles infectées par *N. apis* présentaient un niveau de JH moins élevé associé à un comportement de butinage précoce comparé aux abeilles non infectées.

La transition de nourrice à butineuse est régulée également par quatre phéromones : la phéromone du couvain (BEP : brood ester pheromone) produite par le couvain âgé (Le Conte *et al.*, 2001, 2006 ; Pankiw, 2004), la (E)- $\beta$ -ocimène produite par le couvain jeune (Maisonnasse *et al.*, 2010a, b), la phéromone mandibulaire de la reine (QMP : queen mandibular pheromone) (Pankiw, 1998) et l'oléate d'éthyle (EO : ethyl oleate) produite par les butineuses (Leoncini *et al.*, 2004). Alaux *et al.* (2011) ont montré des modifications dans le taux des composés de la QMP chez des reines infectées par *N. ceranae*. Par exemple, un taux de QMP plus élevé pourrait être associé à la qualité reproductive de la reine. En effet, il a été observé que des reines inséminées avec le sperme d'un seul mâle avaient des niveaux plus élevés de QMP, comparé à des reines inséminées avec le sperme de plusieurs mâles. Ce dernier cas est plus proche de ce qui se passe dans la nature. De plus, les reines avec un taux plus important de QMP sont moins attractives pour les ouvrières (Richard *et al.*, 2007).

## Effets de *N. ceranae* sur l'état nutritionnel et énergétique de l'abeille

Les microsporidies capturent l'ATP de l'environnement cellulaire de leurs hôtes. Elles ne possèdent pas de mitochondries mais un organe réduit dénommé « mitosome » qui au cours de l'évolution a perdu la fonction de respiration cellulaire (Williams, 2009). C'est pourquoi, pendant leur développement et la multiplication intracellulaire, *N. ceranae*, comme d'autres parasites du même groupe, sont entourés par des mitochondries de la cellule hôte ce qui faciliterait l'acquisition d'ATP (Higes *et al.*, 2007) (Fig. 7). Pour d'autres microsporidies, il a été suggéré un libre passage d'ATP de la cellule hôte vers la surface du parasite où des transporteurs amèneraient l'ATP à l'intérieur du parasite. Ce mécanisme a été observé grâce au séquençage du génome d'*E. cuniculi* (microsporidie parasite de l'homme) qui code pour des protéines qui se placent sur la membrane cellulaire et transportent l'ATP présent dans le cytoplasme de la cellule hôte. Il a été proposé que la proximité nécessaire pour le passage d'ATP entre le parasite et la mitochondrie est basée sur un lien protéinique. Un mécanisme similaire est aussi peut-être responsable du rapprochement du parasite aux mitochondries (Williams, 2009). Les microsporidies ont conservé la voie glycolytique pour la production d'énergie à partir des carbohydrates qui doit être complémentaire à l'acquisition d'ATP (Weidner *et al.*, 1999). La glycolyse est sensée être la source d'énergie permettant la germination de la spore qui n'a pas de nutriments stockés. Des concentrations très élevées de tréhalose et l'activité de l'enzyme tréhalase qui transforme le tréhalose en glucose ont été détectées dans les spores de *N. apis* (Weidner *et al.*, 1999).



**Figure 7.** Emplacement de *N. ceranae* « méronte » entouré par des mitochondries dans le cytoplasme de la cellule hôte, d'où il tire l'énergie sous forme d'ATP (basé sur Higes *et al.*, 2007).

Les perturbations de la composition biochimique des tissus par les parasites intracellulaires sont de grande importance car souvent ces derniers altèrent le contenu cellulaire en électrolytes, carbohydrates, protéines et acides aminés libres. Egalement, les changements dans l'environnement cellulaire de l'hôte dus au parasitisme peuvent influencer à leur tour les activités métaboliques du parasite (Weidner *et al.*, 1999). Récemment Aliferis *et al.* (2012) ont étudié le profil métabolique dans l'hémolymphe des abeilles infectées par *N. ceranae*. Ils ont observé une diminution générale des réserves d'acides aminés dans l'hémolymphe peut-être due à un compromis avec les mécanismes de défense (production d'AMPs, augmentation du stress oxydatif). Il pourrait s'agir également de la réduction de la biosynthèse d'acides aminés dans le corps gras de l'abeille dû à des changements physiologiques ou à l'utilisation des acides aminés par *N. ceranae* dans son métabolisme. Egalement, il a été suggéré que la perte de l'homéostasie des carbohydrates présents à des niveaux très faibles dans l'hémolymphe, pourrait être à la base de la perte des capacités de vols des abeilles parasitées. Dans l'étude d'Aliferis *et al.* (2012), un des carbohydrates dont la concentration était la plus élevée était le glucose. Cette concentration élevée a été attribuée au fait d'avoir analysé des abeilles au repos. Mayack et Naug (2010), ont observé des niveaux de glucose faibles chez des abeilles qui revenaient de butiner et donc avaient dépensé le glucose comme énergie pour le vol.

### **Mortalités d'abeilles liées à *N. ceranae***

La virulence d'un parasite peut être définie comme le degré auquel l'infection fait diminuer la reproduction et la survie de son hôte (Fries et Camazine, 2001). Dans le cas de *N. ceranae*, des études montrent des degrés opposés de virulence chez l'abeille (Carreck, 2012). Ces différences ont été observées dans des conditions de laboratoire (Alaux *et al.*, 2010a; Forsgren et Fries, 2010; Higes *et al.*, 2007; Martín-Hernández *et al.*, 2009; Suwannapong *et al.*, 2010; Vidau *et al.*, 2011) tout comme dans des conditions naturelles (Chauzat *et al.*, 2010 ; Borneck *et al.*, 2010 ; Gisder *et al.*, 2010 ; Higes *et al.*, 2008a ; vanEgelsdorp *et al.*, 2009). D'une part la préférence de *N. ceranae* pour les conditions climatiques de certaines régions pourrait expliquer la diversité des résultats (Fenoy *et al.*, 2009; Martín-Hernández *et al.*, 2009), d'autre part, des facteurs génétiques du parasite et de l'hôte pourraient aussi être à la base de ces différences (Chen *et al.*, 2009 ; Genersch, 2010 ; Huang *et al.*, 2012; Williams *et al.*, 2008). Des interactions avec d'autres facteurs pourraient également conduire à des différences de mortalité, par exemple, l'interaction de *N. ceranae* avec des pesticides présents dans l'environnement (Alaux *et al.*, 2010a, Pettis *et al.*, 2012) ou l'interaction entre *N. ceranae* et le paysage qui apporte aux abeilles un régime moins riche en ressources mellifères qui peut rendre les abeilles plus susceptibles au parasite (Porrini *et al.*, 2011). Dans ce contexte, le chapitre 4 contribue à mieux comprendre la diversité d'effets de *N. ceranae* chez l'abeille.

**Tableau 1.** Mortalité des abeilles infectées expérimentalement avec des spores de *N. ceranae*, et élevées au laboratoire.

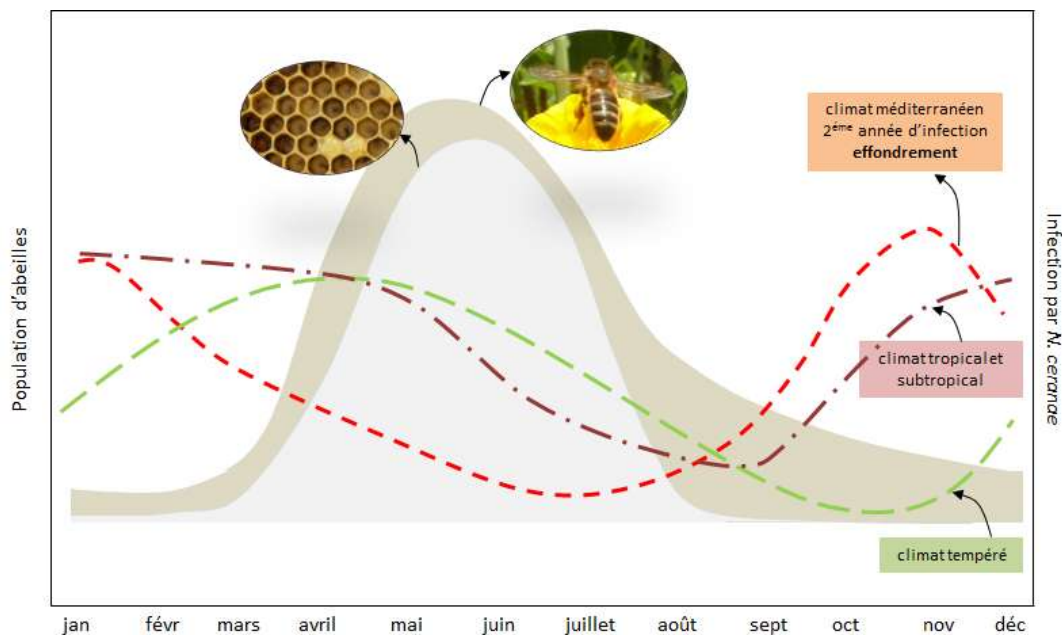
Type d'abeille	% mortalité	Jours à la mortalité	Dose infectieuse (spores par abeille)	Âge à l'infection	Alimentation	Référence
<i>A. mellifera</i>	< 22	14	100 1 000 10 000	Adultes	Sirop du sucre	Forsgren et Fries (2010)
<i>A. m. ligustica</i> x <i>A. m. mellifera</i>	30	10	200 000	1 jour	Pollen Candy (sucre et miel) Sirop	Alaux <i>et al.</i> (2010a)
<i>A. mellifera</i>	44	15	100 000	Adultes du nid	Sirop du sucre	Paxton <i>et al.</i> (2007)
<i>A. florea</i>	50	16	10 000	2 jours	Sirop Pollen	Suwannapong <i>et al.</i> (2010)
	50	14	20 000			
	50	14	40 000			
<i>A. ceranae</i>	50	14	10 000	2 jours	Sirop Pollen	Suwannapong <i>et al.</i> (2011)
	65	14	20 000			
	65	14	40 000			
<i>A. m. iberiensis</i>	11	7	1 000	5 jours	Sirop Promotor L <sup>®2</sup>	Martín-Hernández <i>et al.</i> (2011a)
	20		10 000			
	67		50 000			
	93		100 000			
<i>A. mellifera</i>	25	10 à 12	(46 000 230 000 1 150 000) <sup>1</sup>	7 jours	Sirop + pain de pollen Sirop+Apipromotor <sup>®3</sup>	Porrini <i>et al.</i> (2011)
	65					
	70					
Buckfast x <i>A. m. mellifera</i>	50	20	125 000	5 jours	Sirop Pollen Candy (Apifonda <sup>®4</sup> ) Sirop	Vidau <i>et al.</i> (2011)
<i>A. m. iberiensis</i>	100	7	125 000	5 jours	Promotor L <sup>®</sup>	Higes <i>et al.</i> (2007)
<i>A. m. iberiensis</i>	100	21	100 000 <sup>5</sup>	1 jour	Promotor L <sup>®</sup>	Higes <i>et al.</i> (2008b)

n.d.: no data; <sup>1</sup> pas d'effet de la dose infectieuse ; <sup>2,3</sup> vitamines et acides aminés ; <sup>4</sup> sucre ; <sup>5</sup> infection en groupe.

Dans des conditions de laboratoires, les différents protocoles utilisés rendent difficile l'interprétation des résultats sur la mortalité des abeilles infectées avec *N. ceranae* (Tab. 1). *A priori* trois facteurs pourraient jouer sur la mortalité. La dose de spores pour l'infection dans le cas de Martín-Hernández *et al.* (2011) cause des différences de mortalité. Egalement, Forsgren et Fries (2010) ont observé que des doses plus faibles que 10 000 spores par abeille n'affectent pas la totalité des abeilles (à 14 jours après l'infection). Au contraire Porrini *et al.* (2011) n'ont pas observé d'effet dose. L'âge des abeilles (Malone *et al.*, 1996 ; Woyciechowski and Moron, 2009) et l'alimentation (Alaux *et al.*, 2010b) sont deux facteurs qui peuvent rendre les abeilles plus au moins susceptibles à l'infection.

## Le développement de l'infection par *N. ceranae* au niveau de la colonie

Les pics d'infection de *N. ceranae* sont sensés varier selon les climats (Fig. 8). Sous des climats tropicaux et sub-tropicaux, il a été identifié un seul pic de *N. ceranae* pendant l'hiver (Chen *et al.* 2012). Tandis que sous des climats tempérés le pic apparaît entre le printemps et le début de l'été et parfois à l'automne (Oliver, 2011). En Allemagne *N. ceranae* présente deux pics d'infection, au printemps et en automne (Gisder *et al.*, 2010). En Espagne *N. ceranae* est présent pendant toute l'année (Martín-Hernández *et al.*, 2012), et les colonies infectées ont des niveaux d'infection qui évoluent avec la progression de la maladie. Ainsi, les colonies qui vont succomber ont un pic d'infection pendant l'automne (Higes *et al.*, 2008a). Sous des climats tropicaux et sub-tropicaux, la charge de spores est corrélée négativement avec la température moyenne (Chen *et al.* 2012). Cette adaptation de *N. ceranae* à la température a été également observée par Martín-Hernández *et al.* (2009). En Suisse le niveau d'infection de *N. ceranae* permet de prédire la survie des colonies. En effet, les colonies avec des niveaux plus élevés de *N. ceranae* en automne ont une de probabilité de survie plus élevée au cours de la saison suivante (Dainat *et al.*, 2012b). De façon similaire, une survie plus élevée accompagnée par un plus fort taux de spores *N. ceranae* ont été observés par Porrini *et al.* (2011) chez des abeilles infectées nourries avec un régime riche en pollen.



**Figure 8.** Variations régionales du développement de l'infection de *N. ceranae* chez *A. mellifera* au cours du temps. Trois courbes de développement de *N. ceranae* en fonction de la date sont représentées : en rouge la courbe d'une colonie en voie d'effondrement typique en Espagne (climat de type méditerranéen) ; en vert la courbe d'une colonie sans risque d'effondrement en Allemagne (climat de type tempéré) ; en marron la courbe d'une colonie sans risque d'effondrement à Taiwan (climat de type tropical – subtropical). En fond, un exemple de la dynamique de la population des abeilles d'une colonie pour un climat avec des hivers froids, abeilles adultes (aire en gris foncé) et couvain (aire en gris clair) (adapté de Higes *et al.*, 2008a ; Chen *et al.*, 2012 ; Gisder *et al.*, 2010).

La variation de température n'est sûrement pas la seule explication des différences dans les pics de *N. ceranae* (Chen *et al.* 2012). Les changements physiologiques et comportementaux pendant les saisons notamment avant l'hiver (Winston, 1987), ainsi que la dynamique de la population d'abeilles adultes et du couvain, doivent probablement influencer le développement de l'infection et la capacité de la colonie de contrebalancer l'infection (Khouri *et al.*, 2011).

## Hypothèse générale et objectifs

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Notre **hypothèse générale** place *N. ceranae* comme un pathogène capable d'induire des changements comportementaux chez *A. mellifera* dus à des altérations physiologiques, ce qui pourrait perturber l'organisation sociale des abeilles et aboutir à la mort de la colonie.

Pour répondre à cette hypothèse, trois **objectifs principaux** ont été définis correspondant aux axes principaux de cette thèse. D'abord, nous avons **cherché des effets de *N. ceranae*** sur la perturbation de l'organisation sociale de la colonie. L'approche choisie a été l'étude des taux de la phéromone oléate d'éthyle (EO) dans des abeilles parasitées, couplée à l'observation du comportement de butinage.

Ensuite, nous avons poursuivi nos recherches à travers l'**exploration des mécanismes physiologiques** à la base des effets observés chez les abeilles parasitées. Nous avons abordé cette étude à deux niveaux : transcriptomique et biochimique, focalisés sur l'intestin qui est l'organe cible du parasite.

Le dernier objectif était la **mise en évidence de différences entre deux isolats de *N. ceranae*** provenant d'origines géographiques variées, pouvant être à la base de différents degrés de virulence, et donc, de la variation des effets du parasite. Pour atteindre cet objectif nous avons conduit une étude de la variabilité génétique de deux isolats de *N. ceranae* et nous avons observé le développement de l'infection.





## Chapitre 2

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

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### 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*)**

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Journal of Chemical Ecology

2010. 36, 522-525.

doi:10.1007/s10886-010-9786-2

Rapid Communication

Version « .txt » du texte publié

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## 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 homeostasis. 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 homeostasis.

## 1. Introduction

Analogous to the hormones that control the organism homeostasis, pheromones in social insects play a key role in the regulation of group homeostasis. However, homeostasis 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 identified 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

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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 replicates 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  $\mu\text{g}/\text{kg}$  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  $-20^{\circ}\text{C}$  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  $\mu\text{l}$  of two internal standard solutions at 10  $\text{ng}/\mu\text{l}$  (arachidic acid methyl ester, and methyl heptadecanoate, Sigma-Aldrich, France). Samples were crushed with a glass rod for 2 min at  $0^{\circ}\text{C}$  and centrifuged for 20 min at  $4^{\circ}\text{C}$  (2,500 g). The supernatant was collected and applied to a silica column (silica gel 60, particle size 40–63  $\mu\text{m}$ , 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  $\mu\text{l}$  under a nitrogen stream, and 1  $\mu\text{l}$  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  $\mu\text{m}$  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  $90^{\circ}\text{C}$  for 1 min, raised to  $195^{\circ}\text{C}$  at  $40^{\circ}\text{C min}^{-1}$ , stabilized for 3 min, then augmented to  $210^{\circ}\text{C}$  at  $1^{\circ}\text{C min}^{-1}$ , stabilized

again for 2 min, then increased to 270°C at 40°C min<sup>-1</sup> 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.

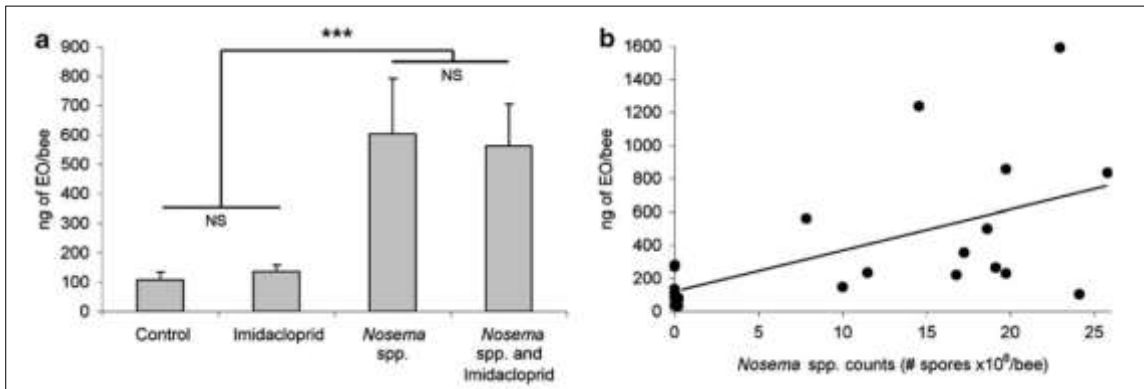
### 2.3. *Nosema* spore counting

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  $\pm$  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**

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Soumis à Journal of Invertebrated Pathology

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## 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).

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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 Kozłowski, 1998). Interestingly, it was observed in laboratory trials that infected bees develop extremely high levels of the primer pheromone “Ethyl Oleate (EO)”

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(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

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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:*

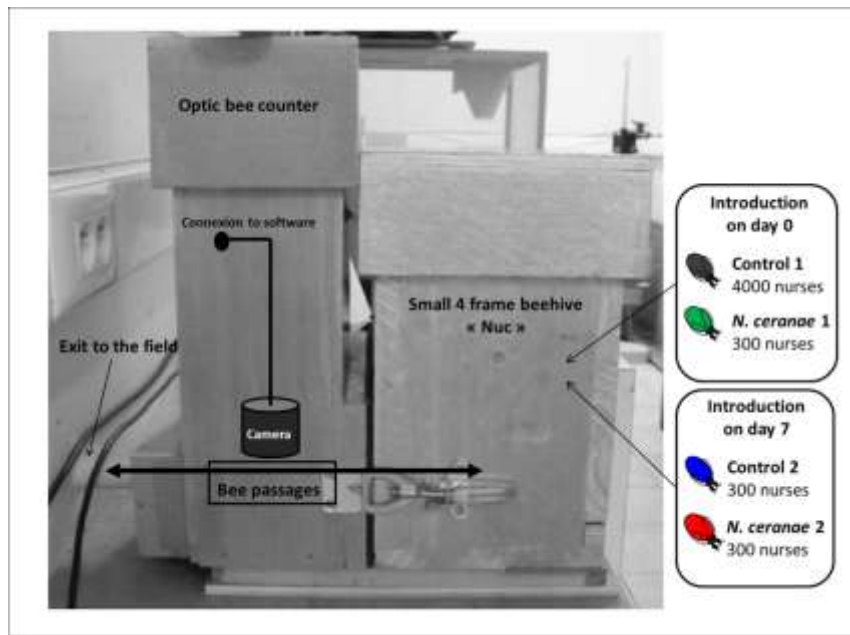
On day 0 of the experiment, each nuc was built with two cohorts composed of new born honey bees. One cohort named “Control 1” was composed of 4000 *N. ceranae*-free bees and a second cohort named “*Nosema* 1” corresponded to 300 *N. ceranae* infected bees. “Control 1” cohort played the role of “background 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  $34^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , 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  $-20^{\circ}\text{C}$  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  $\{0, \dots, T\}$ ). 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:

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

where  $\alpha_i$  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  $0, \dots, T$  is fixed at one so that the parameters  $\alpha_i$  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:

$$P [\text{bee of cohort } i \text{ is active at day } t]$$

$$= P [\text{bee of cohort } i \text{ is no more juvenile but not yet retired at day } t]$$

$$= P [\text{bee of cohort } i \text{ is no more juvenile at day } t] \times P [\text{bee of cohort } i \text{ not yet retired at day } t]$$

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  $Y$ ,

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

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

And, therefore,

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

The variance of  $N(i,t)$  is assumed to be proportional to its expectation:

$$V[N(i,t)] = \sigma^2 E[N(i,t)], \text{ where } \sigma^2 \text{ is an unknown positive parameter.}$$

*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

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

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  $\delta_t$  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  $E[N(i,t)]$  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  $\alpha_i$ ,  $\beta_i$  and  $\gamma_i$  as well as the estimates of the variance parameter  $\sigma^2$  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  $0.77 \pm 2.13$  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  $\pm$  SD expressed in millions of spores per bee.

Cohorts	14-day-old bees	21-day-old bees
<i>Nosema</i> 1	6.956 $\pm$ 4.272	8.708 $\pm$ 3.379
<i>Nosema</i> 2	10.007 $\pm$ 4.943	7.637 $\pm$ 2.360

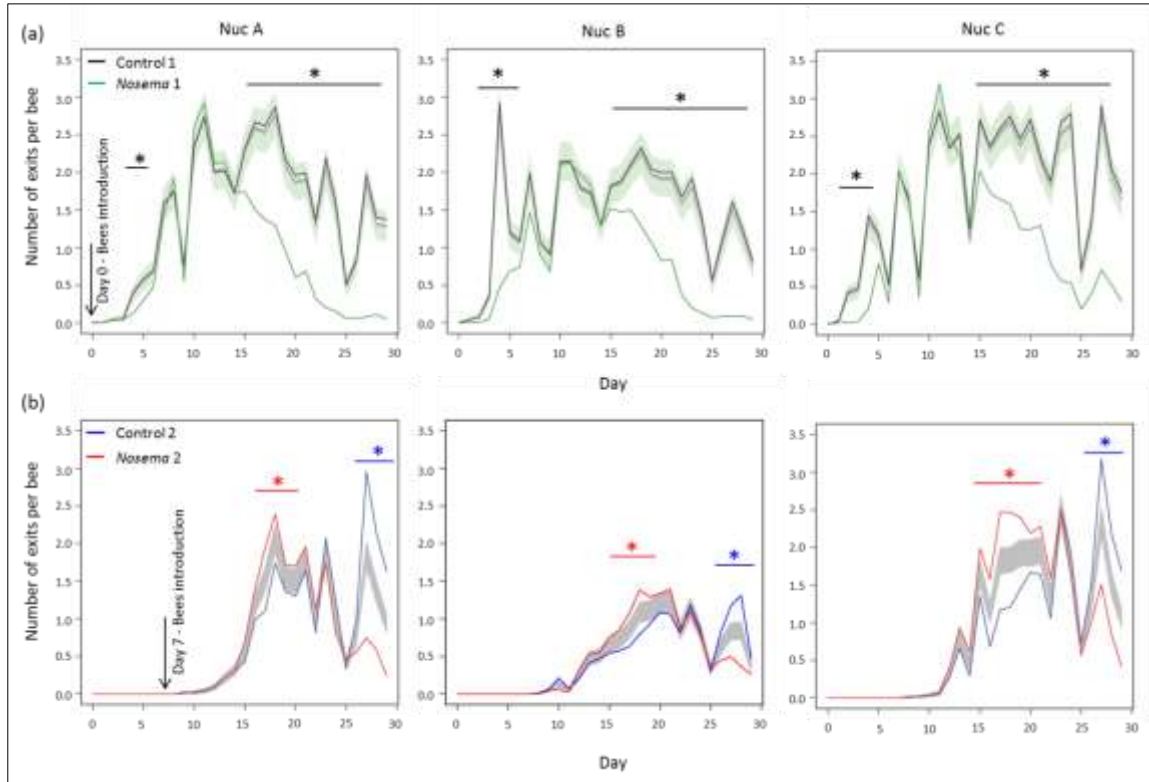
### 3.2. Flight activity

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 ( $\alpha$ ) and the age to exit “juvenile state” ( $\beta$ ) 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.

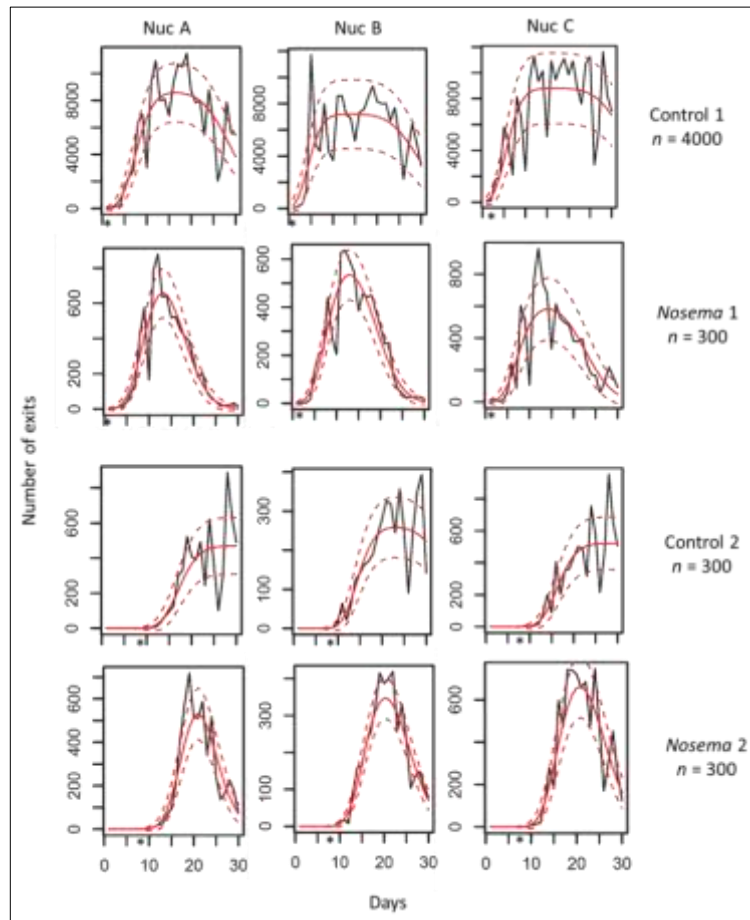


**Figure 2.** Graphical test for the daily number of exits per bee, applied to two time series of numbers of exits (Control and *Nosema* cohorts). 95% confidence envelopes were calculated using Bonferroni’s correction (Miller, 1981). When the observed time series goes out from the 95% confidence envelope, 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 envelopes 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 ( $\alpha$ )	Age to exit “juvenile state” * in days ( $\beta$ )	N° exit per day per bee ( $Tau$ )	SD Lifespan in days ( $\alpha$ )	SD Age to exit “juvenile state” in days ( $\beta$ )	SD N° exit per day per bee ( $Tau$ )
A	Control 1	28.92	8.38	2.16	0.26	0.56	1.14
	<i>Nosema</i> 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
	<i>Nosema</i> 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
	<i>Nosema</i> 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
	<i>Nosema</i> 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
	<i>Nosema</i> 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
	<i>Nosema</i> 2	18.98	9.68	2.63	0.10	0.56	1.30

$\alpha$ ,  $\beta$  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, 3<sup>rd</sup> 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).

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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

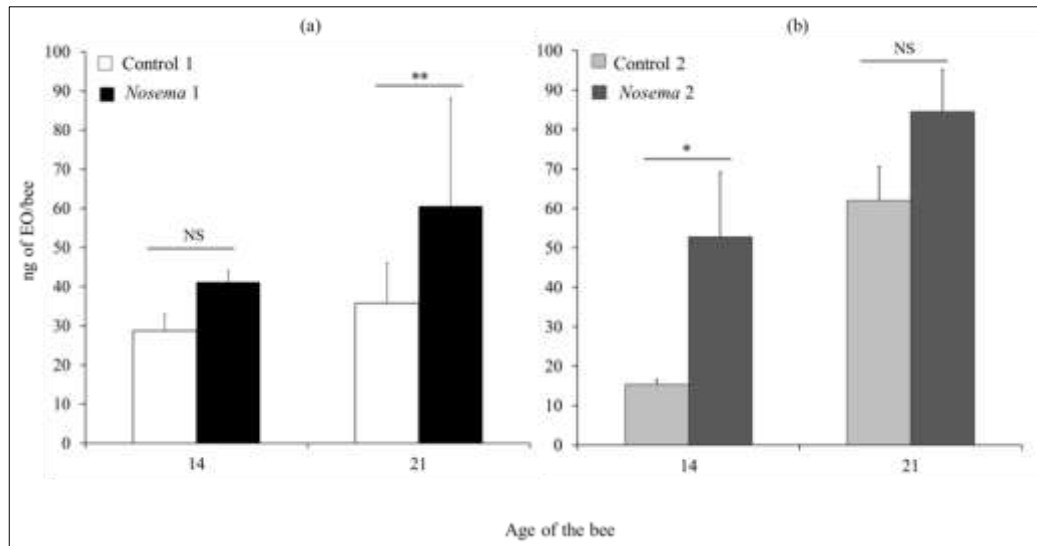
Comparison between the EO levels of the 3 nucs revealed a good repeatability of the experiment as no significant differences were found between Control cohorts ( $p = 0.6041$  and  $0.3738$ , for bees of 14 and 21-day-old respectively). While *Nosema* cohorts of the 3 nucs had almost similar EO levels,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

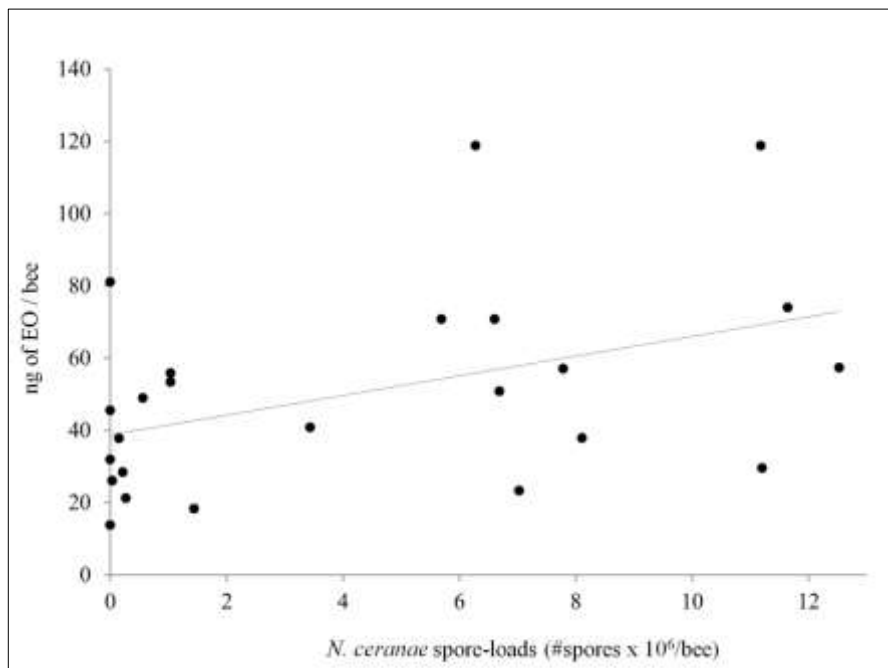


**Figure 4.** EO levels on honey bees. Infected bees showed higher mean levels of EO than non-infected bees, these differences were statistically significant in bees from (a) Control 1 and *Nosema* 1 of 21-day-old, and in bees from (b) Control 2 and *Nosema* 2 of 14-day-old. Control 1 and *Nosema* 1 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  $\pm$  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 et 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	<i>Nosema</i> 1	Control 2	<i>Nosema</i> 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

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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.

### **Acknowledgments**

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 parasite 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 par *N. ceranae* dans l'intestin de l'abeille.



## *Chapitre 3*

# **Mécanismes pathologiques de *Nosema ceranae* chez l'abeille: une approche moléculaire et biochimique**

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### **Présentation**

Après la détection de *N. ceranae* sur *A. mellifera* en 2005, et avec la diminution de la population d'abeilles au niveau mondial, les efforts de recherche ont été orientés principalement vers l'étude de la prévalence et de l'épidémiologie de *N. ceranae* dans différentes régions du monde. Egalement de nombreux travaux ont été focalisés sur l'étude du développement du parasite dans l'intestin de l'abeille au niveau histologique, de la multiplication et la production des spores dans différentes conditions et de la mortalité des abeilles. Un investissement important a été fait aussi dans la mise au point de méthodes de diagnostics microscopiques mais surtout moléculaires pour la détection de *N. ceranae* et sa différenciation de *N. apis*.

Cependant, la connaissance des mécanismes de base de l'infection notamment au niveau moléculaire, reste assez limitée avec des travaux sur l'activation du système immunitaire et les niveaux de tolérance à l'infection (e.g. Antúnez *et al.*, 2009; Bourgeois *et al.*, 2012 ; Chaimanee *et al.*, 2012 ; Huang *et al.*, 2012).

Basés sur des pistes des recherches issues des études sur les interactions hôte - parasite chez d'autres modèles biologiques, insectes et mammifères, nous avons développé une **hypothèse spécifique** sur les mécanismes d'infection et la réponse de l'abeille à *N. ceranae*. Cette hypothèse considère que si l'épithélium de l'intestin agit comme une première ligne de défense face à une infection, comme le cas de *N. ceranae*, on pourrait s'attendre à l'activation de voies métaboliques du système immunitaire et la production d'espèces réactives de l'oxygène d'action antibactérienne.

Dans le chapitre 3 nous développons ainsi notre deuxième objectif qui est l'exploration des mécanismes à la base des effets de *N. ceranae*. Cela par l'observation des différences entre des intestins d'abeilles infectées et d'abeilles saines en utilisant des méthodes moléculaires (transcriptomique) et biochimiques.



**Article n°3:**

**Gut pathology and responses to the microsporidium *Nosema ceranae*  
in the honey bee *Apis mellifera***

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PLoS ONE

2012. 7, e37017.

doi:10.1371/journal.pone.0037017

Version « .txt » du texte publié

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## Résumé

Le microsporidium *N. ceranae* est un nouveau parasite prévalent de l'abeille européenne (*Apis mellifera*). Bien qu'à présent ce parasite est en train d'atteindre une distribution mondiale dans son nouvel hôte, les mécanismes par lesquels il affecte les abeilles et comment elles y répondent ne sont pas très bien connus. Nous avons donc fait une large caractérisation des effets du parasite au niveau moléculaire à l'aide des outils génétiques et biochimiques. Les modifications dans le transcriptome au niveau de l'intestin ont été caractérisées sept jours après l'infection avec le système de puces d'ADN (« tiling microarray »). Puis nous avons testé la réponse de l'intestin à l'infection à travers la mesure de l'activité des enzymes antioxydantes et de détoxification (superoxyde dismutases, glutathion peroxydases, glutathion réductase, et glutathion-S-transférase). Au niveau de l'expression des gènes, la réponse de l'intestin à l'infection est basée sur l'augmentation du stress oxydatif concomitant avec la production des enzymes antioxydantes, ce qui correspond à une réponse défensive et protectrice particulièrement observée dans l'intestin des mammifères et des insectes. Néanmoins, au niveau d'activité enzymatique, la réponse protectrice n'a pas été vérifiée, et seulement la glutathion-S-transférase a montré une activité plus élevée chez les abeilles infectées. Le stress oxydatif était associé avec la transcription élevée d'un transporteur du sucre dans l'intestin. Finalement, l'effet délétère exercé par le parasite se traduit par l'inhibition des gènes impliqués dans l'homéostasie et le renouvellement cellulaire des tissus intestinaux (« *Wnt signaling pathway* »), un effet qui a été confirmé au niveau histologique. La dégénérescence et l'empêchement du renouvellement de l'épithélium intestinal pourrait donc expliquer la mort prématurée des abeilles. En conclusion, notre approche intégrée donne non seulement un nouvel aperçu des effets pathologiques de *N. ceranae* et de la réponse de l'intestin, mais elle démontre également que l'intestin de l'abeille constitue un bon modèle pour l'étude des réponses de défense d'un hôte vis à vis de son parasite.

## Abstract

The microsporidium *Nosema ceranae* is a newly prevalent parasite of the European honey bee (*Apis mellifera*). Although this parasite is presently spreading across the world into its novel host, the mechanisms by which it affects the bees and how bees respond are not well understood. We therefore performed an extensive characterization of the parasite effects at the molecular level by using genetic and biochemical tools. The transcriptome modifications at the midgut level were characterized seven days post-infection with tiling microarrays. Then we tested the bee midgut response to infection by measuring activity of antioxidant and detoxification enzymes (superoxide dismutases, glutathione peroxidases, glutathione reductase, and glutathione-S-transferase). At the gene-expression level, the bee midgut responded to *N. ceranae* infection by an increase in oxidative stress concurrent with the generation of antioxidant enzymes, defense and protective response specifically observed in the gut of mammals and insects. However, at the enzymatic level, the protective response was not confirmed, with only glutathione-S-transferase exhibiting a higher activity in infected bees. The oxidative stress was associated with a higher transcription of sugar transporter in the gut. Finally, a dramatic effect of the microsporidia infection was the inhibition of genes involved in the homeostasis and renewal of intestinal tissues (Wnt signaling pathway), a phenomenon that was confirmed at the histological level. This tissue degeneration and prevention of gut epithelium renewal may explain early bee death. In conclusion, our integrated approach not only gives new insights into the pathological effects of *N. ceranae* and the bee gut response, but also demonstrates that the honey bee gut is an interesting model system for studying host defense responses.

## 1. Introduction

The Microsporidia constitute a group of obligate intracellular single-cell spore-forming parasites that can infect a variety of insect taxonomic orders [1], including honey bees. Indeed, honey bees, which are important for the development and maintenance of natural ecosystems and agriculture, are commonly infected by microsporidia from the genus *Nosema*. Host infection takes place after ingestion of mature spores that germinate in the midgut by polar tube extrusion and injection of the sporoplasm inside the epithelial cell cytoplasm [2]. The European (*Apis mellifera*) and the Asian (*A. cerana*) honey bees were originally parasitized by *N. apis* and *N. ceranae*, respectively [3,4], however recent natural infections of *N. ceranae* in the European honey bee have been found across the world (see [5,6,7] for reviews and [8]). In its new host, *N. ceranae* is considered to cause major health problems characterized by an immune suppression [9], a degeneration of gut epithelial cells [2] and a reduction of bee lifespan [2,10,11,12]. Yet, laboratory assays comparing the virulence of *N. apis* and

*N. ceranae* gave contradictory results, with one study showing that *N. ceranae* is more virulent than *N. apis* [2] and a second one revealing a lack of difference in their virulence [13]. However, those divergent results might be explained by genetic differences in both the host and the parasite isolates [14,15]. In the field, *N. ceranae* has been found to be highly virulent and a potential cause of colony collapse in Spain [6,16] but epidemiological studies performed in the US [17,18] and in Germany [19,20] failed to associate this new parasite to colony losses. Those geographic differences might reflect the better adaptation of *N. ceranae* to elevated temperature as compared to *N. apis* [21,22]. Finally, if this parasite might not act on its own it can potentially interact with others stress factors since pesticide exposure increase its proliferation [23,24] and its impact on bee health [25,26].

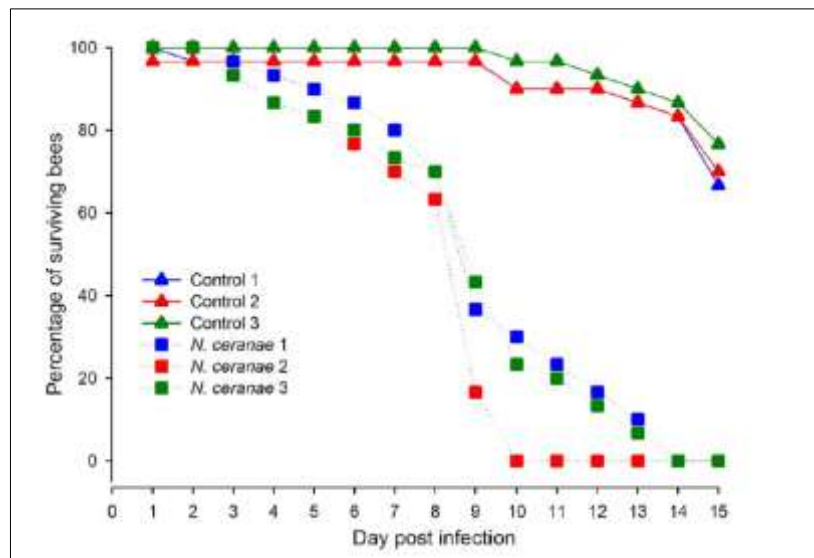
Even though, lots of information has been gathered on the prevalence, development and epidemiology of this emergent parasite [5,6,7], little is known about how *N. ceranae* cause host damages and the mechanisms by which bees protect themselves. Yet this information is vital for designing effective diagnoses and therapeutics. Since the genome sequences of *A. mellifera* [27] is available, it seems promising to use this information to develop novel insights into the gut defense response in insects. Since ingestion is the main entry route of many pathogens, the intestinal epithelium is the first line of defense protecting the host against invasion and dissemination of pathogenic microorganisms. If the classic innate immune system plays a central role in the defense against a broad spectrum of microorganisms [28], one of the most immediate epithelial response in mammals to combat the pathogen involves the generation of antimicrobial reactive oxygen species (ROS) [29,30]. After the ingestion of microbe-contaminated food, insects can rapidly mount an immune response involving different molecular pathways (*NF- $\kappa$ B*, *Toll* and *immune deficiency* pathways) [31,32], but the production of ROS is also a key feature of this protective response [30,33,34]. A concurrent elimination of residual ROS is also observed to protect the host [35], since the homeostasis of redox (reduction-oxidation) balance mediated by antioxidant enzymes is essential to the host survival.

In order to investigate how honey bee gut cells respond to infection by *N. ceranae* and how the parasite affects gut epithelium, we performed a transcriptomic analysis of infected and non-infected bees using a recently developed honey bee tiling microarray. As a complementary approach, we tested the activity of the antioxidant system, required for host protection against gut infection in *Drosophila* [35], by determining the activity of major antioxidant enzymes: the superoxide dismutases (SODs) and glutathione peroxidases (GPs). The indirect antioxidant functions carried out by enzymes such as glutathione reductase (GR), which allows the recovery of reduced glutathione from oxidized glutathione (product of glutathione reactions catalyzed by GP), and glutathione-S-transferases (GST), which catalyze the conjugation of glutathione xenobiotics [36] were also analyzed. Another enzyme

that may play a key role in the maintenance of midgut homeostasis and that presents a strong activity in the midgut tissue in insects [37] is the alkaline phosphatase (AP), which, in the gut of mammals, is involved in the dephosphorylation of bacterial lipopolisaccharides (reducing their toxicity), nutrient absorption and the reduction of gut inflammation [38]. Thus, we determined the influence of *N. ceranae* infection on its activity in honey bees. Finally, to connect molecular changes induced by *N. ceranae* infection to higher-order modifications, we determined the impact of the parasite on the host midgut epithelium and mortality.

## 2. Results and Discussion

The experimental infection was performed in Spain with the local subspecies of honey bee *A. m. iberiensis*. Before analyzing the pathological effects of *N. ceranae* on *A. mellifera* bees, we first verified that the parasite significantly reduced the lifespan of the host, as observed by previous studies [2,10]. The cumulative mortality rate of honey bee workers infected with *N. ceranae* was significantly higher than that of control bees (log-rank test:  $p < 0.001$ ). All infected bees died within 14 days post infection in each trial (Fig. 1), showing a consistent negative effect of the parasite on bees from the different colonies used for assessing the transcriptomic and enzymatic responses to the spore infection.



**Figure 1.** Mortality induced by *N. ceranae*. Data show the percentages of surviving bees per replicate ( $n = 3$ ) and per day in cages composed of 30 bees each (90 bees total/treatment). Cages with *N. ceranae* infected bees achieved one-hundred percent of mortality at day 14 post infection, while in control groups mortality remained low.

### 2.1. *Nosema ceranae* induces oxidative stress in the midgut

We used a tiling microarray based on gene predictions and annotation from the honey bee genome sequencing project [27] to identify at the gut level the pathological impact of *N. ceranae* on worker bees at seven days post-infection. A total of 9,293 genes were expressed in the gut, which represents approximately 70% of the genes so far identified from the honey bee genome. Out of those genes, the transcription of 336 genes was found to be significantly altered by the proliferation of *N. ceranae* spores (see Table S1 in “Annexe 3.2” for the list of genes). Since we analyzed bees from 3 different colonies (i.e. different genetic backgrounds) that were each headed by a multi-drone inseminated queen, those genes might represent the “general” gut pathology and/or response to the parasite.

When challenged by microorganism infections, insects can rapidly mount a potent immune response involving different molecular pathways [31]. For example, ingestion of bacteria activates the *Imd* pathway in the gut [32]. An activation of the innate immune system was therefore expected after challenging the bees with microsporidia, as it was found in drones [39]. However, no immune gene was more highly transcribed in workers after a seven-day infection. On the contrary, 2 genes (*basket* (GB16401) and *u-shaped* (GB16457)) that are involved in *Drosophila* immune responses [40,41] were downregulated (Table S1 in “Annexe 3.2”), perhaps indicating an immune suppression by the parasites, as suggested by Antúnez *et al.* [9]. Several studies have shown that one of the most immediate immune response of the gut involves the production of reactive oxygen species (ROS) to fight bacterial infection both in mammals [30,42] and insects [34,35,43]. ROS, which are efficient antimicrobial molecules, are generally derived from oxidation-reduction process. We performed a Gene Ontology analysis to explore which functional components were affected by *N. ceranae*, and found that genes involved in “oxidation reduction” were significantly overrepresented in the gene set upregulated upon spore infection (Table 1). This increase of oxidation reduction in the gut epithelia of bees parasitized by *N. ceranae* would therefore indicate an enhanced generation of ROS in response to the infection and suggests that ROS production is a general gut immune response to microorganism infection, including microsporidia.

Since residual ROS can cause inflammatory disease, a balance between synthesis and elimination of ROS via antioxidants is necessary to protect the host gut [29,34,35,43]. Therefore, the antioxidant system may play an essential role during gut infection. Interestingly, the functional analysis also revealed a “response to oxidative stress” in the bee gut (Table 1, see Table S2 in “Annexe 3.3” for the list of genes associated with each Gene Ontology category), notably with the upregulation of the *catalase* (GB30227) and *glutathione peroxidase-like 2* (*Gtpx2* (GB18955)) genes, which have both antioxidant properties. A similar catalase, the *immune-regulated catalase* has been



shown to be a key player of the *Drosophila* defense system during microbe infection in the gut epithelia [35]. Two cytochromes P450 were induced in the bee gut (*CYP6AS4* (GB15793) and *CYP6BC1* (GB10466)). *CYP6AS4* has been shown to metabolize quercetin contained in honey and pollen [44], but it is not known whether they might contribute to the production or elimination of ROS. We further tested whether the protective response in the bee gut increased in response to *N. ceranae* infection by measuring the activity of major antioxidant and detoxification enzymes: superoxide dismutase (SOD), glutathione peroxidase (GP), glutathione reductase (GR) and glutathione-S-transferase (GST). The activity of SOD and GR was not significantly different between control and infected bees (Mann-Whitney U tests:  $p = 0.931$  and  $p = 0.558$ , respectively; Fig. 2). Unexpectedly, the general activity of GP was significantly decreased by the spore infection ( $p = 0.002$ ). We observed the opposite pattern for *Gtpx2* at the transcriptome level (see above). The lower GP activity may either reflect a post-translational modification or the expression level of the other GP found in the bee genome (*Gtpx1* (GB14138)) but its expression was not significantly different. The gut might also respond to a potential inhibition of GP activity upon spore infection by increasing the transcription of *Gtpx2*. Further experiments are needed to explore the GP pattern. Finally, the GST activity was induced by *N. ceranae* ( $p = 0.04$  Fig. 2), as recently found by Vidau *et al.* [26].

**Table 1.** Functional analysis of honey bee genes affected *N. ceranae* parasitism.

	Gene Ontology Term (GO)	# genes	p-value
	GO:0005886 - plasma membrane	32	1.62e-09
	GO:0030182 - neuron differentiation	23	2.48e-06
	GO:0048666 - neuron development	20	7.66e-06
	GO:0007409 - axonogenesis	14	6.03e-05
	GO:0007424 - open tracheal system development	12	1.85e-04
	GO:0007411 - axon guidance	11	1.85e-04
	GO:0001894 - tissue homeostasis	5	2.88e-04
Down	GO:0048729 - tissue morphogenesis	14	3.83e-04
	GO:0007169 - transmembrane receptor protein tyrosine kinase signaling pathway	9	3.79e-04
	GO:0050905 - neuromuscular process	4	7.85e-04
	GO:0007167 - enzyme linked receptor protein signaling pathway	10	0.001
	GO:0010647 - positive regulation of cell communication	6	0.0018
	GO:0007242 - intracellular signaling cascade	14	0.0019
	GO:0006468 - protein amino acid phosphorylation	13	0.0023
	GO:0002009 - morphogenesis of an epithelium	12	0.0026
	GO:0006979 - response to oxidative stress	5	7.14e-04
Up	GO:0055114 - oxidation reduction	11	0.0027
	IPR005829 - sugar transporter, conserved site	4	0.004

The lists of genes regulated by *N. ceranae* parasitism were analyzed for statistical enrichment of associated Gene Ontology (GO) and InterPro (IPR) terms ( $p < 0.005$ ), relative to the representation of these terms for all expressed genes.

Different GST genes have been found to be significantly induced in the intestinal tissue after an oral bacterial infection, suggesting that GSTs might be involved in the protection of gut epithelium against pathogens [32]. The gut protection after *N. ceranae* challenge was therefore not really confirmed at the enzymatic level but a previous study reported an increase of the total antioxidant capacity in infected queens one week post-infection [45].

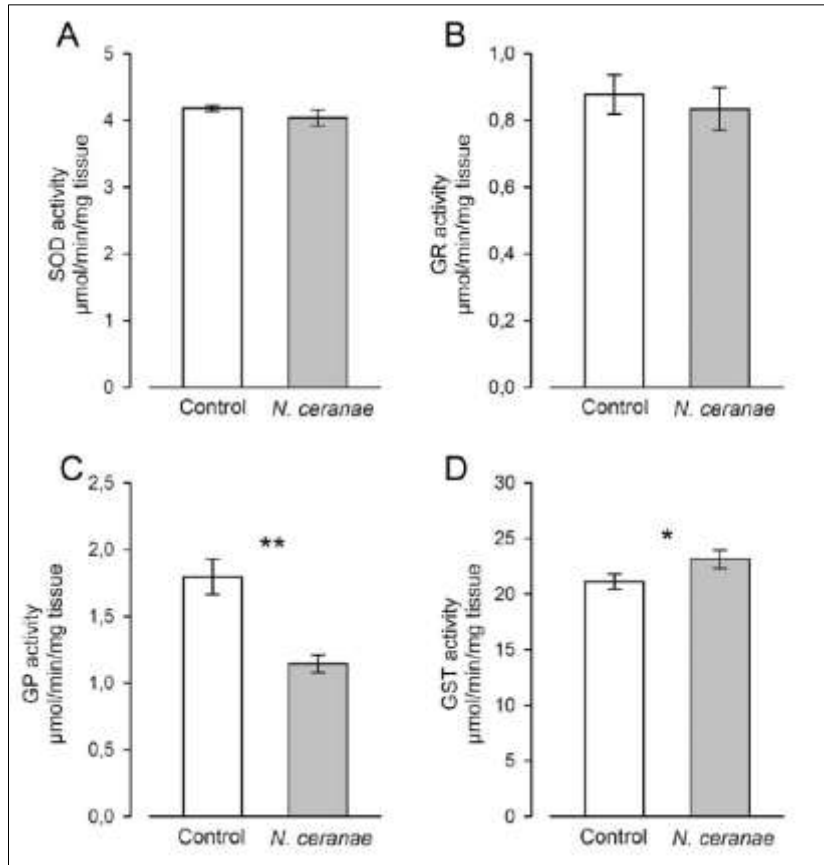
## **2.2. *Nosema ceranae* impairs cell signalling and tissue integrity in the midgut**

A significant number of genes involved in cell signaling (e.g. of GO terms: “positive regulation of cell communication”, “enzyme linked receptor protein signaling pathways”, “transmembrane receptor protein tyrosine kinase signaling pathway”) was inhibited by the parasites (Table 1). Cell-cell communication enables cells to perceive and correctly respond to their environment during tissue development and repair or the regulation of tissue homeostasis. Accordingly, the function “tissue homeostasis” was downregulated in parasitized bees, as well as biological processes implicated in “morphogenesis of an epithelium” and “open tracheal system development” (Table 1).

The deregulation of “protein amino acid phosphorylation” by the parasite (Table 1) might be involved in the degeneration of the gut tissue. Since protein phosphorylation regulates many aspects of cell life, the modification of the phosphorylation states of intracellular proteins might be a cause or a consequence of the disease state [46]. These results suggest that the proliferation of *N. ceranae* caused a degeneration of the gut epithelium. Interestingly, the effect of *N. ceranae* on tissue morphogenesis and integrity at the molecular level was confirmed at the histological level (Fig. 3) as in a previous study [2]. The epithelial cells of infected bees showed major signs of degeneration, which are linked to the downregulation of biological process like “positive regulation of cell communication” and “tissue homeostasis and morphogenesis” (Table 1). Gut cells are usually renewed via the multiplication and differentiation of stem cells in the basal cell layer that, once differentiated, move toward the lumen. In insects, this renewal of intestinal stem cells is controlled by the canonical Wnt signaling pathway, which includes the main downstream components *frizzled* and *armadillo* [47,48]. Our results indicated that four main genes (*frizzled2* (GB12765), *groucho* (GB13456), *basket* (GB16401) and *armadillo* (GB12463)) from this pathway were inhibited by the parasite, suggesting that *N. ceranae* development inhibited the self-renewal of intestinal cells of the host.

This finding might be surprising, since the reaction of the gut to microorganisms involved not only the activation of the immune system, but also integrated responses controlling self-renewal and differentiation of stem cells, essential to the gut tissue homeostasis [32]. However, similar results have been found with *Encephalitozoon* microsporidia that induces a disruption of the cell cycle of the host

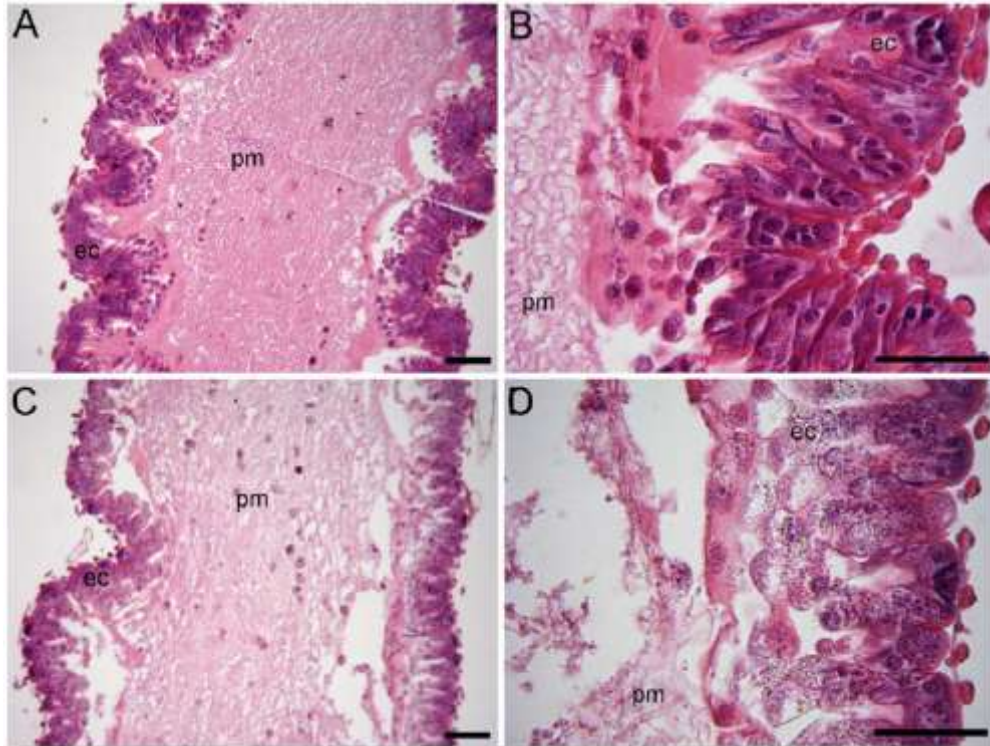
cells [49] but without killing the host. The dramatic lifespan reduction of bees parasitized by *N. ceranae* could likely be explained by greater changes in the host cell cycle as compared to changes induced by non-lethal microsporidian species.



**Figure 2.** Activity of antioxidant enzymes in the midguts of bees challenged by *N. ceranae*. Differences in enzymatic activity of A) superoxide dismutase (SOD), B) glutathione reductase (GR), C) glutathione peroxidase (GP) and D) glutathione-S-transferase (GST) were estimated by a Mann-Whitney U test. Means  $\pm$  SE are shown for 4 pools of 3 midguts per replicate ( $n = 3$  replicates, 36 bees total/treatment). \* and \*\* denote significant differences at  $p < 0.05$  and  $p < 0.01$ , respectively.

We explored whether genes affected by *N. ceranae* in the honey bee gut were connected through functional networks. The network analysis was performed by testing our gene list in GeneMania, which determines whether genes are connected through physical (protein) or genetic interactions based on a large set of functional association data [50]. We found that 34 out of the 336 genes affected by *N. ceranae* were connected within a single network, characterized by 63% of physical interaction and 37% of genetic interaction (Fig. 4, see Table S3 in “Annexe 3.4” for the GB name of honey bee genes). All of those genes from this network were downregulated by the parasite, which represents 25% of the total number of downregulated genes. Interestingly, most of the genes were involved in tissue and neuron development and included the 4 main genes of the Wnt signaling pathway (*frizzled2*, *groucho*, *basket* and *armadillo*), although we cannot exclude other effects of the

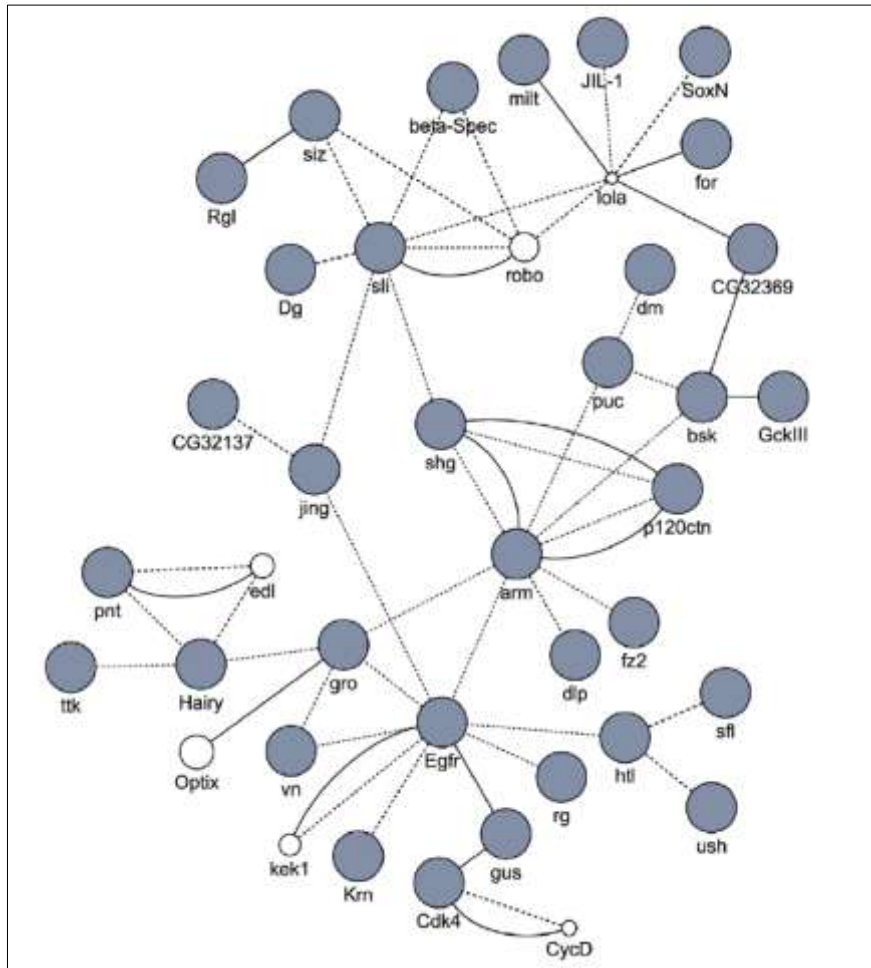
observed changes in expression of these genes, since a gene can be involved in different biological processes.



**Figure 3.** Histology of honey bee midguts 7 days post-infection. Light microscopy analysis of control (A, B) and *N. ceranae* infected midguts (C, D) stained with Hematoxylin-Eosin. In control guts, the peritrophic membrane (pm) and epithelial cells (ec) are homogenous, while in parasitized the guts peritrophic membrane and epithelial cells show signs of degeneration and lyses, respectively. Similar lesions were observed in each infected bees ( $n = 2$  bees per replicate and treatment, giving  $n=6$  bees per treatment). A) and C) x100, B) and D) x400. Scale bar: 10  $\mu\text{m}$ .

The microsporidian might therefore induce the degeneration of the epithelial cells of the bee gut through the inhibition of this network. The functional analysis also revealed a negative impact of the parasite on neuron development and differentiation and neuromuscular process (Table 1). In insects, the enteric nervous system (ENS) of the gut is composed of interconnected ganglia and nerve plexuses that contribute to the regulation of feeding and swallowing and gut peristalsis and metabolism [51]. The impairment of the ENS is further confirmed by the inhibition of some genes involved in the circulation of  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  (*Ca<sup>2+</sup>-channel-protein-b-subunit* (GB17403), *Calcium ATPase at 60A* (GB17876), *Na pump a subunit* (GB20055), *Na<sup>+</sup>-driven anion exchanger 1* (GB19698)) that are important for neuromuscular transmission in insects [52,53]. In particular mutation of the *Na pump a subunit* causes a pronounced neurodegeneration in the nervous system and reduces life span [52,54]. Therefore, our results showed that the pathology induced by the microsporidia development is characterized by an impairment of both the epithelium and the ENS of

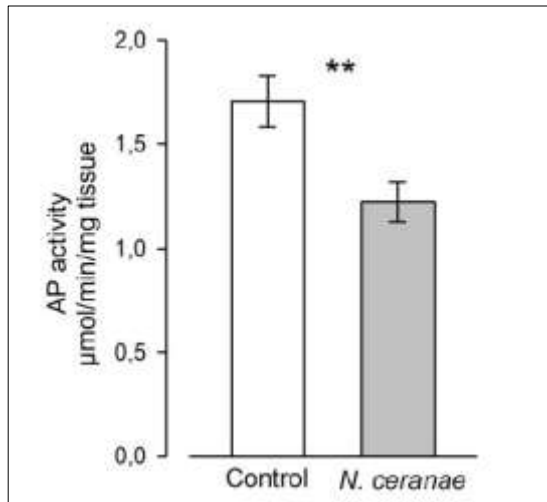
the gut. It is also noteworthy that the corticotrophin-releasing hormone (CRH)- binding protein, which is highly conserved between insects and vertebrates [55] was upregulated in parasitized bees (Table S1 in “Annexe 3.2”). In mammals, the CRH plays an important role in mediating stress responses in the gut (i.e. increase in motility, transit, defecation, diarrhea) [56]. The upregulation of this gene might thus represent a stress response to the *N. ceranae* infection.



**Figure 4.** Network of genes downregulated by *Nosema* in the bee gut. The composite functional association network derived from different genomic and proteomic data sources was generated with GeneMania using the *Drosophila* orthologs of bee genes. Physical and genetic interactions between genes are indicated by dot and solid lines, respectively. Grey and white circles represent implemented genes (known genes affected by *Nosema*) and new genes predicted to be functionally associated to the known genes, respectively. The size of the predicted gene circle provides an indication of its interaction score. Except *ETS-domain lacking (edl)*, the predicted genes *roundabout (robo)*, *longitudinal (lola)*, *Cyclin D (CycD)*, *Optix*, *kekkon-1 (kek1)* had bee orthologs: GB17658, GB12094, GB14028, GB16761 and GB17490.

Finally, to further understand the pathological impact of the parasite, we measured the activity of the alkaline phosphatase (AP). Its biological role in insect gut is not well known. However, in mammals, the activity of the AP plays a pivotal role in gut health [38] since it is involved in the regulation of nutrient absorption [57], the detoxification of bacterial lipopolysaccharide [58], prevents

bacterial invasion [59] and effectively reduces intestinal inflammation caused by bacteria [60]. In addition, there are numerous structural and functional homologies between insect and mammal APs [37]. Here, its activity was significantly decreased by *N. ceranae* (Fig. 5, Mann-Whitney U test:  $p = 0.007$ ), suggesting a reduction of gut protection or health. Similarly, Antúnez *et al.* [9] found that this microsporidian induces an immune suppression in bees, which would affect the host susceptibility to others pathogens.



**Figure 5.** Activity of alkaline phosphatase (AP) in the midguts of bees challenged by *N. ceranae*. Means  $\pm$  SE are shown for 4 pools of 3 midguts per replicate ( $n = 3$  replicates, 36 bees total/treatment). \*\* denotes significant differences at  $p < 0.01$  using a Mann-Whitney U test.

### 2.3. *Nosema ceranae* increases sugar metabolism in the midgut

The  $\alpha$ -glucosidase gene (GB19017), which is involved in sugar metabolism, was upregulated in infected bees (Table S1 in “Annexe 3.2”). This protein hydrolyzes sucrose from the flower nectar to glucose and fructose in the hypopharyngeal glands [61,62] suggesting an increase in sucrose breakdown in the midgut. Since carbohydrates represent the main source of energy, specialized transporter proteins are needed to transport these molecules across the plasma membrane of cells. This was confirmed by a higher transcription of genes that might be involved in the transport of trehalose (glucose-glucose), the main major carbohydrate energy storage molecule in insects (GB12932: *facilitated trehalose transporter Tret1-like*, GB13688: *trehalose transporter 1* and GB17752: *facilitated trehalose transporter Tret1-like*, Table 1 and Table S1 in “Annexe 3.2”). The increase of sugar metabolism is not yet clear, but previous studies clearly showed that *N. ceranae* infected bees have a higher sugar demand and consumption [11,25,63,64]. This observation might be explained by the fact that microsporidia, including *N. ceranae*, are usually amitochondriate, and thus have a high dependency on host ATP [65,66], especially for their germination. This dependence of microsporidia on host energy likely causes the increase in sucrose needs in bees, which would stimulate the expression of sugar transporter and  $\alpha$ -glucosidase. However, we cannot exclude that the increase in

sugar metabolism is an energy requirement for the bee gut epithelium to enhance the production of ROS in response to infection (see above).

#### 2.4. Expression of *Nosema ceranae* and other microorganisms' genes

Since the tiling arrays were spotted with *N. ceranae* probes based on gene predictions and annotation from the microsporidia genome sequencing project [66] but also with gene sequences of other microorganisms, we could measure in parallel the transcriptome of the parasite and other microorganisms. In order to consider a gene to be significantly expressed we took into account the median value of all the probe fluorescence signals in a gene, but due to the weakness of the fluorescence signal for most of the probes, we did not find any microsporidia genes to be expressed in infected bees. However, the signal intensities of 174 single probes (representing 81 genes) were more highly expressed in infected bees (Table S4 in “Annexe 3.5”). Therefore, only a small fraction of the parasite genome was found to be expressed on the array, possibly representing actively transcribed and/or the most highly expressed *N. ceranae* genes at this moment of its life cycle. This is rather low in comparison to the 2,614 protein-coding genes found in the parasite genome [66], but it might be possible that RNA from spores could not be isolated due to spore wall protection, which could lead to poor hybridization and weak signal on the arrays. However, during the germination of spores, the rigid wall ruptures, allowing the polar filament to infect the cytoplasm of host cells. Alternatively, the observed signal might come from few spores that were actively germinating, since the spore count level off around 8 days post-infection [13,67]. By conducting an Expressed Sequenced Tags (EST) survey of the microsporidian *Encephalitozoon cuniculi*, a parasite of the mosquito *Edhazardia aedis*, Gill *et al.* [68] also identify a small number of transcripts (133 unique genes).

We then checked whether the populations of other microorganisms were affected by *N. ceranae* infection. Only one gene of the bacteria *Paenibacillus larvae* (Plarl\_010100002528,  $M = 0.48$ ,  $q = 0.099$ ), three genes of the fungus *Ascospaera Apis* (2 upregulated: *Ascospaera\_26355*,  $M = 0.52$ ,  $q = 0.097$ ; *Ascospaera\_07711*,  $M = 0.56$ ,  $q = 0.052$ ; 1 downregulated: *Ascospaera\_15598*,  $M = -0.52$ ,  $q = 0.094$ ) and the *Lactobacillus* sp. 16S rRNA (EF032161:  $M = 0.66$ ,  $q = 0.05$ ) were differentially expressed between infected and control bees. Therefore we could not really draw conclusion on changes of the microorganism population. The differential expression of bacterial RNA that is usually nonpolyadenylated might be surprising at first sight, since for the microarray analysis the RNA was amplified with a reversetranscription reaction using an initial dT-priming step. However, studies have long reported the presence or the differential expression of RNA believed to be non-polyadenylated, like rRNA, in cDNA libraries (for a review, see [69]) and cDNA microarray analysis relying on a polyadenylated tail reverse-transcription reaction (see [70,71]), respectively, which suggest that polyadenylation has a functional role those RNA. This phenomenon could be explained

by the fact that in prokaryotes and organelles both mRNAs and non-coding RNAs can be polyadenylated [72]. In those systems, polyadenylation has a functional role and is wellknown to promote RNA decay by the degradosome [72,73]. Further experiments are needed to better characterize the *N. ceranae* gene expression and the gut microorganism modifications in infected bees. Finally, we did not find any effects on virus infection but recently, a negative correlation between *N. ceranae* spore loads and deformed wing virus infection was found in the bee midgut [74].

### 3. Conclusions

In summary, we captured the molecular events defining the bee gut response to *N. ceranae* infection, notably characterized at the gene expression level by the generation of oxidative stress. This gut immune response previously identified in *Drosophila* appears thus to be a more general phenomenon in insects. However, this mechanism does not seem to be sufficient for preventing bee mortality. Tissue degeneration and cell renewal impairment induced by infection would be two of the main factors leading to serious mortality during continuous intestine-*Nosema* infection. These pathological effects were captured late after the initial infection, while bees were dying, which gave some clues about the factors that caused the death of bees. However, future experiments will also benefit from similar analysis at the beginning of the infection or while the spores are proliferating in order to develop a complete picture of *N. ceranae* pathology. In sum, the honey bee gut is an interesting model system for studying host-pathogen relationship; and since both *N. ceranae* and *N. apis* are cross-infective across hosts in both *A. cerana* and *A. mellifera* [75,76], cross-comparison of molecular events during infection would provide great insights into the evolution of host-microsporidia interactions.

### 4. Materials and Methods

#### 4.1 *Nosema ceranae* infection and bee rearing

In order to determine the transcriptomic and enzymatic changes induced by *N. ceranae* in the midgut of honey bees, we performed experimental infections. They were performed at the Regional Apicultural Center in Marchamalo, Central Spain, with *A. m. iberiensis*. Frames of capped brood were obtained from healthy colonies located 20 km away from CAR to provide a supply of newly emerged honey bees free of *Nosema* for all trials (*Nosema*-free honey bees confirmed by PCR following method from Martín-Hernández *et al.* [77]). After emergence bees were kept in an incubator at 33°C ( $\pm 1^\circ\text{C}$ ) (Memmert Mod. IPP500) until they were 7-day old. Then, bees were starved for 2 hours and then anesthetized with CO<sub>2</sub> to facilitate individual feeding with 2  $\mu\text{l}$  of 50% sucrose solution containing 100,000 fresh spores of *N. ceranae*. This dose, currently found in naturally infected bees



[78] and ten times higher than the minimal infectious dose required to infect all the bees [13], was chosen to guarantee a successful infection of each bee and reduce the variability in infection between bees. Purified spores of *N. ceranae* were obtained from experimentally infected honey bees and the spore concentration was calculated using haemocytometer chamber [2]. Controls were fed only with the sucrose solution. After that, bees were introduced in cages and reared in two separated incubators at 33°C ( $\pm 1^\circ\text{C}$ ), one containing *N. ceranae* infected bees and the other one containing non-infected bees in order to avoid cross contamination. They were fed *ad libitum* with water, a solution of sucrose (50% w/w in water) and 2% Promotor L (Calier Laboratory), a commercial mixture of amino acids and vitamins.

Three experiments consisting of control and *N. ceranae*-infected groups were performed to assay the effect of the microsporidia on mortality (30 bees per cage), transcriptomic (20 bees) and enzymatic activity (50 bees). Each experiment was repeated 3 times (one repetition corresponding to one cage) on 3 colonies randomly distributed within the apiary in order to avoid any bias due to colony genetics and to analyze the “general” host/pathogen interactions. In the mortality assay, dead bees were counted daily and the mortality experiment ended when all bees infected with *N. ceranae* were dead; results were compared using a log-rank test. For the transcriptome and enzymatic activity analysis of the nosemosis type C, bees were flash frozen in liquid nitrogen and store at  $-80^\circ\text{C}$  seven days post-infection, just before the infection level (spore count) reaches a plateau [13,67]. At this time *N. ceranae* infection is largely developed in the midgut, while bees are still alive [2]. A few days later, bees died *en masse* (Fig. 1).

#### 4.2. RNA labeling, array hybridization and statistical analysis

Gene expression was analyzed in honey bee midguts. Twelve bees per group were treated with RNA later Ice (Ambion) following kit instructions and midguts were dissected on dry ice. Pools of four midguts were homogenized in Trizol (Invitrogen). Whole infected midguts containing *Nosema ceranae* or control midguts were homogenized with a hand motor-driven grinder (Kondes) and RNA extraction was carried out as indicated in the Qiagen RNeasy kit (Qiagen) for total RNA with on-column DNase I treatment (Qiagen). For each group, RNA isolated from three samples was equally pooled giving a larger pool of 12 midguts that was used for microarray analysis. Three pools of *Nosema* infected bees were then directly compared to three control pools (one pool of each per colony) using a set of four custom NimbleGen HD2 tiling microarrays containing sequences from the following draft genomes (Table S5, *see online version doi:10.1371/journal.pone.0037017*): the honey bee *A. mellifera* version Amel\_4.0, NCBI AADG000000000 interrogating 9,295 annotated gene models and 12,581 transcripts [27]; the bacterium *Paenibacillus larvae*, NCBI AARF000000000 interrogating 4,718 of 5,019 annotated genes [79]; the filamentous fungus *Ascospaera apis*, NCBI

AARF00000000 [79]; the microsporidian *Nosema ceranae*, NCBI ACOL00000000 interrogating 2,295 of 2,678 annotated genes [66]; and 10 viruses including *Varroa destructor* virus 1, NCBI AY251269 [80], Deformed wing virus, NCBI NC\_004830 [81], Sacbrood virus, NCBI NC\_002066 [82], *Kakugo* virus, NCBI NC\_005876 [83], Chronic bee paralysis virus RNA 1, NCBI NC\_010711 [84], Chronic bee paralysis virus RNA 2, NCBI NC\_010712 [84], Kashmir bee virus, NCBI NC\_004807 [85], Black queen cell virus, NCBI NC\_003784 [86], Israel acute paralysis virus of bees, NCBI NC\_009025 [87], and Acute bee paralysis virus, NCBI NC\_002548 [88] and 16 s ribosomal RNA sequences from different bacteria, including probiotics (Table S5 for a list, *see online version doi:10.1371/journal.pone.0037017*). Note that only 1,540 out of 9,244 unmapped *A. mellifera* sequences are represented on the microarrays, those being the first 1540 sequences of the “GroupUn” set from assembly 4.0. The probes on the microarrays range in size between 50 to 60 bp, and are tiled across the unique sequences of genomes at median distances of 25 bp (mean = 33 bp); only 76,315 probes are tiled at distances >100 bp and 4,285 probes are tiled at distances > 1000 bp. The microarray platform is deposited at NCBI GEO under the MIAME-compliant accessions GPL11147, GPL11148, GPL11149, GPL11150.

Total RNA concentration and integrity was determined using the Nanodrop ND-1000 (ThermoScientific) and the Bioanalyzer 2100 with the RNA6000 Nano Kit (Agilent Technologies), respectively. Total RNA was primed with oligo-dT-T7 primer and converted to amplified RNA using MessageAmpII aRNA Amplification Kit (Ambion) according to the manufacturer’s recommendations. Then, amplified RNA was primed with Random hexamer Primer (Promega) and converted to double stranded (ds) cDNA using Double-stranded cDNA Synthesis kit (Invitrogen). After treating samples with RNaseA, ds cDNA was purified with ChargeSwitch PCR Clean-Up Kit (Invitrogen). Next, ds cDNA (1000 ng) was labeled using NimbleGen Dual Color Labeling Kit (Roche NimbleGen) in triplicate following the kit’s instructions to produce enough labeled product for 3x2.1 M microarray chips. Hybridization and post-hybridization washing was performed using Hybridization Kit (Roche NimbleGen) and Wash Buffer Kit (Roche NimbleGen) according to manufacturer’s recommendations.

Arrays were scanned using an Axon GenePix 4200A Professional scanner (Molecular Devices, Sunnyvale, CA), 5 micron resolution and images analyzed with NimbleScan 2.5 Software (Roche NimbleGen) and raw signal intensities extracted as PAIR files. First, replicate arrays were quantile normalized [89] and to each probe the median value of the replicate probe values was assigned. The fluorescence signal of random probes, designed to reflect the genome nucleotide composition by Markov modeling, was used to determine a false positive rate threshold. Probes were considered positive if their fluorescence signal was higher than the 99th percentile of the fluorescence signal of the random probes. The data analysis to measure differential expression of genes was

performed using the statistical software package R (R-Project 2009) [90] and Bioconductor [91] with additions and modifications. The signal distributions across chips, samples and replicates were adjusted to be equal according to the mean fluorescence of the random probes on each array. All probes including random probes were quantile-normalized across replicates. Expression level scores were assigned for each predicted gene based on the median log<sub>2</sub> fluorescence over background intensity of probes falling within the exon boundaries. We calculated the per-gene, per-treatment differential expression (DE) levels with LIMMA R package [92] producing M, A expression estimates, t-statistic, probability, and probability adjusted for multiple testing by using the Benjamini-Hochberg method. The data are deposited at NCBI GEO under the MIAME-compliant accession GSE25455.

#### **4.3. Functional analysis**

We tested whether molecular functions or biological processes from the list of genes differentially expressed after *N. ceranae* parasitism were represented by larger numbers of genes than expected by random. *Drosophila melanogaster* orthologs were identified by reciprocal best BLASTX match to honey bee genes, and Gene Ontology (GO) terms were assigned based on annotation of *Drosophila* genes. Then, DAVID 6.7 bioinformatic resources was used to identify overrepresented terms (molecular function and biological process) [93].

#### **4.4. Network analysis**

Network analysis was performed by analyzing all differentially expressed genes in GeneMania, which uses a large set of functional association data including protein and genetic interactions [50]. The algorithms underlying the bioinformatic tools consists of two parts: 1) a linear regression-based algorithm that calculates a single composite functional association network from multiple data sources and 2) a propagation algorithm for predicting gene function given the composite functional association network. This principle enable to predict new genes functionally associated with known genes (see [50] for details). In GeneMania, *Drosophila* was selected and the physical (protein-protein interaction data) and genetic (genetic interaction data) network options were enabled. See GeneMania website for detailed instructions. Cytoscape (version 2.7.0) was used to display the networks.

#### **4.5. Verification by reverse-transcriptase PCR**

Confirmation of some of the results obtained from tiling array analysis was performed with quantitative PCR (qPCR) on selected genes affected by *N. ceranae* parasitism. The qPCR analysis was carried out with 6 RNA pools of 4 bees per treatment, which included the RNA stocks used for tiling array analysis. For cDNA synthesis, 500 ng of RNA per sample was reverse-transcribed using the SuperScript III kit (Invitrogen, France). The transcript abundance was measured for the transcription

factor *Hairy* [94]), *Slit*, which is involved in neuron differentiation [95] and  $\alpha$ -glucosidase [96] with an Mx3000P QPCR Systems (Agilent) and the SYBR green detection method (Agilent). qPCR values of the selected genes were normalized to the housekeeping gene *eIF3-S8*, that did not vary in expression levels on the tiling array ( $q$ -value = 0.99). Relative expression was calculated by raising 2 to the power of the difference in Ct values. Primer sequences (5' to 3') were: *Hairy* (GB14857) forward: CCAGCGCGACACTCGAAGCT, reverse: AAACCTGCCAACCTCGCCGG; *Slit* (GB19929) forward: AGGCATCACGCGGAGAACGC, reverse: CGGCGGGCAACCGAGTATCC;  $\alpha$ -glucosidase (GB19017) forward: TTGCTGCCAGGTGTTGCCGT, reverse: TTGGAATGGCGTTCTCGCGGG; *eIF3-S8* (GB12747) forward: TGAGTGTCTGCTATGGATTGC, reverse: TCGCGGCTCGTGGTA from [97]. Results were consistent with the microarray results (Fig. S1 in “Annexe 3.1”).

#### 4.6. Enzyme activity analysis

The enzymatic activity of superoxide dismutase, glutathione reductase, glutathione peroxydase, glutathione-S-transferase and alkaline phosphatase was measured at day 7 post infection. Measures were carried out on honey bee midguts that were dissected and stored at -80°C until analysis. All analysis were done with 3 replicates/treatment and using 4 pools of 3 midguts per replicate. The effect of *N. ceranae* parasitism on enzyme activities was assayed using Mann-Whitney U tests.

*Enzyme extraction.* Samples were homogenized at 4°C with a TissueLyser (Qiagen) (5x10 s at 30 Hz) in phosphate buffer pH 7.4 (40 mM sodium phosphate, 10 mM NaCl, 1% (w/v) Triton X-100, containing a mixture of 2 mg/ml of antipain, leupeptin and pepstatin A, 25 units/ml of aprotinin and 0.1 mg/ml of trypsin inhibitor) to make a 10% (w/v) extract. The homogenates were then centrifuged at 15,000 g for 20 min at 4°C and resulting supernatants were used for further analysis of enzymatic activities and protein contents.

*Superoxide dismutase.* SOD activity was measured indirectly as the rate of reduction of nitroblue tetrazolium when superoxide anion radical was generated during oxidation of xanthine by xanthine oxidase as described by Boldyrev *et al.* [98]. The reaction mixture contained 50 mM sodium carbonate dissolved in 50 mM phosphate K/Na buffer (pH 7.8 at 25°C), 0.1 mM EDTA, 0.1 mM xanthine, 0.0833 U/ml xanthine oxidase and 0.025 mM nitroblue tetrazolium. The enzyme activity was assayed at 560 nm for 5 min at 25°C.

*Glutathione reductase.* Glutathione reductase activity was measured in a reaction mixture containing 50 mM Phosphate Na/K buffer (pH 7.4 at 25°C), 1 mM EDTA, 0.16 mM NADPH and 0.8 mM

oxidized glutathione (substrate) as described by Boldyrev *et al.* [98]. The enzyme activity was assayed at 340 nm for 5 min at 25°C.

*Glutathione peroxidase.* Glutathione peroxidase activity was monitored as described by Boldyrev *et al.* [98] at 340 nm in a reaction mixture containing 50 mM phosphate K/Na buffer (pH 7.8), 1 mM EDTA, 0.12 mM NADPH, 0.85 mM reduced glutathione as a substrate, 0.5 unit/ml glutathione reductase and 0.2 mM tert-butyl hydroperoxide. The reaction was followed for 10 min at 25°C.

*Glutathione-S-transferase.* Glutathione-S-transferase activity was monitored by following the conjugation of reduced glutathione to 1-chloro-2,4-dinitrobenzene using a method adapted from Habig *et al.* [99]. GST activity was measured by adding enzymatic extract to the reaction mixture containing 1 mM EDTA, 2.5 mM reduced glutathione, 1 mM 1-chloro-2,4-dinitrobenzene and 100 mM Na/K-phosphate pH 7.4. GST activity was followed spectrophotometrically at 340 nm during 5 min at 25°C.

*Alkaline phosphatase.* AP activity was monitored at 410 nm in a medium containing 20 µM of MgCl<sub>2</sub>, 2 mM of *p*-nitrophenyl phosphate as a substrate and 100 mM Tris-HCl pH 8.5 [100]. The enzyme activity was assayed for 5 min at 25°C.

#### **4.7. Histology**

We also checked the tissue integrity of 2 bees per replicate and treatment, giving a total of n=6 bees per treatment. Midguts from infected and non-infected honey bees were fixed in formalin-acetic-alcohol 70° (5/5/90) for 24 hrs at 5–6°C, then rinsed 3x1 hr in water, dehydrated in ethanol and stored in butanol. Tissues were embedded in paraffin (Histowax, Histolab - Products AB) and sectioned at 7 µm thickness with a microtome (Leitz 1512). The following sections were stained with Hematoxylin-Eosin and photographed using a light microscope VWR (TR500 High End Tri).

#### **Acknowledgments**

We thank Javier Almagro, Jesús García for their assistance with bee rearing; Almudena Cepero, Virginia Albendea, Carmen Abascal, Carmen Rogerio and Teresa Corrales for assistance in laboratory experiments, and members of the INRA lab for comments that improve the manuscript. We also thank Jay Evans (USDA-ARS Bee Research Lab), Daniel Weaver (Bee Power, LP) and Kimberly Walker (Roche NimbleGen Inc.) for guidance on the tiling microarray design.

**Author Contributions**

Conceived and designed the experiments: CD YLC CA. Performed the experiments: CD JLB MH JL RMH CB MC MB. Analyzed the data: CD JLB JKC JHC CM CA. Contributed reagents/materials/analysis tools: MH JKC LPB YLC. Wrote the paper: CD JLB RFAM YLC CA.

**Funding**

This work was co-funded by a BEEDOC grant (FP7, RTD REG/E.4(2009)D/561221), grants from INRA Santé des Plantes et de l'Environnement department (AAP SPE 2010) and Junta de Comunidades de Castilla-La Mancha (Consejería de Agricultura, Medio Rural y Marino, Programa Nacional Apícola 2011-2013). C. Dussaubat was supported by a CONICYT/French Embassy of Chile grant and a COLOSS short-term mission (COST Action FA0803), R. Martín-Hernández by the Instituto de Recursos Humanos para la Ciencia y la Tecnología (Fundación Parque Científico y Tecnológico de Albaceta, Spain), CM by the BEEDOC grant (FP7, RTD REG/E.4(2009)D/561221) and C. Alaux by an INRA young researcher position (INRA Santé des Plantes et de l'Environnement department). Work at the Center for Genomics and Bioinformatics was supported in part by the METACyt Initiative of Indiana University, funded in part through a major grant from the Lilly Endowment, Inc. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Supporting Information - Annexe 3**

- Annexe 3.1:** **Figure S1.** Validation of microarray results with qPCRs.
- Annexe 3.2:** **Table S1.** List of honey bee genes differentially expressed after *N. ceranae* parasitism.
- Annexe 3.3:** **Table S2.** List of genes associated with each Gene Ontology category.
- Annexe 3.4:** **Table S3.** List of genes integrated within the gene network downregulated by *N. ceranae* parasitism.
- Annexe 3.5:** **Table S4.** List of *N. ceranae* probes significantly expressed.

**Note:**

For **Table S5** please refer to online version doi:10.1371/journal.pone.0037017.

## Synthèse

Au cours du développement de cette thèse nous avons d'abord constaté le potentiel de *N. ceranae* à induire des changements dans le comportement de butinage associé à la production d'une phéromone modificatrice, l'EO.

Dans une deuxième partie, notre recherche s'est intéressée à la compréhension des mécanismes cellulaires activés dans une étape avancée de l'infection. Nous avons mis en évidence des changements de niveau de l'expression de gènes dans l'intestin de l'abeille qui donnent lieu à la générésence d'un état de stress oxydatif connu pour agir contre des microorganismes pathogènes. Néanmoins, la dégénération de l'épithélium et l'inhibition du renouvellement cellulaire, observés dans l'intestin via la sous expression à niveau moléculaire de voies métaboliques spécifiques, semblent surpasser les mécanismes de réponse à l'infection, ce qui pourrait induire la mort des abeilles.

Cependant, la problématique exposée tout au début de cette thèse nous avait montré la diversité des effets lorsqu'une colonie fait face à l'infection de *N. ceranae*. Ces effets vont d'une mortalité totale de la colonie, jusqu'au la présence du parasite sans être impliqué directement dans l'effondrement des colonies. L'existence de souches de *N. ceranae* avec différents degrés de virulence, des populations d'abeilles avec divers niveaux de tolérance ou des facteurs de l'environnement, pourraient être à la base de la diversité des effets observés.

La recherche décrite dans le prochain chapitre vise à contribuer à mieux comprendre cette diversité des réponses des colonies à *N. ceranae* observée dans différentes régions.





## **Chapitre 4**

# **Facteurs de variation des effets de *Nosema ceranae* chez l'abeille : une approche de la virulence**

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### **Présentation**

De nombreuses recherches scientifiques se sont intéressées au rôle de *N. ceranae* dans la mortalité des colonies d'abeilles dans différents pays, notamment parce que les effets du parasite ne sont pas consistants dans tous les cas. L'hypothèse des souches de *N. ceranae* portant différents degrés de virulence a été proposée par la communauté scientifique pour expliquer les différences de virulence rencontrées en Espagne par rapport à la France et aux pays du nord de l'Europe, mais n'a jamais été testée. Ainsi l'**hypothèse spécifique** de ce chapitre est que différents degrés de virulence des isolats de *N. ceranae*, provenant du centre de l'Espagne et du sud de la France, sont à la base de la diversité des effets observés dans ces deux régions.

Pour tester cette hypothèse nous avons comparé la virulence des deux isolats de *N. ceranae* de différentes origines géographiques chez *A. mellifera iberiensis*. Cette comparaison a été basée sur le taux de mortalité induit par les deux isolats chez des abeilles infectées au laboratoire et la charge de spores développée au cours de l'infection. Nous avons également conduit une étude permettant d'estimer la proximité génétique des deux isolats.



**Article n°4:**

**Comparative study of *Nosema ceranae* (Microsporidia) isolates  
from two different geographic origins**

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Journal of Veterinary Microbiology

doi:10.1016/j.vetmic.2012.09.012.

Version « .txt » du texte publié

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## Résumé

Le parasite intestinal de l'abeille *Nosema ceranae* (Microsporidia) est à la base de la perte de colonies dans quelques régions, tandis que dans d'autres sa présence ne cause pas directement de la mortalité. C'est le cas de l'Espagne et de la France respectivement. L'hypothèse que nous formulons vise à expliquer les différences entre les réponses de l'abeille à *N. ceranae* par une différence de degré de virulence de souches de *N. ceranae* provenant de différentes origines géographiques. Afin de tester cette hypothèse nous avons d'abord comparé la variabilité génétique d'un fragment de l'ADNr qui pouvait mettre en évidence des différences entre les deux isolats de *N. ceranae*, l'un provenant d'Espagne et l'autre provenant de la France. En parallèle, nous avons comparé la capacité d'infection des deux isolats de *N. ceranae* chez *Apis mellifera iberiensis*, basé sur les lésions anatomopathologiques conséquentes du développement de *N. ceranae* dans l'intestin. Nous avons comparé également la charge de spores dans l'intestin et le taux de survie des abeilles. Nos résultats suggèrent que les deux isolats de *N. ceranae* utilisés dans cette étude n'ont pas une origine génétique spécifique. Ces résultats sont en accord avec le développement de l'infection, la survie des abeilles et la charge de spores qui n'ont pas présenté de différences entre les abeilles infectées avec les deux isolats de *N. ceranae*. Il se peut que les variations dans les réponses des abeilles à l'infection dans les deux régions, ne soient pas dues aux différences entre les isolats de *N. ceranae*, mais soient plutôt liées au degré de tolérance des sous espèces ou hybrides locaux d'abeilles, ou aux conditions expérimentales (cas des essais conduits en laboratoire). Des recherches plus approfondies pourront aider à estimer la contribution de chaque facteur dans la réponse des abeilles à l'infection.

## Abstract

The intestinal honey bee parasite *Nosema ceranae* (Microsporidia) is at the root of colony losses in some regions while in others its presence causes no direct mortality. This is the case for Spain and France, respectively. It is hypothesized that differences in honey bee responses to *N. ceranae* infection could be due to the degree of virulence of *Nosema* strains from different geographic origins. To test this hypothesis, we first performed a study to compare the genetic variability of an rDNA fragment that could reveal differences between two *N. ceranae* isolates, one from Spain and one from France. Then we compared the infection capacity of both isolates in *Apis mellifera iberiensis*, based on the anatomopathological lesions due to *Nosema* development in the honey bee midgut, *Nosema* spore-load in the midgut and the honey bee survival rate. Our results suggest that there is no specific genetic background of the two *N. ceranae* isolates, from Spain or France, used in this study. These results agree with the infection development, honey bee survival and spore-loads that were similar between honey bees infected with both *N. ceranae* isolates. Probably, differences in honey bee response to infection are more related to the degree of tolerance of honey bee subspecies or local hybrids to *N. ceranae*, or experimental conditions in the case of laboratory trials, than to differences between *N. ceranae* isolates. Further studies should be done to estimate the contribution of each of these factors on the response of the honey bees to infection.

## 1. Introduction

*Nosema* spp. are obligate unicellular parasites that belong to the phylum Microspora. They are characterized by the production of a resistant spore that contains a polar filament which serves to transmit the genetic material to the host cell (Wittner and Weiss, 1999). Microsporidiosis of adult honey bees caused by *Nosema apis* and *Nosema ceranae* is a common worldwide disease with both, a direct negative impact on colony strength and productivity (Fries, 1988; Higes *et al.*, 2010a; Mayack and Naug, 2009) and an indirect effect by interacting with other environmental stressors weakening colony health (Alaux *et al.*, 2010b; Pettis *et al.* 2012; vanEgelsdorp *et al.*, 2009). While it has been known for a century that *N. apis* infects the European honey bee, *N. ceranae* was first isolated in 1996 in the Asian honey bee *Apis ceranae* (Fries *et al.*, 1996) and recently detected in the European honey bee *Apis mellifera* in 2005 (Higes *et al.*, 2006; Huang *et al.*, 2007). Among the factors related to *Nosema* pathology, the existence of different *N. ceranae* isolates from distant geographic areas (Chen *et al.*, 2009) that exhibit different degrees of virulence may explain the differences in the response of the honey bee to infection (Genersch, 2010). Contradictory results on *N. ceranae* virulence have been

obtained in a large number of laboratory experiments studying *N. ceranae* effects (Forsgren and Fries, 2010; Higes *et al.*, 2007; Martín-Hernández *et al.*, 2009; Suwannapong *et al.*, 2010) as well as in field surveys of commercial apiaries (vanEgelsdorp *et al.*, 2009; Higes *et al.*, 2010b).

More precisely, there are apparent differences in honey bee mortality due to *Nosema* spp. between Spain and France. A large study carried out between 2002 and 2006 showed the presence of *Nosema* spp. in France in 50 to 79% of the surveyed apiaries from different regions (Chauzat *et al.*, 2010a, 2010b). *N. ceranae* was found to be predominant over *N. apis* (Chauzat *et al.*, 2007). In all surveyed years, the presence of the parasite was not significantly related to any acute mortality (Chauzat *et al.*, 2010a, 2010b), with the exception of apiaries from a region in France not considered in the previous study where *N. ceranae* appeared to have a central role in massive losses during 2005 – 2006 (Borneck *et al.*, 2010). In contrast, a study of the epidemiological factors involved in colony losses in different regions of Spain suggested that *N. ceranae* is a key factor in colony depopulation (Higes *et al.*, 2010b). *N. ceranae* was much more common than *N. apis* and it was present in 95% of the samples from depopulated colonies, compared to 5% of asymptomatic beehives (Higes *et al.*, 2010b). Laboratory experiments also showed a similar tendency. In France, experimental infections resulted in 10% and 50% bee mortality, 10 and 20 days post-infection respectively (Alaux *et al.*, 2010b; Vidau *et al.*, 2011); whereas in Spain, higher mortality has been observed over shorter study periods, the most dramatic being 100% of bee mortality 8 days post-infection (Higes *et al.*, 2007).

In consequence, we tested the hypothesis that different degrees of virulence of *N. ceranae* isolates from each country could explain this differential effect of *N. ceranae*. We compared the effects on the honey bee of two *N. ceranae* isolates, originating from the National Institute of Agricultural Research (INRA) of Avignon, South of France, and the Regional Apicultural Centre (CAR) in Central Spain. In order to avoid the confounding effects of methodology we used one honey bee subspecies (*Apis mellifera iberiensis*) and a single laboratory protocol. We carried out a genetic study of a variable fragment of rDNA, looking for similar sequences or characteristic fragments of both *N. ceranae* isolates that could define strains from two different geographic origins; at the same time, we performed cage experiments to compare the development of infection of both *N. ceranae* isolates in the honey bee through the observation of histopathological lesions in the midgut and the measurement of daily mortality and spore counts.

## 2. Materials and methods

In October 2010, naturally-infected forager honey bees from two colonies were collected from Central Spain (Regional Apicultural Center, CAR, at Marchamalo), and the South of France (National Institute of Agronomic Research, INRA, from Avignon), to obtain fresh *N. ceranae* spores to be used in experimental infections. *Nosema ceranae* from Spain are known to cause rapid mortality of honey bees (Higes *et al.*, 2008, 2010b), while *N. ceranae* from France have not been related to mass colony depopulation (Chauzat *et al.*, 2010a, 2010b).

Both sets of bee samples were collected from the entrance of the hive on the same day at the two different geographic locations and maintained at room temperature, whether in Spain, in France or during the transport of the French samples to the CAR laboratory in Spain where the infections were carried out.

### 2.1. Obtaining *N. ceranae* spores for experimental infection

Bee samples from both geographic locations obtained as described above, were processed in parallel, in exactly the same way, to obtain fresh mature spores as described in Botías *et al.* (2011). Briefly, the abdomens of all bees were homogenized in 25 ml H<sub>2</sub>O PCR grade for 2 min at high speed in a Stomacher® 80 Biomaster (Seward, West Sussex, UK) using strainer bags (BA6040/STR, Seward). The homogenate was recovered in a tube and 15 ml of H<sub>2</sub>O PCR grade was again added to the strainer bag to repeat the homogenization under the same conditions. Honey bee homogenates were centrifuged (6 min at 800g), the supernatant was discarded as the spores remained in the sediment. The pellet was resuspended in 1 ml of distilled water. To confirm the *Nosema* species of the spores, an aliquot of each homogenate was analyzed by PCR as previously described (Martín-Hernández *et al.*, 2011b) using 218MITOC FOR/218MITOC REV and 321APIS FOR/321APIS REV primers specific for *N. ceranae* or *N. apis* respectively and COI-F/COI-R primers for *A. mellifera* COI, as internal control of each reaction. All the PCR reactions were carried out in a Mastercycler® ep gradient S (Eppendorf®, Hamburg, Germany). Each PCR product was analyzed in a QIAxcel System (Qiagen, Hilden, Germany), using a QIAxcel DNA High Resolution Kit (Qiagen, No. 929002) to detect positive and negative reactions. Negative controls were analyzed in parallel to detect possible contaminations in all phases of this technique. Once the *N. ceranae* species were confirmed, the spores were purified with Percoll® to obtain fresh pure spores suspension for artificial infection. During extraction and before infection, spores were kept at room temperature. The spore number was counted using a hemocytometer chamber and a phase contrast microscope.

## 2.2. Genetic variability of an rDNA fragment from two *N. ceranae* isolates

Up until now no reliable genetic markers have been found to be suitable to distinguish between strains of *N. ceranae*. In fact, two genetic markers can produce different phylogenetic trees of the same sample, leading to incongruous results (Ironside, 2007). Because of this, we performed a study on the variability of an rDNA fragment between samples as in Sagastume *et al.* (2011), through the amplification by PCR and cloning of the product, to look for a decrease in variability that consequently occurs because of geographic isolation.

Total DNA was previously extracted from honey bee samples naturally infected by *N. ceranae* from Central Spain and South of France (see above). PCR was performed with the pair of primers named NOS3-UPPER (5'ACTGGCTTAACTTCGGAGAG 3') and NOS3-LOWER (5'AAGTAATACCGTTACCCGTCA 3') which amplify a 890 bp fragment that contains the intergenic spacer (IGS) and part of the small subunit (SSU) of rDNA. In order to ensure the reliability of our results, PCR was performed using a low initial DNA concentration and a high fidelity polymerase with proof-reading capability that works with an elongation time of one minute, enough to synthesize 890 bp in each cycle of PCR (Meyerhans *et al.*, 1990; Bradley and Hillis, 1997). PCR reactions started with 5 µL per tube of 1/10 diluted DNA, 0.4 µM of each nucleotide, 0.4 µM of each primer, 1.5 units of Expand High Fidelity Plus PCR System (Roche, cat no. 3300226) and its 5X buffer, 12 µg of BSA (Roche Diagnostic, cat no. 10711454001) per tube and sterilized distilled water for a final volume of 25 µl. PCR was performed in a Eppendorf Mastercycler EpGradient Pro S thermocycler with the following program: 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 57.2°C for 30 sec, and 68°C for 1 min, plus a final step at 68°C for 7 min. The PCR products were kept at 4°C, and 5 µL of each one were resolved on standard 2% agarose gels (Invitrogen E-GEL 2% Agarose GP, cat no. G8008-02) and visualized by ethidium bromide staining. 4 µL of each PCR product were cloned in *Escherichia coli* plasmid pCR2.1-TOPO® with TOPO TA Cloning® Kit (Invitrogen, cat no. K4500-01) following the manufacturer's instructions. From 4 to 6 clones were obtained per PCR product and the plasmid DNA was extracted and purified using the QIAprep Spin Miniprep Kit (Qiagen, cat no. 27106). A sample of each plasmid was digested with EcoRI (New England Biolabs, R0101S) and separated in 1% agarose gel electrophoresis in order to check the correct size of the insert. The inserts from the different clones were sequenced, using the commercial primers M13, at the Alcalá de Henares University, Unidad de Biología Molecular of the Faculty of Environmental Sciences, Spain.

Fourteen sequences from GenBank (<http://www.ncbi.nlm.nih.gov/nucleotide>) were used to compare our results (Table 1). All the sequences were aligned following the CLUSTAL W algorithm



(Thompson *et al.*, 1994) with the program BIOEDIT 7.0.5.2 (Hall, 1999), and the analysis of polymorphic sites and haplotypes generation were carried out with the program DNASP (Rozas *et al.*, 2003).

**Table 1.** *Nosema ceranae* DNA sequences from GenBank (GB). The GB sequences shown here are haplotypes that include clones from samples with different origins (Sagastume *et al.*, 2011).

GB accession	Haplotype characteristics
GU131055	Central Spain
GU131067	Central Spain
GU131109	Slovenia
GU131117	France
GU131110	Slovenia
GU131114	North Spain
GU131082	North Spain; Germany
GU131086	Germany
GU131090	Slovakia
GU131111	North Spain
GU131112	North Spain
GU131113	North Spain
GU131115	North Spain
GU131116	France

### 2.3. Experimental infection

Newly emerged honey bees of the local subspecies *A. m. iberiensis* were obtained as described in Higes *et al.* (2007) and Martín-Hernández *et al.* (2009) from a pool of three *N. ceranae* and *N. apis*-free honey bee colonies with queens naturally mated. Briefly, frames of sealed brood were kept in an incubator at  $34^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , and when new worker bees started to emerge, they were carefully removed and confined to cages in an incubator for five days at  $33^{\circ}\text{C} \pm 1^{\circ}\text{C}$  and fed ad libitum with sugar syrup composed of 50% sucrose solution and 2% Promotor L (Calier Lab) through an individual feeder attached to the cage. Three experimental groups of honey bees were assigned as follow: honey bees infected with *N. ceranae* from Avignon (South of France), honey bees infected with *N. ceranae* from Marchamalo (Central Spain) and a group of non-infected honey bees as control. Each group was composed of three replicates of 40 honey bees, 30 of them destined to follow mortality and the other 10 selected at random for optic and electronic microscopy preparations. The five-day-old bees were starved for 2 hours and then anesthetized with  $\text{CO}_2$  to be fed individually using a micropipette with 1  $\mu\text{l}$  of a spore solution containing 40 000 spores per bee. After infection honey bees were fed ad libitum

with the same sugar syrup from the beginning. Two similar incubators (Memmert® Mod. IPP500,  $\pm 0.1^\circ\text{C}$ ) were maintained at  $33^\circ\text{C}$ , one contained the *N. ceranae* infected honey bees and the other the uninfected honey bees.

#### **2.4. Histopathological study**

Two honey bees per cage were collected at random both at days 12 and 16 post-infection to be used for histology preparations as described in Higes *et al.* (2007) and Martín-Hernández *et al.* (2009). After dissecting out the alimentary canal, the ventriculus and attached Malpighian tubes were divided into sections and fixed in 10% buffered formalin for 24 hours. The tissues obtained were then embedded in paraffin, and 4  $\mu\text{m}$  thick sections were stained with haematoxylin-eosin to perform a complete histopathological study as described in Higes *et al.* (2007). For transmission electron microscopy (TEM) we utilized the method described in Higes *et al.* (2007) and Martín-Hernández *et al.* (2009). From each replicate cage the ventriculus and attached Malpighian tubes of two honey bees were processed. Tissue was prefixed in a 2% glutaraldehyde - 2.5% paraformaldehyde solution for a maximum of one hour, before it was washed three times in phosphate buffer (PBS, pH 7.4) and post-fixed in 1% osmium tetroxide at room temperature. After again washing in PBS and dehydrating with an ascending series of acetone, the ventriculus was embedded in Epon-Araldite resin. Semi-thin (0.5  $\mu\text{m}$ ) sections were cut with a Reichert-Joung ultracut E microtome, stained with 1% methylene blue in 4% sodium borate water, and observed with an Olympus Vanox AHB53 photomicroscope. After light microscopy selection of representative tissue areas, the Epon block was trimmed before ultrathin (60 nm) sections were obtained. For TEM studies, grids were double contrasted with 2% uranyl-acetate in water and lead citrate Reynolds solution for 10 min each, then examined and photographed with a Jeol 1010 electron microscope at an accelerating voltage of 80-100 kV.

#### **2.5. Mortality and spore counts**

Dead honey bees were counted and removed daily. To compare honey bee survival between groups we used a logrank test. Spores were counted in all dead bees of the infected cages as described in Martín-Hernández *et al.* (2009) and compared through time using ANCOVA.

### **3. Results**

#### **3.1 Genetic variability of an rDNA fragment from two *N. ceranae* isolates**

A total of 10 different sequences were obtained after cloning PCR products of the two *N. ceranae* isolates rDNA, 4 clones from INRA of Avignon, South of France, named “France 1, 2, 3, 4”, and 6 clones from CAR, Central Spain, named “Spain 1, 4, 5, 6, 7, 8” (GenBank accessions JQ595451 to

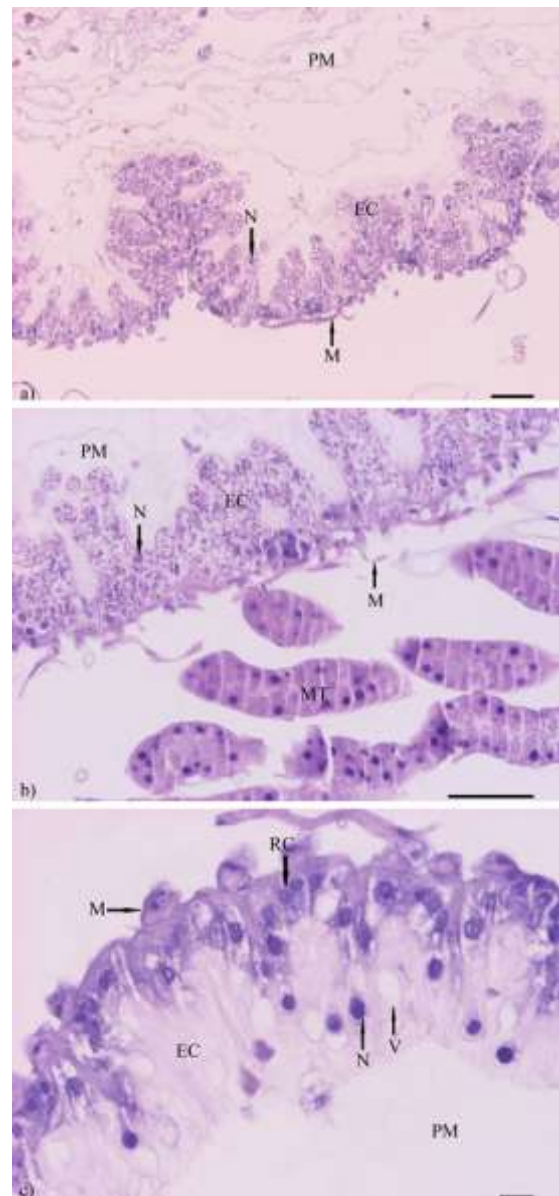
JQ595460). We compared them with 14 partial sequences from GenBank (Table 1). All the aligned sequences are shown in Table 2, in which we observed sequences with many polymorphic sites that appeared with different frequencies. Singletons were not discarded. Different sequences and variable sites were analyzed by haplotype generations. We did not find matching sequences, nor characteristic areas that define each sample. One haplotype per sequence was obtained and no evidence of common SNPs (Single Nucleotide Polymorphism) between clones from the same sample (France or Spain) was observed.



### 3.2. Histopathological study

#### 3.2.1. Light microscopy

Epithelial cell morphology in control bees showed no alterations due to methodology. There were no histological differences in the lesions produced by both *N. ceranae* groups, CAR and INRA of Avignon, in the honey bee midgut epithelium in samples taken at days 12 and 16 post-infection. At day 12 almost all the midgut was already infected and the degree of infection increased slightly until day 16. Either at the tips or at the bottom of epithelium folds there were cells that contained *N. ceranae* intracellular stages. As described previously by Higes *et al.* (2007), few unaltered epithelial cells were observed, while parasitized cells showed evidence of degeneration, such as extensive lyses and a perithrophic membrane that appeared broken and fragmented (Fig. 1).



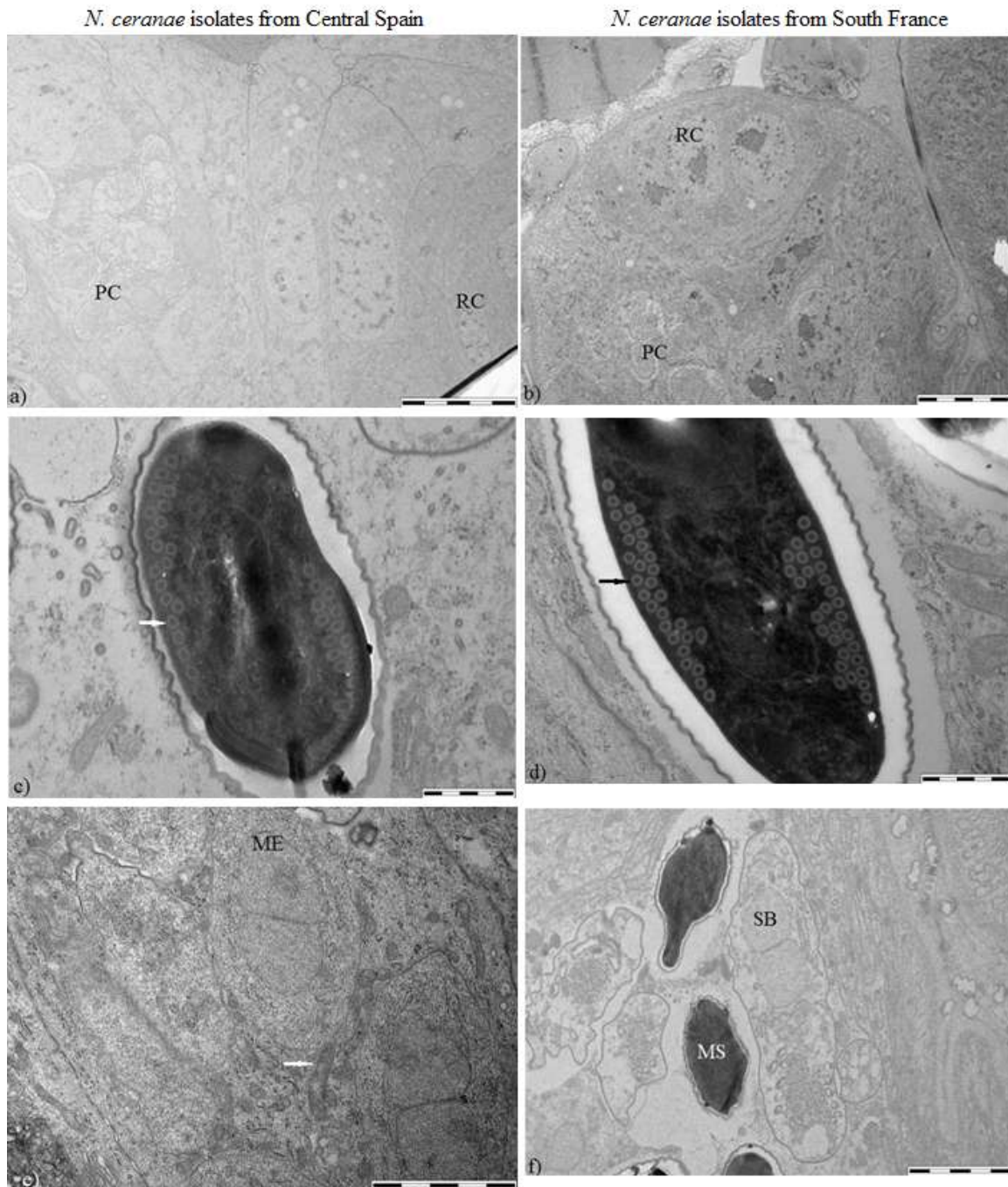
**Figure 1.** Midgut sections under light microscopy. At day 12<sup>th</sup> post-infection both *N. ceranae* infected groups showed similar lesions, a) *N. ceranae* from CAR (Central Spain) and b) from INRA of Avignon (South of France): infected midgut epithelial cells (EC) contained different *N. ceranae* stages and presented evidence of extensive lyses, the perithrophic membrane (PM) appeared broken and fragmented, the circular and longitudinal musculature (M) of the midgut was unchanged, no spores were found in the epithelium of Malpighian tubules (MT), small intestine or rectum (not shown in the picture); c) non-infected midgut epithelial cells with non-altered perithrophic membrane and intact cell morphology nucleus (N), vacuoles (V) and regenerative cells (RC). Bar scale: a) and b) 50  $\mu$ m, c) 10  $\mu$ m.

### 3.2.2. Electron microscopy

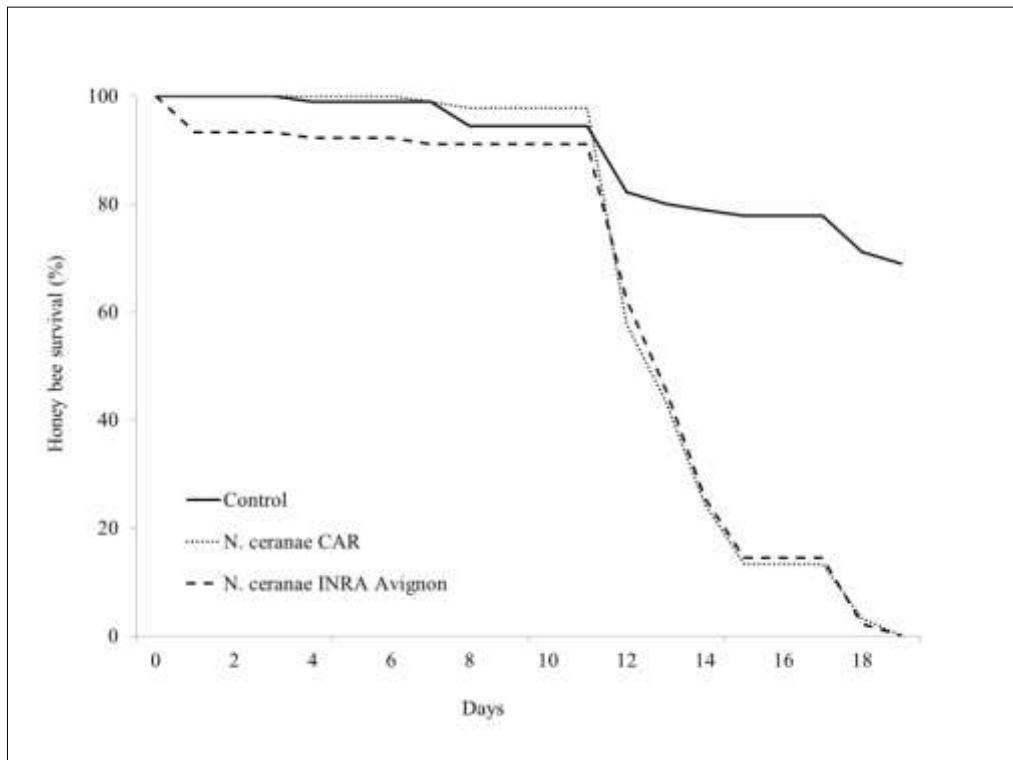
No differences were observed between spores or other *N. ceranae* stages developed from both isolates. At day 16 post-infection the majority of the epithelial cells were parasitized with the exception of regenerative cells in which it was not possible to distinguish any *N. ceranae* stage, however they were visible in the more developed neighboring cells. Sporoblasts and mature spores were observed in the cytoplasm; the mature spores showed the same number of coils of the polar filament, between 21 and 29, most frequently with coil numbers between 21 and 24 (Fig. 2). These observations are consistent with those of Higes *et al.* (2007, 2008) and García-Palencia *et al.* (2010), as they previously described infected cells as appearing enlarged with the nucleus apically displaced, the cytoplasm containing a larger number of mitochondria and showing evidence of degeneration, such as the presence of vacuoles and lysosomes, most of them secondary and irregularly shaped with heterogeneous electron-dense areas. All stages of *N. ceranae* showed diplokaryotic nuclei that were in direct contact with the host cell cytoplasm and no evidence of grouping or enclosure within a vacuolar membrane was evident. Immature and mature stages in invaginations of the nuclear membrane were also visible, and both empty spores and germinating spores were observed inside infected cells. A predominance of basophilic mature spores in the epithelial cells at the tips of the folds was observed, while, in contrast, cells at the bottom of the folds contained less basophilic but larger stages, indicating more cells parasitized by the vegetative stage.

### 3.3. Mortality and spores count

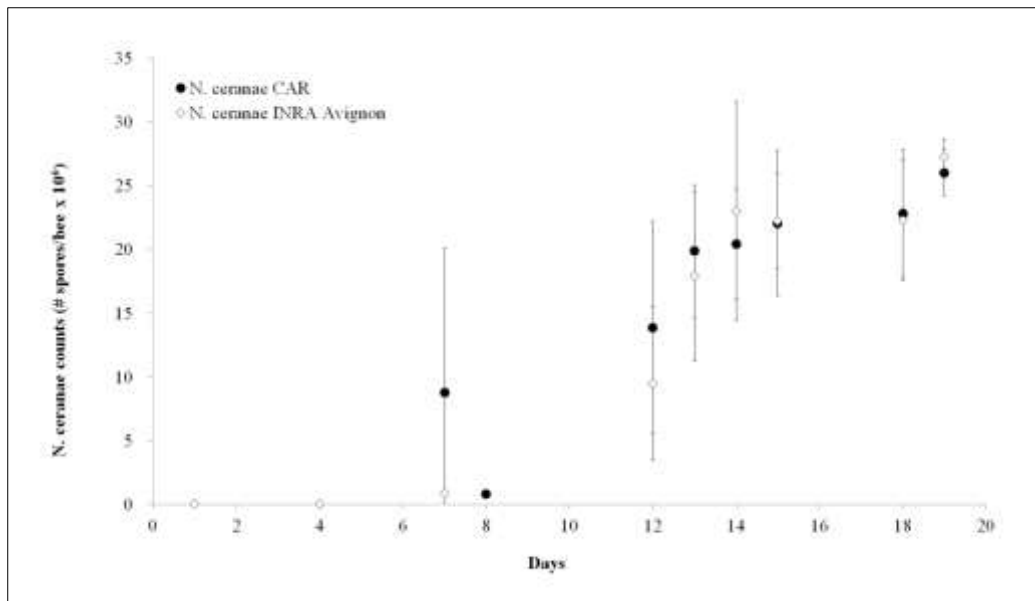
Survival curves (Fig. 3) were not significantly different between infected bees ( $X^2 = 3.346 \times 10^4$ ,  $p$ -value = 0.9854,  $N=90$ ), but they were significantly higher compared to controls ( $X^2 = 132.289$ ,  $p$ -value < 0.0001). There were no differences between cages within all groups with the exception of one cage from CAR (Central Spain) which showed a higher mortality than others. The difference in mortality started when infected honey bees achieved a mean spore count greater than  $9 \times 10^6$  per bee, and from that point, spore counts in dead bees increased with time from days 12 and 13 post-infection until all infected groups were dead by day 19 post-infection (Fig. 4). Spore count significantly increased with time ( $F$ -test = 81.792,  $p$ -value < 0.0001) but was not different between the two groups of *N. ceranae* ( $F$ -test = 0.001,  $p$ -value = 0.9771) with mean spore counts from CAR and INRA-Avignon of  $17.92 \times 10^6$  ( $\pm 7.78 \times 10^6$ ) spores/bee and  $16.12 \times 10^6$  ( $\pm 9.62 \times 10^6$ ) spores/bee, respectively. As expected throughout the study control bees were *Nosema*-negative.



**Figure 2.** Midgut sections under electron microscopy show similar effects in *N. ceranae* from CAR (Central Spain) and *N. ceranae* from INRA of Avignon (South of France) infected-bees: a) and b) not infected regenerative cells (RC) surrounded by other parasitized cells (PC); c) and d) mature spores showed same number of coils (arrow) of the polar filament, between 21 and 29, been more frequent to observe spores with coil number between 21 and 24, intracellular germination was observed in both cases; e) meronts (ME) surrounded by mitochondria (white arrow), f) sporoblasts (SB) and mature spores (MS) of a *N. ceranae* in the cytoplasm. Bar scale: a) and b) 5  $\mu$ m, c) and d) 0.5  $\mu$ m, e) and f) 2  $\mu$ m.



**Figure 3.** Survival of honey bees infected with *N. ceranae* from CAR (Central Spain) and *N. ceranae* from INRA of Avignon (South France) compared to control bees. Each curve represents a pool of 3 cages with 30 bees each ( $N = 90$ ). When combining the results within the two infected groups and controls, survival curves were not significantly different between infected bees ( $\chi^2 = 3.346 \times 10^4$ ,  $p$ -value = 0.9854), but they were significantly higher compared to control ( $\chi^2 = 132.289$ ,  $p$ -value < 0.0001).



**Figure 4.** Spore count per bee and day (mean  $\pm$  SD). Dead honey bees were removed daily from each cage and spores counted. Spore count significantly increased with time but was not different between bees infected with *N. ceranae* from CAR (Central Spain) and *N. ceranae* from INRA of Avignon (South France).



#### 4. Discussion

In our study, we hypothesized that two different *N. ceranae* strains, each one from a different geographic origin, would differ in their virulence. We designed a genetic study amplifying a high variable region of ribosomal DNA. This fragment corresponded to an intergenic spacer (IGS) and to the first part of the small subunit gene (SSU). By now, many sequences of this fragment are available at the GenBank and were used to analyze our results. The IGS is a non-coding region of the DNA that allows the accumulation of mutations. Generally, this kind of variability can be typical of a geographic origin. In addition, a small but conserved degree of variability characterizes the beginning of the SSU gene; i.e. the insertion-deletion (INDEL) GATT at around 100 bp of distance from the beginning of the gene. In 2011 Sagastume *et al.* showed that this region (IGS+SSU) is not only highly variable, in and of itself, but also undergoes genetic recombination that contributes to increase this variability. In addition, they showed that some of these SNPs (single-nucleotide polymorphism) and INDELs are transmitted as whole blocks, generating haplotypes that can be common to multiple sequences. Thus, if the *N. ceranae* spores from South of France or from Spain are a case of genetic differentiation within the same species, we should expect: (i) a decrease in the variability of this fragment, particularly in the SSU gene; (ii) a higher probability of finding similar sequences within the same sample; (iii) groups of SNPs and common haplotypes in function of the geographic origin, that is supposed to contribute to a specialization of *N. ceranae*. We did not find those characteristic zones in our results and the variability of the fragment remained very high. This result suggests that there is no specific genetic background of the *N. ceranae* isolates from Spain or France used in this study.

Results from the genetic characterization were consistent with the anatomopathological lesions observed in the bee's midgut epithelium and the survival rate that were similar in both *N. ceranae* infected-groups but different from control. Indeed *N. ceranae* produced the same pathological lesions at cellular and tissue level in both infected-groups as a consequence of the spore germination in the midgut and subsequent multiplication in the epithelium. Our observations agree with precedent studies carried out by Fries *et al.* (1996) in *A. ceranae*, Higes *et al.* (2007) in *A. m. iberiensis* and Suwannapong *et al.* (2010) in *A. florea*. As well, García-Palencia *et al.* (2010) observed in *A. m. iberiensis* the same lesions in natural infections verifying the previous histopathological studies carried out in laboratory conditions. Spores were not detected in the Malphigean tubes or in the intestinal muscles using either optic or electronic microscopy, which suggests tissue-specificity. However, other studies have found *N. ceranae* in organs other than the honey bee midgut based on PCR signal (Copley and Jabaji 2012). More histological data would be needed to understand the presence of *N. ceranae* in other tissues and the relevance for the host-parasite relationship.

In our experiment all infected honey bees died within 19 days. Similar rates of mortality in the same or shorter periods were also observed using comparable laboratory conditions in *A. m. iberiensis* (Higes *et al.*, 2007 and Martín-Hernández *et al.*, 2011a for spore loads per bee over 50 000). In contrast other authors obtained lower mortality rates using a variety of honey bee types such as Buckfast (Vidau *et al.*, 2011), *A. mellifera* hybrid *ligustica* x *mellifera* (Alaux *et al.*, 2010b), *A. mellifera* (Forsgren and Fries, 2010), however it is difficult to compare results since these trials were ended before achieving 100% of mortality and using different rearing methods. The genetic origin of the honey bees could be a key in the understanding of differential mortality of *N. ceranae* infected bees. Indeed, selection of honey bees for low levels of *Nosema* spp. infection seems to be successfully achieved in Denmark as a result of years of selection (Huang *et al.*, 2012) suggesting that different degrees of host susceptibility exist.

In our experiment spore count in dead bees showed no differences between infected-groups. The consistency of spore count suggests a similar pattern of infection development, which in turn supports the idea that the *N. ceranae* isolates are the same strain. Huang *et al.* (2012) also used *N. ceranae* isolates from apiaries at INRA Avignon in France, but tested them on two different strains of bees. As mentioned before, one bee strain was selected for tolerance to *Nosema* spp. in Denmark, and the other one was the subspecies *A. mellifera carnica* from Germany. Interestingly, tolerant bees, drones in that case, could resist higher spore-loads and present lower mortality than non-tolerant drones. Again, genetic background of the honey bee seems to be a key factor when studying response to *N. ceranae* infection.

Differences in experimental procedures between laboratories might also explain different results when performing *N. ceranae* infections. First, fresh, purified spores are preferable for experiments, given that refrigeration and freezing may reduce spore viability and virulence (Fenoy *et al.*, 2009). Incubation temperature may contribute to differences in honey bee response to infection since less spores are produced at extreme incubation temperatures, like 25°C and 37°C, compared to 33°C; in field conditions sensitivity to low temperature may also explain a higher virulence of *N. ceranae* in warmer regions (Martín-Hernández *et al.*, 2009). The age of the honey bees at infection may also influence the infection development (Malone, Giacon and Newton, 1996), however in our experiment we used 5-day-old bees and the rate of mortality was higher than some experiments using younger bees (Alaux *et al.*, 2010b) as well as same-age honey bees (Vidau *et al.*, 2011) or older honey bees (Forsgren and Fries, 2010). In addition, nutritional sources during artificial rearing are not standardized and they can be more or less favorable to honey bee health (Alaux *et al.*, 2010a) and so the development of infection could differ.

## 5. Conclusion

We tested two *N. ceranae* isolates from different geographical origins, France and Spain, where the effects of this parasite on the honey bee are different, giving rise to the hypothesis that they should differ in virulence. The study of the genetic variability of an rDNA fragment of both *N. ceranae* isolates in addition to similarities observed in histopathological lesions of infected honey bee midgut and honey bee survival rate, suggest that differences in the honey bee response to infection could be more related to honey bee genetics (degree of susceptibility to *N. ceranae*) or experimental conditions than to differences between *N. ceranae* isolates. Future studies should be done to estimate the contribution of each of these factors on the effects of *N. ceranae* on honey bees.

## Acknowledgement

We thank A. Cepero, V. Albendea, T. Corrales, M.C. Rogerio, C. Abascal from the CAR laboratory for their technical support, C. Alaux for comments that improved the manuscript and C. McDonnell for English revision and comments. This work was co-funded by Consejería de Agricultura de Castilla - La Mancha, INCRECYT (FEDER funds) and BEEDOC grant (FP7, RTD REG/E.4(2009)D/561221). C. Dussaubat was supported by COLOSS Short Term Scientific Mission (STSM) COST Action FA0803 and CONICYT/French Embassy of Chile grants.

## Synthèse

Les résultats de cette recherche montrent que la variabilité des effets de *N. ceranae* dans différentes régions, notamment les isolats du sud de la France et du centre de l'Espagne, ne s'expliquent pas par l'existence des souches plus ou moins virulentes de *N. ceranae* comme c'est proposé dans notre hypothèse spécifique. Ces différences sont donc dues plus probablement à une base génétique des abeilles liée aux degrés de tolérance à l'infection qui s'exprime lorsque les conditions environnementales sont favorables et que les pratiques apicoles sont appropriées. Ces dernières ont un rôle dans le développement et la transmission des maladies infectieuses chez les abeilles, comme par exemple, l'utilisation des antibiotiques contre *Nosema* spp. (« fumagiline » provenant du *Penicillium fumigatus*) qui empêche la sélection naturelle, ainsi que les grandes densités de populations des ruchers commerciaux qui favorisent la transmission des pathogènes entre colonies.

Une synthèse de l'ensemble des résultats des chapitres 2, 3 et 4 sera présentée dans la discussion générale, structurée de façon à répondre à l'hypothèse globale proposée dans l'introduction de cette thèse.

## *Chapitre 5*

### **Discussion générale**

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Dans le cadre du phénomène global de la mortalité des abeilles, et de l'incertitude sur le rôle de *N. ceranae* dans ces mortalités, nous avons voulu apporter une approche pluridisciplinaire pour contribuer à la compréhension de cette relation hôte-parasite.

Notre **hypothèse générale** est que *N. ceranae* est un pathogène capable d'induire, chez *A. mellifera*, des changements comportementaux dus à des altérations physiologiques, qui pourraient perturber l'organisation sociale des abeilles et aboutir à la mort de la colonie.

Afin de tester cette hypothèse générale, nous avons établi trois objectifs spécifiques (i) chercher des effets de *N. ceranae* sur la perturbation de l'organisation sociale de la colonie, (ii) explorer les mécanismes à la base des effets chez les abeilles parasitées, et (iii) mettre en évidence les différences de virulence d'isolats de *N. ceranae* pour expliquer la variation des effets du parasite chez l'abeille.

A partir de nos recherches nous avons obtenu trois résultats majeurs. D'abord, au niveau social, nous avons constaté des modifications dans la structure organisationnelle des abeilles après l'infection par *N. ceranae*, ces modifications sembleraient contribuer à la survie de la colonie constituant probablement un mécanisme d'immunité sociale. Ensuite, l'étude des effets physiologiques de *N. ceranae* chez l'abeille en tant qu'individu, nous ont permis de mettre évidence deux mécanismes pathogéniques importants dans l'intestin de l'abeille qui peuvent induire leur mort: l'inhibition du renouvellement cellulaire de l'épithélium et l'induction d'un état de stress oxydatif. Finalement, nos résultats suggèrent que l'expression du potentiel de *N. ceranae* à induire la mort de colonies dépend des caractéristiques de l'hôte et de l'environnement. La contribution de chacun de ces résultats à la vérification de l'hypothèse générale sera développée par la suite.

## **I. Effets de *N. ceranae* chez l'abeille au niveau social**

### **Théorie sur une réponse d'immunité sociale**

L'abeille possède un nombre réduit de gènes d'immunité individuelle par rapport à des insectes solitaires (Honey Bee Genome Sequencing Consortium, 2006). Cela semble être compensé par d'autres moyens de réduction de la pression des pathogènes (Evans *et al.* 2006). En effet, dans le contexte de leur vie sociale les abeilles ont développé des mécanismes de défense collective contre les parasites (immunité sociale), basés sur des comportements de coopération entre les individus (Cremer *et al.*, 2007). Les changements phéromonaux et de comportements, observés lors de l'infection par *N. ceranae* (articles n°1 et n°2), nous ont permis de proposer un mécanisme d'immunité sociale chez les ouvrières qui permettrait la réduction des risques de transmission du parasite de la façon suivante :

- Effet de *N. ceranae* sur l'individu :

Les abeilles infectées ont montré (article n°2) une maturation comportementale accélérée, une activité de vol intense, une durée de vie raccourcie et une production élevée de la phéromone EO en comparaison avec les abeilles saines (Fig. 1a). Premièrement, ces comportements contribueraient à diminuer la transmission par voie orale du parasite car la nourriture ne serait pas un véhicule de spores pour deux raisons : (i) l'augmentation du butinage serait due aux besoins énergétiques individuels des abeilles infectées plutôt qu'à la récolte de nourriture pour la colonie (Mayack et Naug, 2010), et (ii) l'abeille aurait tendance à moins partager sa récolte par trophallaxie (Naug et Gibbs, 2009 ; Smith, 2012). Deuxièmement, l'augmentation de la mortalité hors de la colonie diminuerait l'inoculum du parasite au sein de la colonie (Kralj et Fuchs, 2010 ; Rueppell *et al.*, 2010).

- Effet de *N. ceranae* sur la société :

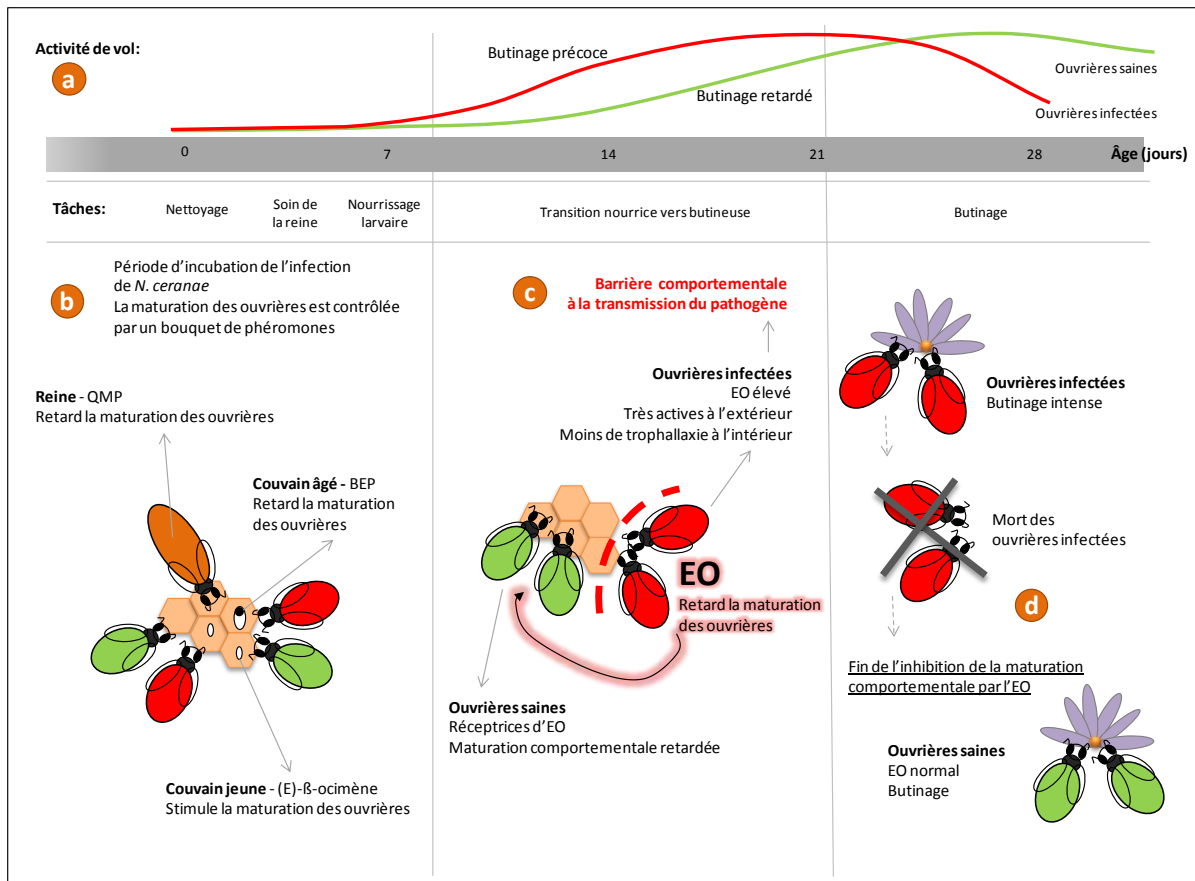
Les niveaux élevés d'EO des abeilles infectées, atteints plus tôt que chez les abeilles saines, auraient comme résultat l'inhibition de la maturation comportementale des abeilles saines. Cela expliquerait pourquoi les abeilles saines étaient moins actives que celles infectées (article n°2). L'entrée en butinage tardive des abeilles non-infectées permettrait de prolonger leur durée de vie (Woyciechowski et Moron, 2009) et de remplacer les abeilles infectées au fur et à mesure qu'elles meurent (Fig. 1a).

Les changements de comportement des abeilles infectées au cours du temps sont représentés dans la Fig. 1. Tout d'abord, les ouvrières peuvent être infectées après l'émergence, lorsqu'elles

réalisent des tâches de nettoyage (Fries, 1997). Puis, pendant le temps du développement de l'infection (Fig. 1b), 8 – 12 jours (Higes *et al.*, 2007 ; Forsgren et Fries, 2010), la maturation comportementale des ouvrières est sous le contrôle de trois autres phéromones (Fig. 1c) (Castillo *et al.*, 2012): la BEP (brood ester pheromone) produite par le couvain âgé, le (E)- $\beta$ -ocimène produit par le couvain jeune, et la phéromone mandibulaire de la reine QMP (queen mandibular pheromone). Ces phéromones agissent principalement pendant les premiers jours de vie des ouvrières lors qu'elles restent dans la ruche avec le couvain pour nettoyer l'intérieur, s'occuper de la reine et puis devenir nourrices. Le couvain jeune qui a besoin d'aliments, émet du (E)- $\beta$ -ocimène qui stimule la transition de nourrices à butineuse. Le couvain âgé, qui n'a plus besoins d'être nourri, émet BEP qui retarde la transition de nourrice à butineuse. En même temps, toutes les abeilles de la colonie sont exposées à la QMP qui retarde également la transition (Castillo *et al.*, 2012). Finalement, lorsque l'infection est bien développée et que les abeilles vieillissent, les différences de comportement entre abeilles infectées et saines vont devenir apparentes (article n°2). Au fur et à mesure que les nourrices vieillissent elles se déplacent vers les zones de stockage (Fig. 1d) dans la périphérie de la colonie. Là, lorsque les abeilles infectées deviennent butineuses plus tôt que les abeilles saines, leurs niveaux plus élevés d'EO retarderaient la maturation comportementale des abeilles saines du même âge. Les abeilles saines remplaceraient les abeilles infectées qui meurent à l'extérieur (Fig. 1e).

Dans ce contexte, la survie de la colonie dépendrait de sa capacité à contrebalancer l'infection principalement par le remplacement des abeilles qui meurent lorsque les lésions intestinales sont irréversibles (article n°3), d'où l'importance (i) de la qualité de la reine, (ii) de la disponibilité de ressources mellifères en quantité et qualité, et (iii) de l'absence d'autres facteurs de stress présents dans l'environnement comme les pesticides et d'autres pathogènes qui peuvent affaiblir la colonie (Cornman, *et al.*, 2012 ; James et Xu, 2012 ; Khoury *et al.*, 2011).

Finalement, il est connu que *N. ceranae* altère en même temps d'autres signaux chimiques. Par exemple, il a été observé une augmentation de l'hormone juvénile III (JH) chez des abeilles infectées (Ares *et al.*, 2012). Comme la transition de nourrice à butineuse est précédée par une augmentation de JH, l'infection pourrait déclencher la transition (Castillo *et al.*, 2012). Chez les reines, *N. ceranae* modifie également le bouquet phéromonal (Alaux *et al.*, 2011, annexe 2) mais les conséquences de ces changements sur le contrôle de la maturation comportemental des ouvrières n'a pas encore été étudié.



**Figure 1.** Hypothèse sur les modifications induites par *N. ceranae* dans le modèle de polyéthisme d'âge de l'abeille pendant l'été (basé sur Castillo *et al.*, 2012). **(a)** Les abeilles infectées (ligne rouge) ont une maturation comportementale accélérée, une activité de vol intensifiée et une durée de vie raccourcie, alors que les abeilles saines du même âge (ligne verte) ont une entrée au butinage tardive et une durée de vie allongée; **(b)** les différences de comportement entre les abeilles infectées et saines commencent à devenir évidentes après la période d'incubation de l'infection; pendant ce temps la maturation comportementale des abeilles est sous le contrôle de trois autres phéromones: BEP (brood ester pheromone), (E)-β-ocimène et QMP (queen mandibular pheromone); **(c)** au fur et à mesure que les abeilles vieillissent, les niveaux d'EO des abeilles infectées (rouges) augmentent et retardent la maturation comportementale des abeilles saines (vertes); les abeilles infectées agiraient comme une « barrière » à la transmission du parasite au sein de la colonie par la diminution de la trophallaxie et donc du partage de la nourriture (véhicule de spores) (Mayack et Naug, 2010; Naug et Gibbs, 2009; Smith, 2012), et également par l'augmentation de la mortalité hors de la colonie qui diminuerait l'inoculum du parasite au sein de la colonie (Kralj et Fuchs, 2010; Rueppell *et al.*, 2010); **(d)** lorsque les abeilles infectées meurent l'inhibition de la maturation comportementale par l'EO diminue et donc les abeilles saines vont augmenter leur activité de vol.



## II. Mécanismes pathogéniques de *N. ceranae* chez l'abeille au niveau individuel

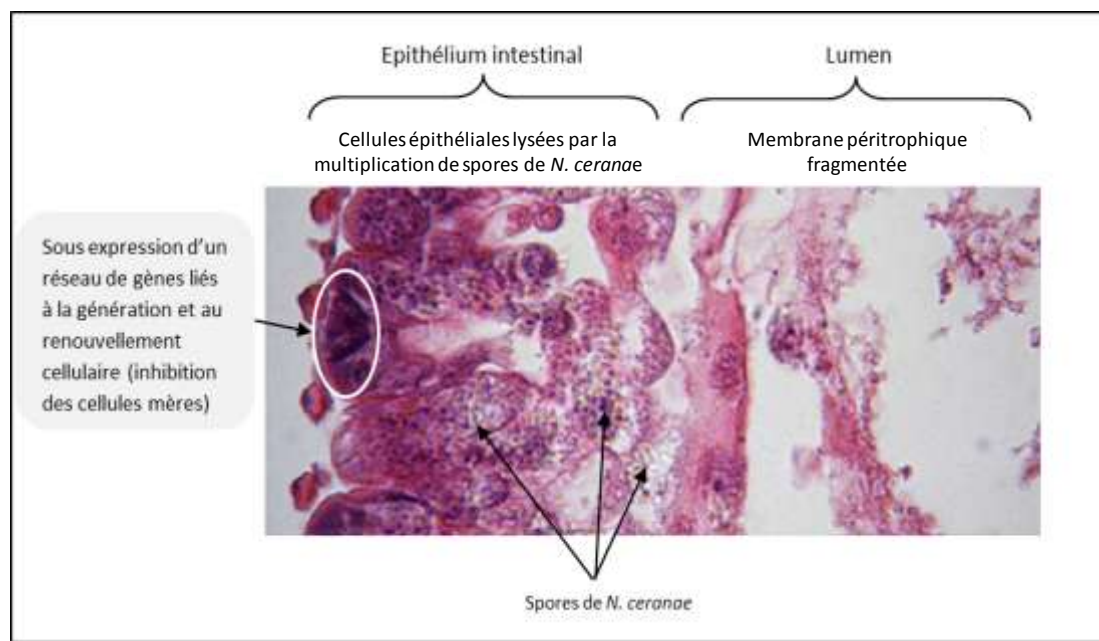
Le passage de nourrices à butineuses comprend un changement physiologique qui est lié au processus de maturation de l'abeille (Winston, 1987). Nous avons constaté que lors de l'infection par *N. ceranae* les abeilles présentent une modification du processus de maturation, l'altération de signaux chimiques et une mortalité prématurée (article n°2). Nous avons conduit une étude au niveau transcriptomique et biochimique, focalisés sur l'intestin qui est l'organe cible du parasite, afin de mieux comprendre les effets sur la physiologie de l'abeille.

### a) Inhibition du renouvellement cellulaire de l'épithélium de l'intestin

La caractéristique principale de la microsporidiose intestinale est la blessure de l'épithélium qui conduit à la mauvaise absorption des nutriments (Kotler et Orenstein, 1999). Cette perte de fonctionnalité expliquerait le stress énergétique et le déficit de nutriments chez l'abeille infectée par *N. ceranae* (Aliferis *et al.*, 2012). Nos travaux (article n°3) ont montré que *N. ceranae* inhibe l'auto-renouvellement cellulaire de l'épithélium de l'intestin de l'abeille, via l'inhibition de l'expression d'un réseau de gènes impliqués dans le développement des tissus et neurones. Ce réseau contient 4 gènes de la voie moléculaire « Wnt signaling pathway » qui contrôlent le renouvellement des cellules mères intestinales (Fig. 2). Ces observations au niveau moléculaire viennent se joindre aux observations d'altérations morphologiques et physiologiques des cellules de l'épithélium que nous avons également observées et qui sont aussi décrites dans de nombreux travaux précédents (Chen et Huang, 2010 ; Fries *et al.*, 2006 ; García-Palencia *et al.*, 2010 ; Gisder *et al.*, 2010 ; Higes *et al.*, 2007, 2008a). Parmi les effets les plus évidents au niveau histologique, on note : l'altération des membranes cellulaires, le déplacement apical et la condensation ou réduction du noyau cellulaire, la lyse de cellules, et l'absence ou la fragmentation de la membrane péritrophique (Higes *et al.*, 2007, Higes *et al.*, 2008a, García-Palencia *et al.*, 2010). Les cellules épithéliales lysées ne peuvent donc pas être remplacées par de nouvelles cellules, cela se traduit par la dégénérescence de l'épithélium et la perte des fonctions de l'intestin.

Il est connu que lors d'une infection intestinale par une microsporidie, la destruction excessive des cellules épithéliales perturbe les mécanismes qui maintiennent l'homéostasie. Ces mécanismes permettent d'optimiser l'absorption des nutriments par l'activation de la prolifération cellulaire de l'épithélium intestinal (Kotler et Orenstein, 1999). Chez les humains par exemple, la mort cellulaire croissante qui caractérise l'infection par une microsporidie, active des mécanismes compensatoires dans les cellules basales de l'épithélium (crypte intestinale) où se trouvent des cellules sécrétoires et

des cellules régénératives. Le résultat est la prolifération rapide des cellules de la crypte qui va restituer l'architecture de l'épithélium mais qui peut aussi causer l'hyperplasie de l'intestin et l'atrophie de cellules apicales (Kotler et Orenstein, 1999). Ce phénomène ne semble pas avoir lieu chez l'abeille vu l'inhibition du mécanisme d'auto-renouvellement cellulaire de l'épithélium de l'intestin que nous avons observé, mais cela reste à confirmer expérimentalement.



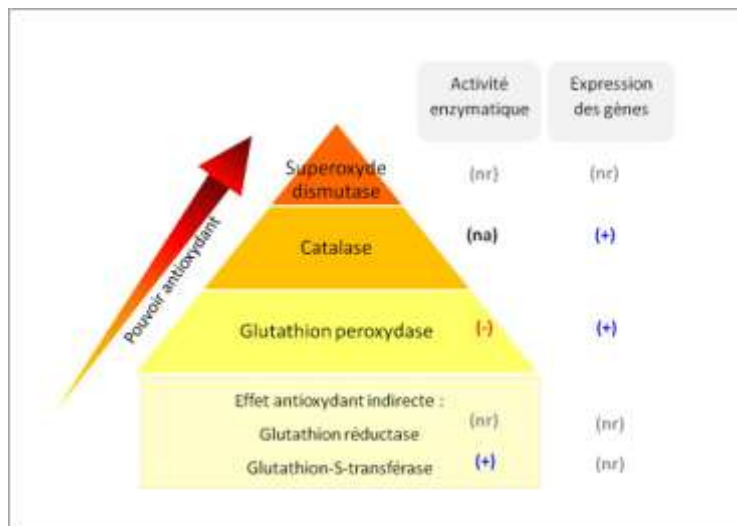
**Figure 2.** Epithélium intestinal d'une abeille où le renouvellement cellulaire est altéré 7 jours après l'infection par *N. ceranae* (article n°3).

### b) Etat de stress oxydatif dans l'intestin

Une des réponses presque immédiate du système immunitaire de l'abeille au niveau de l'intestin est la production d'espèces réactives de l'oxygène (ROS, reactive oxygen species) qui ont une action antimicrobienne (James et Xu, 2012). Cela confère à l'intestin une fonction de barrière primaire contre l'invasion de microorganismes pathogènes. Face à l'infection par *N. ceranae* nous avons observé dans l'intestin la surexpression des gènes impliqués dans les processus d'oxydation – réduction (par exemple la respiration cellulaire ou d'autres fonctions du métabolisme) d'où sont issus les ROS comme produits secondaires (Nikolenko *et al.*, 2012). La surexpression de ces gènes pendant l'infection suggère que cette réponse serait due à l'activation du système immunitaire de l'abeille (Fig. 3).

Néanmoins, les ROS ne sont pas spécifiques et une surproduction (état de stress oxydatif) peut détruire les microorganismes pathogènes mais également peut provoquer des dommages dans les

cellules hôtes. Les ROS peuvent réagir avec des macromolécules d'importance biologique comme les lipides, les protéines, les acides nucléiques et les hydrocarbures, ce qui conduit éventuellement à la mort cellulaire (Cadet *et al.*, 2005). Pour éviter le dommage cellulaire l'organisme possède des mécanismes de neutralisation de l'excès de ROS via principalement la production d'enzymes antioxydantes (Cadet *et al.*, 2005). Parallèlement à la surexpression des gènes liés à la production de ROS, nous avons observé l'activation au niveau moléculaire des mécanismes de neutralisation. Notamment la surexpression des gènes de deux des enzymes du système antioxydant, la catalase et le glutathion peroxydase (GP) (*Glutathione peroxidase like 2*). Dans le même sens, l'activité de l'enzyme glutathion-S-transférase (GsT), qui participe au processus de neutralisation de ROS (Turrens, 2003), était plus élevée chez les abeilles infectées que chez les abeilles saines. Néanmoins, ce n'est pas le cas de l'activité d'autres enzymes jouant un rôle dans la neutralisation des ROS. L'activité de la glutathion peroxydase (GP) a diminué au lieu d'augmenter comme nous l'attendions. Dans les cas de la superoxyde dismutase (SOD) et de la glutathion réductase (GR) l'activité n'était pas différente de celle des abeilles saines (Fig. 3). Nos résultats suggèrent que l'abeille ne possède pas de mécanismes suffisants pour la neutralisation du stress oxydatif et donc, la surproduction de ROS pourrait causer la mort des cellules de l'épithélium de l'intestin de l'abeille. En plus d'être la conséquence d'une réponse immunitaire, la surproduction de ROS peut aussi être liée directement à l'accélération du métabolisme de l'abeille (Nikolenko *et al.*, 2012) due au stress énergétique induit par *N. ceranae* (Aliferis *et al.*, 2012). Finalement, une autre interprétation de nos résultats serait que la production de ROS n'était pas suffisante pour contrer l'infection, c'est pourquoi l'activité enzymatique est restée faible, et par conséquent, le développement de *N. ceranae* a provoqué la destruction cellulaire dans l'épithélium de l'intestin de l'abeille.



**Figure 3.** Réponse au stress oxydatif au niveau biochimique (gauche) et moléculaire (droite). L'activité des enzymes qui participent à la neutralisation de ROS sur le tissu du tube digestif lors de l'infection par *N. ceranae*, est montrée : (+) augmentation de la réponse ; (-) diminution de la réponse ; (nr) pas de réponse ; (na) non analysée. De bas en haut une représentation générale du niveau d'implication de l'enzyme dans la neutralisation de ROS (basé sur Beaudeau et Vasson, 2005).

A partir de notre travail, au moins quatre aspects méritent d'être approfondis, (i) les différences entre la réponse antioxydante au niveau moléculaire et biochimique, (ii) l'activité enzymatique de la catalase que nous n'avons pas analysée, mais qui contribuerait à une vision plus complète des mécanismes de neutralisation de ROS car son action s'enchaîne aux autres enzymes étudiées (Thérond et Bonnefont-Rousselot, 2005), (iii) la variation de la réponse à l'infection entre les différentes castes d'abeilles vu, par exemple, que la capacité antioxydante totale des reines augmente lors de l'infection (Alaux *et al.*, 2011, annexe 2), et (iv) les conséquences du déséquilibre ROS – antioxydants dans la signalisation cellulaire, car le rôle de la réponse antioxydante n'est pas limité seulement à la survie de la cellule, le mécanisme de réduction-oxydation est impliqué également dans la signalisation cellulaire des multiples fonctions physiologiques, comme par exemple la prolifération cellulaire (Kamata et Hirata, 1999).

En plus de la production de ROS, l'intestin possède également d'autres mécanismes pour prévenir l'attaque de microorganismes. Par exemple, l'enzyme phosphatase alcaline (AP, alkaline phosphatase) est essentielle pour la santé de l'intestin, notamment dans la régulation de l'absorption des nutriments, la détoxification de lipopolysaccharides bactériens et la réduction de l'inflammation due à l'action des bactéries (Lalles, 2010). Ces fonctions étudiées principalement chez les mammifères, peuvent être extrapolées par homologie chez les insectes. Nous avons observé que ce mécanisme de protection est également inhibé lors de l'infection par *N. ceranae*. De plus, nos résultats suggèrent aussi la suppression de l'immunité ce qui avait été observé dans des études précédentes (Antúnez *et al.*, 2010 ; Chaimanee *et al.*, 2012). Ces observations révèlent les effets de *N. ceranae* sur les différents mécanismes de défense de l'abeille lorsque l'infection est au point de développement maximum (7 jours).

Ces effets de *N. ceranae* chez l'abeille ne sont pas une exception parmi les microsporidies car en général, les réponses de défenses des hôtes n'empêchent pas la progression de l'infection qui peut soit devenir chronique, soit provoquer la mort. De plus, de nombreuses données suggèrent que certaines microsporidies peuvent développer des mécanismes de survie et être capables de moduler/supprimer l'immunité de leurs hôtes (Texier *et al.*, 2010).

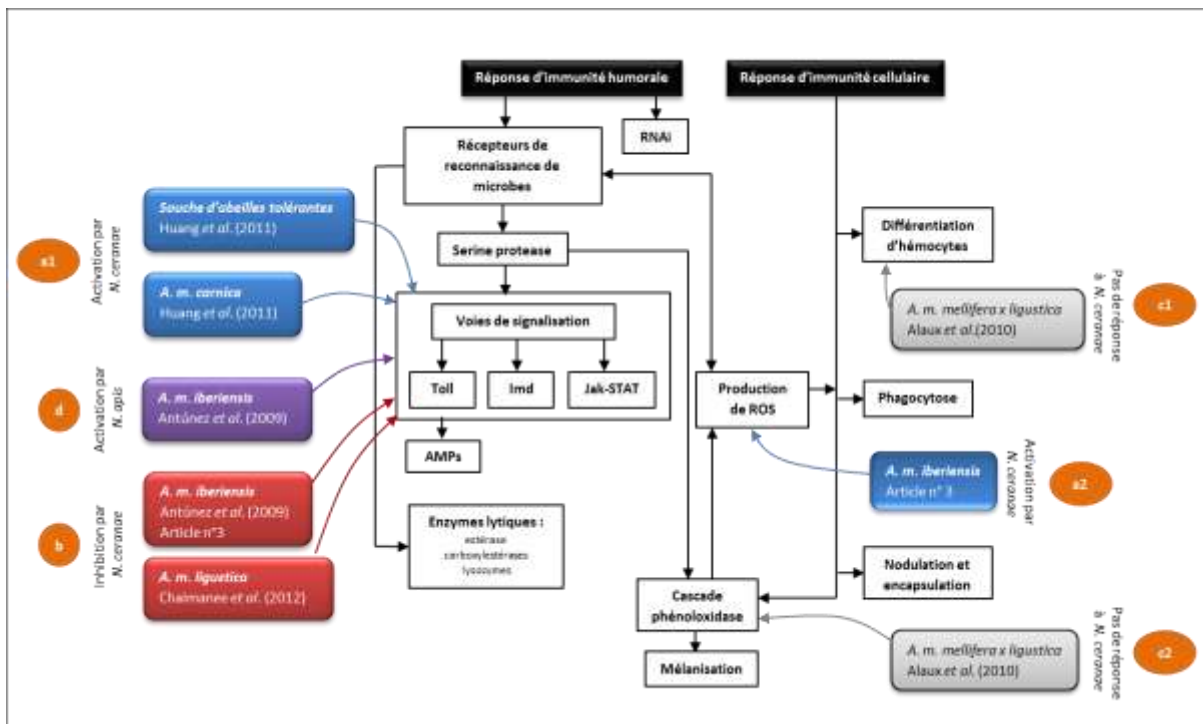
### III. Variation des effets pathogènes de *N. ceranae* chez l'abeille

L'inhibition du renouvellement cellulaire et le stress oxydant observés dans l'intestin des abeilles infectées par *N. ceranae* sont des effets assez importants pour provoquer la mort (article n°3). En même temps, la colonie aurait des mécanismes pour contrer la perte des individus malades et assurer la survie du groupe (article n°2). Cependant, il a été observé que les colonies d'abeilles peuvent supporter le parasitisme de *N. ceranae* sans succomber ou bien dans d'autres cas, qu'elles peuvent s'effondrer au bout d'une ou deux saisons (Carreck, 2012). Dans ce contexte nous avons voulu expliquer les différences de mortalité des abeilles à travers la comparaison de la virulence de deux isolats de *N. ceranae* d'origines géographiques différentes (article n°4). Or, lorsque ces isolats ont été testés dans les mêmes conditions expérimentales (même souche d'hôte et même environnement), les différences de développement de l'infection et de mortalité ont disparu. Cette étude sur ces deux isolats de *N. ceranae* suggère que le pouvoir pathogène de *N. ceranae* s'exprime sous certaines conditions. Une de ces conditions pourrait être l'origine génétique de l'abeille qui détermine sa résistance ou sa susceptibilité au parasite. Par exemple, un jour après l'infection avec *N. ceranae*, les voies moléculaires de l'immunité s'activent au lieu d'être supprimées chez des mâles d'*A. mellifera* des lignées sélectionnées pour leur tolérance à *N. apis* (Huang *et al.*, 2012). Le même effet, mais de plus courte durée, a été observé chez des mâles d'*A. mellifera carnica* (Huang *et al.*, 2012). Par ailleurs, l'infection par *N. apis* active également très rapidement cette réponse chez *A. mellifera iberiensis* alors que *N. ceranae* l'inhibe (Fig. 4) (Antúnez *et al.*, 2009). En effet, il est connu que les réponses de défenses de l'abeille aux pathogènes sont caractérisées par des différences entre espèces d'abeilles. Par exemple, il existe des différences d'expression des protéines antimicrobiennes, de la dynamique d'activité de l'enzyme phénoloxydase (qui participe au processus de mélanisation), et de la réponse des hémocytes entre des sous espèces d'abeilles (Nikolenko *et al.*, 2012).

D'autres facteurs pourraient être également impliqués dans les variations de la réponse à l'infection comme (i) la nutrition, (ii) l'interaction parasite-pesticides, (iii) les coinfections de parasites, et (iv) l'adaptation à l'environnement. Les facteurs qui affaiblissent la santé de l'abeille, comme une mauvaise nutrition, permettraient au parasite opportuniste de s'installer sur un hôte immunodéprimé (Alaux *et al.*, 2010b ; Porrini *et al.*, 2010). Cependant, la présence de *N. ceranae* n'est pas seulement le résultat de l'affaiblissement des colonies, car elle est présente également dans des colonies considérées comme fortes (Cornman *et al.*, 2012). Dans d'autres cas, ce serait plutôt des effets de synergie entre les pathogènes et les pesticides qui agiraient sur la santé de l'abeille (Alaux *et al.* 2010a; James et Xu, 2012 ; Pettis *et al.*, 2012 ; Vidau *et al.*, 2011 ). De plus, le métabolisme des sous espèces d'abeilles est adapté aux conditions de l'environnement d'origine, ainsi, les mêmes

conditions climatiques ont des effets différents sur la biosynthèse, la stabilité et l'activité des protéines, ce qui peut expliquer les différents degrés de susceptibilité aux maladies (Nikolenko *et al.*, 2012).

Nous n'avons pas observé de différences de virulence entre les deux isolats de *N. ceranae* étudiés (article n°4). Cependant, la vaste distribution géographique de *N. ceranae* et la diversité des sous espèces d'abeilles et lignées locales, laisse penser qu'il pourrait exister des souches de *N. ceranae* ayant des degrés de virulence différents ou des abeilles dotées de différents degrés de résistance. En effet, lorsque des pathogènes virulents causent des niveaux très élevés de mortalité, comme dans certaines régions dans le cas de *N. ceranae* (Carreck, 2012), il est possible d'attendre une adaptation très rapide du niveau de la résistance de l'hôte (Schmid-Hempel, 1998). De plus, les microorganismes entomopathogènes présentent des souches caractérisées par des degrés de virulence spécifiques. Un mécanisme de différenciation serait par exemple, une adaptation locale qui conduirait à la diminution de la virulence selon la distance géographique entre le parasite et son hôte (Schmid-Hempel, 1998). Ce type d'adaptation locale, qui renforce les réponses de défense, met en valeur l'intérêt de l'étude et de la conservation des différents écotypes d'abeilles (Nikolenko *et al.*, 2012).



**Figure 4.** Variation des effets pathogènes de *N. ceranae* sur l'immunité de l'abeille selon la sous espèce d'abeille (modifié à partir de James et Xu, 2012). En noir un schéma du système immunitaire. **(a1, a2)** en bleu : activation par *N. ceranae* du système immunitaire au niveau moléculaire; **(b)** en rouge : suppression par *N. ceranae* du système immunitaire au niveau moléculaire ; **(c1, c2)** en gris : pas de réponse à *N. ceranae* du système immunitaire au niveau cellulaire ; **(d)** en violet : activation du système immunitaire par *N. apis* au niveau moléculaire.

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## IV. Conclusion générale et perspectives

L'approche pluridisciplinaire choisie pour étudier l'impact de *N. ceranae* sur la santé des abeilles nous a permis de mettre en évidence (i) des modifications dans la structure sociale des abeilles après l'infection qui contribuaient à la survie de la colonie constituant probablement un mécanisme d'immunité sociale, (ii) des effets sur la physiologie de l'abeille à travers de mécanismes pathogéniques qui peuvent provoquer la mort des individus, et (iii) des variations des effets de *N. ceranae* probablement dues aux caractéristiques de l'hôte et de l'environnement.

D'abord nous avons constaté des modifications dans la structure sociale des abeilles après l'infection par *N. ceranae*. Ces changements sembleraient contribuer à la survie de la colonie constituant probablement un mécanisme d'immunité sociale. Ce mécanisme géré par un signal phéromonal, permettrait de diminuer la transmission du parasite au sein de la colonie et de prolonger la survie des abeilles saines. La vérification d'un tel mécanisme lors de l'infection par *N. ceranae*, mais également dans le cas d'autres parasites, permettrait d'orienter les pratiques apicoles vers le renforcement de la colonie afin de contrebalancer le coût de ces comportements d'immunité sociale. Par exemple, il serait fondamental de stimuler la production du couvain qui va remplacer les butineuses infectées lors de leur mort prématurée. Par ailleurs, il serait important de surveiller le niveau des réserves d'aliments dans la zone de stockage de la colonie car la population jeune augmente et la récolte apportée au nid par les butineuses malades diminue, ceci afin d'éventuellement nourrir la colonie. Cependant, *N. ceranae* n'altère pas seulement les signaux chimiques des ouvrières mais également ceux des reines (Alaux *et al.*, 2011, annexe 2) et probablement ceux des mâles (Nikolenko *et al.*, 2012). L'effet conjoint des signatures chimiques dans les trois castes mérite d'être étudié plus profondément car les phéromones sont à la base de l'organisation de la colonie et régulent la division des tâches des ouvrières (Leoncini *et al.*, 2004), le succès reproductif des reines (Richard *et al.*, 2007) et la reconnaissance entre les individus de la colonie (Dani *et al.*, 2005).

Ensuite, notre travail nous a permis de mettre en évidence des mécanismes pathogéniques du parasite et de suggérer des effets sur la physiologie de l'abeille qui peuvent expliquer la détérioration irréversible de la santé de l'abeille en tant qu'individu. La suppression des mécanismes du système immunitaire permettrait la progression de l'infection jusqu'au point où la dégénérescence de l'épithélium intestinal et l'inhibition du renouvellement cellulaire seraient irréversibles et provoqueraient la mort de l'hôte. Par conséquent, la survie de la colonie dépendrait de sa capacité à contrebalancer l'infection principalement par le remplacement des abeilles qui meurent. Une approche

complémentaire à nos travaux, qui contribuerait à mieux comprendre le degré d'implication de ces mécanismes dans la mortalité des abeilles, serait de tenter la réduction de l'expression des gènes du réseau lié à la voie moléculaire impliquée dans le renouvellement cellulaire et d'observer les effets sur l'intestin de l'abeille au cours du temps. Une autre approche serait également l'étude de la dynamique du processus infectieux qui permettrait d'établir les points de l'infection où les effets délétères du parasite seraient peut-être encore réversibles. Identifier le moment où la prolifération des cellules épithéliales de l'intestin est interrompue, ou bien celui où l'activité antioxydante diminue, permettrait d'envisager des traitements préventifs. Par exemple, la stimulation opportune de la capacité antioxydante par des aliments pourrait renforcer le système immunitaire et contribuer à contrebalancer les effets de l'infection dans une phase initiale (Alaux *et al.*, 2010b; Porrini *et al.*, 2011).

Finalement, nos résultats nous ont permis de suggérer que les variations des effets de *N. ceranae* sont dues probablement aux caractéristiques de l'hôte et de l'environnement. La vaste distribution géographique de *N. ceranae* et la diversité génétique des abeilles dans différentes régions, permet de soupçonner l'existence de variations de virulence ou de résistance. Dans ce cadre, l'étude des niveaux de résistance de l'abeille et de la virulence des souches de *N. ceranae* sont encore nécessaires, car les pratiques apicoles peuvent empêcher le développement naturel de résistance de l'abeille. Par exemple, certaines pratiques apicoles contribueraient à la transmission horizontale du parasite entre les colonies (dérive et pillage) ce qui favoriserait le développement de la virulence du parasite (Fries et Camazine, 2001).

Les effets de *N. ceranae* chez l'abeille peuvent aussi être influencés par les interactions entre divers facteurs présents dans l'environnement. Ainsi, l'étude des interactions devient pertinente car, dans des conditions naturelles, les coinfections et la présence de pesticides ont le potentiel d'affaiblir les colonies. Jusqu'à présent des études sur les effets des interactions pathogène-pathogène (Costa *et al.*, 2011) et pathogène-pesticides ont été conduites chez les ouvrières (Alaux *et al.*, 2010a ; Aufauvre *et al.*, 2012 ; Vidau *et al.*, 2011 ; Pettis *et al.*, 2012). Or, non seulement les ouvrières sont exposées à ces risques mais également les reines et les mâles d'une colonie (Traver *et al.*, 2011, 2012). C'est pourquoi nous nous sommes intéressés aux effets de l'interaction entre *N. ceranae* et un pesticide chez les reines dans des conditions naturelles. Ce travail actuellement en cours, permettra de mieux comprendre les déficiences des performances des reines que décrivent les apiculteurs (Botías *et al.*, 2012c ; vanEngelsdorp *et al.*, 2012).

En résumé, *N. ceranae* peut causer la mort à travers de mécanismes pathogéniques qui altèrent la physiologie de l'intestin de l'abeille. Cependant, nos résultats suggèrent que la colonie pourrait contrer l'infection, par exemple, par de mécanismes d'immunité sociale. Or, la réponse générale à



l'infection et par conséquent la survie de la colonie, dépendrait des caractéristiques de l'hôte en combinaison avec les conditions de l'environnement.

Le phénomène d'effondrement de colonies à l'échelle mondiale a stimulé la recherche autour du rôle des pathogènes, des pesticides et d'autres facteurs qui pourraient être à la base de cette mortalité. La détection récente chez *A. mellifera* de nouveaux parasites comme *N. ceranae* et des virus, a posé la question de la diversité et de la charge de l'ensemble des parasites que les abeilles peuvent naturellement tolérer ainsi que celle des facteurs de l'environnement qui favorisent ou mettent en danger la santé d'une colonie. En effet, l'adaptation génétique de l'abeille à différents environnements influence la dynamique de population, son statut sanitaire et sa productivité (Büchler *et al.*, 2012). Dans le futur, l'étude de l'adaptation des écotypes d'abeilles à leurs environnements d'origine pourrait donner des clés sur les facteurs qui contribuent à maintenir le système abeille – environnement en équilibre.



## **Annexe 1**

### **Recherche complémentaire**

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#### *Article en co-auteur*

### **Interactions between *Nosema* microspores and a neocotinoid weaken honeybees (*Apis mellifera*)**

Journal of Environmental Microbiology

2009. 12, 774-782

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Baldy, A., Belzunces, L., Le Conte, Y.

**\*Contribution à cet article** : mis au point de la méthode d'extraction et purification de spores de *Nosema* à utiliser dans les infections expérimentales. Suivi de la charge de spores durant l'expérience. Commentaires au manuscrit.

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## Interactions between *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*)

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### Summary

Global pollinators, like honeybees, are declining in abundance and diversity, which can adversely affect natural ecosystems and agriculture. Therefore, we tested the current hypotheses describing honeybee losses as a multifactorial syndrome, by investigating integrative effects of an infectious organism and an insecticide on honeybee health. We demonstrated that the interaction between the microsporidia *Nosema* and a neonicotinoid (imidacloprid) significantly weakened honeybees. In the short term, the combination of both agents caused the highest individual mortality rates and energetic stress. By quantifying the strength of immunity at both the individual and social levels, we showed that neither the haemocyte number nor the phenoloxidase activity of individuals was affected by the different treatments. However, the activity of glucose oxidase, enabling bees to sterilize colony and brood food, was significantly decreased only by the combination of both factors compared with control, *Nosema* or imidacloprid groups, suggesting a synergistic interaction and in the long term a higher susceptibility of the colony to pathogens. This provides the first evidences that interaction between an infectious organism and a chemical can also threaten pollinators,

interactions that are widely used to eliminate insect pests in integrative pest management.

### Introduction

The current decline in abundance and diversity of wild bees as well as honeybees has been reported in several regions of the world (Biesmeijer *et al.*, 2006; National Research Council of the National Academies, 2007). The magnitude of this pollinator crisis is believed to not only have a deep impact on agriculture and its related economy (Gallai *et al.*, 2009) but also on plant diversity (Biesmeijer *et al.*, 2006) and landscapes (Ricketts *et al.*, 2008). The most spectacular pollinator decline concerns honeybee colonies, which are disappearing en masse in USA and Europe (Faucon *et al.*, 2002; Higes *et al.*, 2005; Oldroyd, 2007; Stokstad, 2007). Although many stressors have been identified as a potential cause or indicator of colonies losses, including viruses (Cox-Foster *et al.*, 2007), microsporidia pathogens (Higes *et al.*, 2008; 2009) and pesticides (Frazier *et al.*, 2008), a combination of multiple agents is more likely to contribute to honeybee losses. Therefore, investigations have to be carried out on integrative effects of different agents.

A large spectrum of pesticides is used to manage crop pests. But as an alternative, and to reduce the harmful effects of chemicals on non-pest organisms and human, new eco-friendly strategies for controlling crop pests have been developed. These biological controls include the use of microbial pathogens like viruses, bacteria and fungi. Modern crop management integrates these different techniques in a compatible manner leading to an integrated pest management (IPM) (Mareida *et al.*, 2003). The most extensively used biological agents are fungi, which are often associated with insects [around 750 species are pathogens of insects (Carruthers and Soper, 1987)]. Entomopathogenic fungi and chemical insecticides used together significantly improve the lethality of control agents. Indeed, when fungi are delivered with sub-lethal doses of pesticides, they interact synergistically in killing insects (Purwar and Sachan, 2006). Among the insecticides, the neonicotinoid imidacloprid is one of the most effective in interacting synergistically with fungi. And IPM using the synergy between imidacloprid and fungal spores is commonly used for killing a variety of insect pests, like termites, thrips and leaf-cutter ants

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(Ramakrishnan *et al.*, 1999; Al Mazraawi, 2007; Valmir Santos *et al.*, 2007).

Interestingly, imidacloprid is a systemic insecticide widely used worldwide on food crops and has been believed to cause honeybee losses in France (Doucet-Personeni *et al.*, 2003). Despite a high percentage of hives containing residues of imidacloprid [e.g. in France, more than one hive in two has residues of imidacloprid and its metabolite 6-chloronicotinic acid in the pollen, 30% in honey and 26% in bees (Chauzat *et al.*, 2009)], the level of exposure is sub-lethal with no obvious effect on mortality (Schmuck *et al.*, 2001; Nguyen *et al.*, 2009). On the other side, a parasitic microsporidia, *Nosema ceranae*, has been associated to bee losses in USA without contributing significantly to it (Cox-Foster *et al.*, 2007), but it is reported to be a cause of bee losses in Spain (Higes *et al.*, 2008; 2009).

Ironically, the combination of pathogens and pesticides that may be effective for insect pest control may result specifically in imidacloprid and *Nosema* acting together to kill bees. Because a single factor would not explain honeybee or more generally pollinator decline, it is highly possible that stressors act in concert. So, we ask the question of whether honeybees are victim of an interaction between infectious organism and a chemical like in IPM.

We looked at interactive effects between biological and chemical stressors on pollinators by analysing the interaction between imidacloprid and *Nosema* in honeybees. As social organisms, honey bees depend not only on the health of individuals, but also on the overall functioning of the hive. Consequently, we tested those integrative effects on honeybee health, at two levels, the individual and colony level. This study was designed to look at a possible effect on: (i) individual mortality and energetic demands; (ii) individual immunity; and (iii) social immunity. Sucrose consumption was calculated to estimate the energetic stress as *Nosema* alters host nutrient store and feeding behaviour (Mayack and Naug, 2009; Naug and Gibbs, 2009). Total haemocyte count (THC) and phenoloxidase (PO) enzymatic activity were analysed as parameters of individual immunity. Phenoloxidase plays a central role in invertebrates' immune reaction, being implicated in the encapsulation of foreign object through melanization (Decker and Jaenicke, 2004). Total haemocyte count gives an indirect measurement of basal cellular immunocompetence and is involved in the processes such as the phagocytosis and the encapsulation of a parasite (Tanada and Kaya, 1993). Those two defence reactions have been observed against fungal pathogens in insects (Chamley, 1984). Finally, glucose oxidase (GOX) enzymatic activity was analysed as a parameter of social immunity. Mainly expressed in the hypopharyngeal glands (HPGs) (Ohashi *et al.*, 1999), GOX catalyses the oxidation of  $\beta$ -D-glucose to D-gluconic acid and hydrogen

peroxide, the latter having antiseptic properties (White *et al.*, 1963). The antiseptic products are secreted into larval food (Sano *et al.*, 2004) and into honey (White *et al.*, 1963; Ohashi *et al.*, 1999) which contributes to colony-food sterilization and therefore to diseases prevention. Indeed, the level of hydrogen peroxide in honey is positively correlated with the inhibition of pathogens development (Taormina *et al.*, 2001; Brudzynski, 2006).

## Results

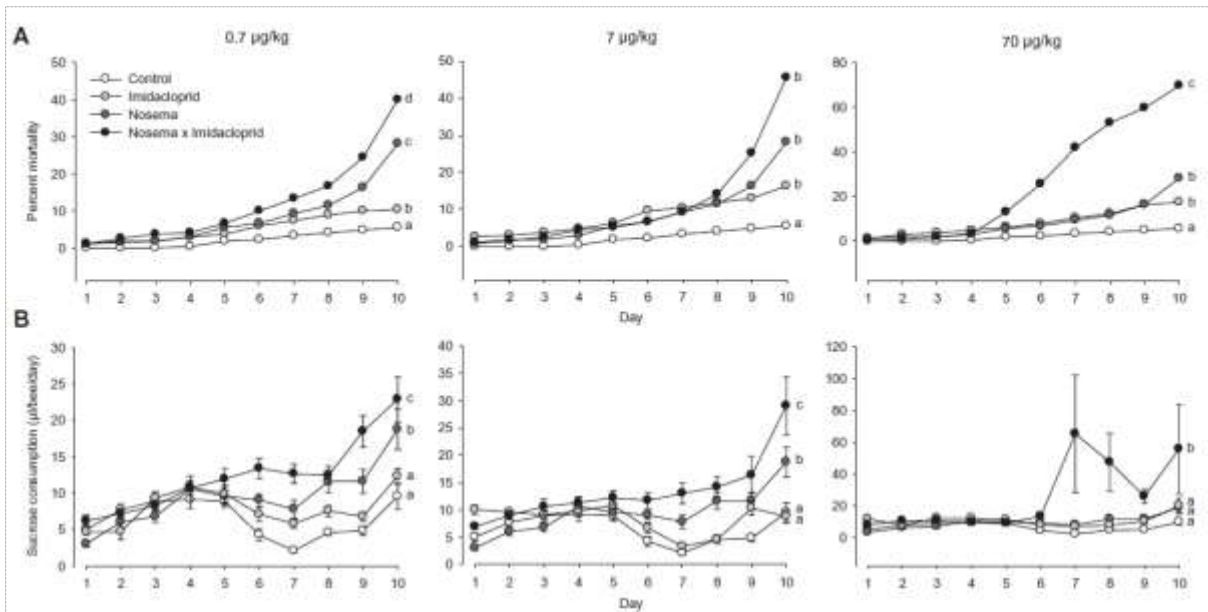
### *Effect of Nosema infection and/or exposure to imidacloprid on bee mortality and energetic demand*

The cumulative mortality rate increased with time in all experimental groups, but remained lower in control groups (-5%) ( $P < 0.001$  for each imidacloprid concentration, Fig. 1A). In addition, an important treatment effect was detected ( $P < 0.001$  for each imidacloprid concentration). Indeed, all three treatment groups exhibited significantly higher mortality rates than the control group (Fig. 1A). The effect of *Nosema* infection and imidacloprid exposure did not differ significantly except for the low concentration of imidacloprid (Fig. 1A). For each imidacloprid concentration, the mortality was the highest in bees when also challenged with *Nosema*. Interestingly, on the last 2 days of rearing, mortality rates of the *Nosema* × imidacloprid group equalled the sum of the mortality rates of the *Nosema* and imidacloprid groups, showing an additive effect, which was significant for the low imidacloprid concentration. The interactive effect was even stronger with the high concentration of imidacloprid showing, in that case, a potentiating effect.

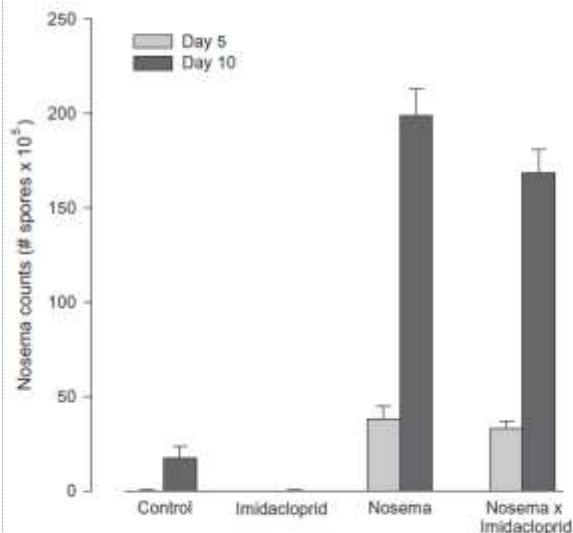
The sucrose consumption measurements, which were performed on the same cages as those used for the mortality assay, showed a similar pattern to the mortality rate. The amount of sucrose solution consumed significantly increased with time ( $P < 0.001$  for each imidacloprid concentration, Fig. 1B) and was affected by the treatments ( $P < 0.001$  for each imidacloprid concentration, Fig. 1B). Bees infected with *Nosema* consumed significantly more sucrose than control and imidacloprid-exposed bees. This amount was the highest in bees both infected with *Nosema* and exposed to imidacloprid (Fig. 1B).

The number of *Nosema* spores also increased with time even in the control groups, meaning that some control bees were likely infected at the beginning of the experiment (Fig. 2). However, the level of *Nosema* infection was significantly different between bees fed with *Nosema* (*Nosema* groups and *Nosema* × imidacloprid groups) and control bees or bees only exposed to imidacloprid ( $P < 0.001$  for each comparison). Interestingly, at day 10, bees exposed to imidacloprid had a slightly lower number of spores than bees non-exposed to imidacloprid





**Fig. 1.** Effect of *Nosema* infection and/or exposure to imidacloprid on bee mortality and energetic demands. A. Effect on mortality. Mortality is expressed as the percentage of cumulated number of dead bees per cage and per day ( $n = 270$  bees). Three colonies were analysed, with three cage replicates for each colony ( $n = 30$  bees per cage). Each letter indicates significant differences between treatments ( $P < 0.05$ ). B. Effect on energetic demand. Sucrose consumption is expressed as the amount of sucrose solution (50% w/v, *ad libitum* delivery) consumed per day and per bee ( $n = 30$  bees per cage) during the 10 h of treatment. The same cages as in A were analysed. Each letter indicates significant differences between treatments ( $P < 0.05$ ).

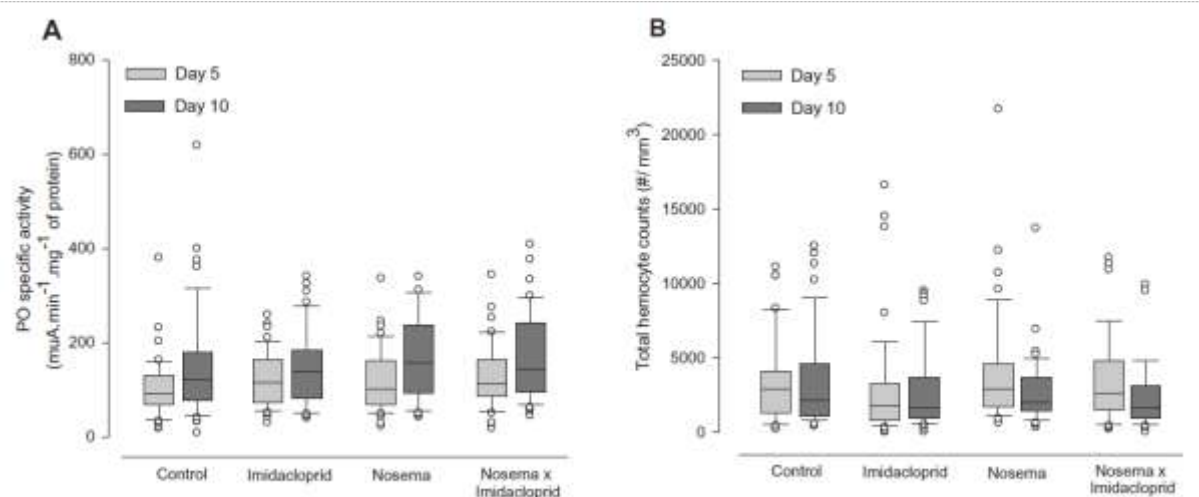


**Fig. 2.** Level of *Nosema* infection in bees fed with *Nosema* and/or exposed to imidacloprid. Level of infection was determined at days 5 and 10 on seven to eight bees per cage for each experimental group ( $n = 382$  bees). Three colonies were analysed, with two cage replicates for each colony. Data show mean  $\pm$  SE.

suggesting a slight inhibiting effect of imidacloprid on spore germination; a difference that was marginally significant between groups of bees individually infected with *Nosema* (control versus imidacloprid:  $P = 0.11$ , *Nosema* versus *Nosema*  $\times$  imidacloprid:  $P = 0.051$ ).

#### Effect of *Nosema* infection and/or exposure to imidacloprid on individual immunity

Phenoloxidase enzymatic activity was normalized to the protein concentration, which did not differ between experimental groups and age but changed between colonies ( $F_{1,388} = 1.06$ ,  $P = 0.31$ ;  $F_{3,388} = 1.88$ ,  $P = 0.13$ ;  $F_{2,388} = 8.75$ ,  $P < 0.001$  respectively). Phenoloxidase specific activity was not affected by *Nosema* infection and/or exposures to imidacloprid (Fig. 3A). Similarly, THC did not change between the different groups (Table 1, Fig. 3B). However, PO-specific activity and THC were found to, respectively, increase and decrease with age as found by Schmid and colleagues (2008) and Wilson-Rich and colleagues (2008) (Table 1, Fig. 3A and B). There was also a significant variation between colony replicates (Table 1).



**Fig. 3.** Effect of *Nosema* infection and/or exposure to imidacloprid on individual immunity.  
 A. Total haemocyte counts at days 5 and 10 on seven to eight bees per cage for each experimental group ( $n = 373$  bees).  
 B. Phenoloxidase activity at days 5 and 10 in eight bees per cage for each experimental group ( $n = 384$  bees). For each parameter, three colonies were analysed, with two cage replicates for each colony. Boxes show 1st and 3rd interquartile range with line denoting median. Whiskers encompass 90% of the individuals, beyond which each outliers are represented by circles.

*Effect of Nosema infection and/or exposure to imidacloprid on social immunity*

The protein concentration in the head changed significantly according to the treatments and colony origin ( $F_{3,175} = 5.78, P < 0.001$ ;  $F_{2,175} = 36.9, P < 0.001$  respectively). Bees from *Nosema* × imidacloprid groups had a lower protein concentration ( $4.4 \pm 1.3 \times 10^{-3} \text{ mg ml}^{-1}$ ) than bees from the control ( $4.9 \pm 1.2 \times 10^{-3}$ ), *Nosema* ( $4.8 \pm 0.9 \times 10^{-3}$ ) and imidacloprid groups ( $4.8 \pm 1.2 \times 10^{-3}$ ) ( $P < 0.01, P < 0.01, P < 0.05$  respectively). A significant effect of treatments on the specific activity of GOX was detected (Table 1, Fig. 4A). The combined effects of *Nosema* infection and exposure to imidacloprid

significantly decreased the GOX-specific activity compared with control, *Nosema* and imidacloprid groups ( $P = 0.013, P < 0.001$  and  $P < 0.01$  respectively; Fig. 4A), demonstrating a synergistic effect between the two stressors. This response of GOX activity was highly consistent because there was no significant difference between colony replicates (Table 1).

The HPG size was also affected by the treatments (Fig. 4B). Bees from the *Nosema* × imidacloprid group possessed smaller HPG than control ( $P < 0.001$ ) and imidacloprid-exposed bees ( $P = 0.004$ ), but were not different from bees infected with *Nosema* ( $P = 0.27$ ). Contrary to the GOX activity results, bees infected with *Nosema* had smaller HPG than control bees ( $P < 0.01$ ) but they were not different from bees exposed to imidacloprid ( $P = 0.09$ ). As for GOX activity, those differences were steady between colony replicates (Table 1).

**Table 1.** Analysis of *Nosema* infection, individual (THC, PO) and social immunity (GOX, HPG) as a function of experimental treatment (control, *Nosema*, imidacloprid and *Nosema* × imidacloprid), age and colony origin.

Parameter	Source of variation	d.f.	F	P
<i>Nosema</i>	Treatment	3, 358	161.3	< 0.001
	Age	1, 358	265.5	< 0.001
	Colony	2, 358	10.9	< 0.001
THC	Treatment	3, 349	1.3	0.274
	Age	1, 349	5.4	0.021
	Colony	2, 349	13.9	< 0.001
PO	Treatment	3, 352	1.57	0.197
	Age	1, 352	10.9	< 0.001
	Colony	2, 352	17	< 0.001
GOX	Treatment	3, 182	4.6	0.004
	Colony	1, 182	1.9	0.168
	Age	2, 180	7.3	< 0.001
HPG	Treatment	3, 180	7.3	< 0.001
	Colony	2, 180	1.2	0.288

**Discussion**

Because current hypotheses about honeybee colony losses strongly suggest multifactorial causes, we addressed for the first time the effect of an interaction between a parasite and a pesticide on honeybee health. Our results demonstrated interactive effects between microsporidia and pesticides that weaken honeybee health.

Malone and Gatehouse (1998) observed that bees could ingest some spores by chewing the wax capping at emergence, which could explain the detection of some spores in control bees. This observation suggests that we



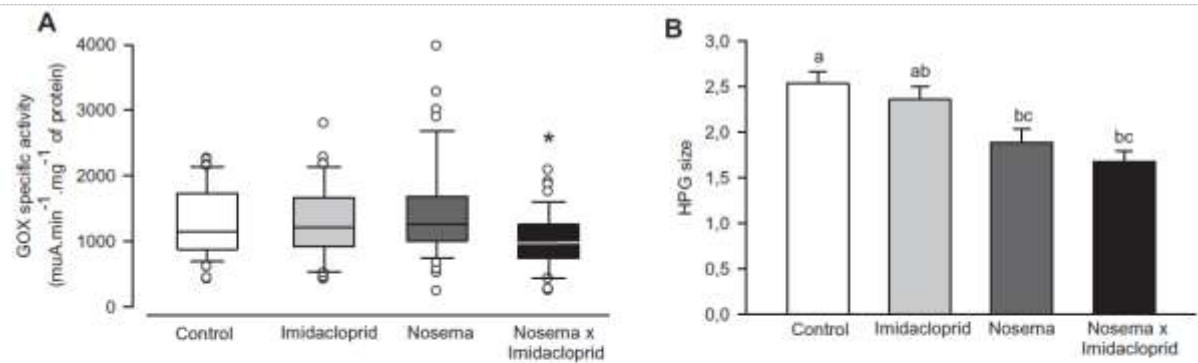


Fig. 4. Effect of *Nosema* infection and/or exposure to imidacloprid on social immunity.

A. Glucose oxidase activity at day 10 on eight bees per cage for each experimental group ( $n = 192$  bees). Boxes show 1st and 3rd interquartile range with line denoting median. Whiskers encompass 90% of the individuals, beyond which each outliers are represented by circles. \*denotes significant difference between *Nosema* × imidacloprid groups and the three others groups ( $P < 0.05$ ).

B. HPG size at day 10 in seven to eight bees per cage for each experimental group ( $n = 191$  bees). For each parameter, three colonies were analysed, with two cage replicates for each colony. The size was indexed from 1 to 5 (see *Experimental procedures*). Each letter indicates significant differences between treatments ( $P < 0.05$ ). Data show mean  $\pm$  SE.

compared lightly to heavily (experimentally) infected bees; however, the mortality rate in the first group was insignificant. Bees that were both infected with *Nosema* and exposed to imidacloprid at concentrations encountered in the environment showed the highest mortality rate. Interestingly, the sucrose feeding followed a similar pattern both regarding the treatment and time effect. This correlation gives some clues about the mechanisms of the interaction between *Nosema* and imidacloprid. *Nosema ceranae* can affect nutrient needs in hosts by using host nutrients and inducing an energetic stress (Mayack and Naug, 2009; Naug and Gibbs, 2009). Microsporidia are usually amitochondriate and unable to perform oxidative phosphorylation, meaning that they have a high dependency on host ATP (Keeling and Fast, 2002; Comman *et al.*, 2009), especially for germination which requires high level of energy. However, microsporidian spores have retained the glycolytic pathway suggesting that they are able to use glycolysis to produce ATP (Keeling and Fast, 2002). This idea is supported by a significant drop in trehalose levels (glucose–glucose disaccharide) in hosts during the germination of *Nosema algerae* (Undeen and Vander Meer, 1994). In our study, this dependence on host energy triggered also an increase in sucrose needs in bees that are challenged by *Nosema* parasitism. Imidacloprid alone did not increase food intake, meaning that it is not particularly attractive to the bees. However, when the food was treated with imidacloprid, the boost in food intake caused by parasitism was associated with an increase in imidacloprid exposure. Although imidacloprid contamination in the hive is usually found at sub-lethal doses, microsporidia infection could have the capacity to expose bees to lethal doses by increasing the intake of contaminated food. This is particularly striking with the

high concentration of imidacloprid used here, where *Nosema* and imidacloprid irremediably potentiates their effects.

Besides their direct impacts on host survival, pathogens can also impose significant costs on immunity. For example, one strategy of pathogens to promote their survival and replication in hosts is to suppress the activity of the immune system, which can involve the depression of PO activity (Yang and Cox-Foster, 2005) and haemocyte population (Ibrahim and Kim, 2006). However, our results showed that PO activity was neither up- nor down-regulated by *Nosema* challenge alone or in combination with imidacloprid. Similarly, THC was not affected by the different treatments. Antunez and colleagues (2009) showed that *Nosema apis* induced a higher expression of the gene coding for PO, but at the enzymatic level, we did not observe higher activity. The lack of immune response might be explained by deficient immunoregulatory activation, a lack of stimulation by microsporidia, or both. However, we cannot exclude that other parameters of individual immunity were activated or immunosuppressed, like antibacterial peptides and other immunity-related enzymes (e.g. glucose dehydrogenase, lysozyme) (Antunez *et al.*, 2009).

Another type of immunity that can be found in social insects and particularly in honeybees is a social immunity, which consists in a cooperation between the individual group members to prevent disease contamination (Cremer *et al.*, 2007; Wilson-Rich *et al.*, 2009). The analysis of the honeybee genome showed that honeybees possess only one-third the number of immune response genes known for solitary insects (Evans *et al.*, 2006). This apparent lack of immune genes could be explained by a highly effective and maybe less costly social immunity



compared with individual immunity (Cremer *et al.*, 2007). In honeybees, collective immune defence is well developed and includes hygienic behaviour, which is an antiseptic behaviour consisting of the ability to detect and remove diseased brood from the hive (Wilson-Rich *et al.*, 2009). The secretion of antiseptics in brood food and honey constitute another type of social immunity. Interestingly, the interaction between parasitism and exposure to pesticides induced an immunosuppression at the social level by causing a significant decline of GOX activity. This enzyme is essential in producing the antiseptic and thus sterilizing larval food (Sano *et al.*, 2004) and honey (White *et al.*, 1963; Ohashi *et al.*, 1999). As a result, if the colony is not able to maintain levels of GOX activity by recruiting more workers for this task, a reduction of antiseptics in the colony would not only affect adult nestmates but also the brood survival, i.e. would weaken the colony in the long term. And even if the colony responds accurately to the need for antiseptic production by a massive worker recruitment, this would reduce worker allocation in others tasks (like food collecting) and thus induce also a cost for the colony.

The mechanisms by which the combination of both stressors causes a reduction in GOX activity are not known. Glucose oxidase is mainly expressed in the HPG (Ohashi *et al.*, 1999), but the size reduction of HPG observed in bees infected with *Nosema*, as also found by Wang and Moeller (1969), is not associated with a decline in GOX activity, suggesting no link between HPG size and GOX activity. One possible explanation is that microsporidia use glucose to generate energy for their development (see above). As a result, the lack of glucose available to the bee could be followed by a decrease in the expression of GOX. However, the similar spore number in *Nosema* groups and *Nosema* × imidacloprid groups does not explain the depression in GOX activity in the last group. So it is reasonable to suppose that the interaction of both stressors might accentuate the energetic stress and induce a cost for GOX production that cannot be overcome.

In order to determine the consistency of our results, we conducted the experiments on three different colonies and observed that colony origin had a significant effect on PO activity and THC. The different responses between colonies could be explained by different colony environment history (pathogens, food sources), genetic background or both. However, the colonies that were used in the experiments came from the same location and were exposed to the same environment, suggesting that genetic variation might influence those individual immunity parameters. Indeed, Evans and Pettis (2005) found considerable genetic variation between colonies regarding the immune responsiveness of colony members. On the contrary, GOX activity was consistent between colo-

nies, which would suggest a lower genetic variation across colonies regarding antiseptic production. A current hypothesis suggests that if social immunity is less costly and more effective than individual immunity, then selective pressure would favour collective defence against disease at the expense of individual defence (Cremer *et al.*, 2007). Consequently, higher selective pressure on social immunity would reduce genetic variation of this trait; however, this needs to be tested.

In summary, the interaction between microspore parasites and pesticide not only caused a higher rate of mortality but also demonstrated the potential to weaken colonies. By focusing either on the effects of pesticides or on parasites alone, their well-established interaction has been completely ignored despite clear evidences in IPM that entomopathogenic fungi act synergistically with sub-lethal doses of pesticides to kill insect pests. Thus, our study paves the way for future studies that will begin to tease apart the multiple factors that strain pollinator health. Therefore, multifactorial analysis should be performed in other pollinator species such as bumblebees, which show similar sensitivity to pesticides as honeybees (Goulson *et al.*, 2008), also are parasitized by *N. ceranae* as well as *N. bombi* (Plischuk *et al.*, 2009), and are also declining (Goulson *et al.*, 2008). With the increase in agricultural dependency on pollinators (Aizen *et al.*, 2008) and the pollinator declines looming worldwide, now, more than ever, studies are needed that reveal the interplay between our efforts at insect control, like the use of insecticides, and the pathogens that naturally infect the insect pollinators on which we depend for our survival.

#### Experimental procedure\*

Experiments were performed at the Institut National de la Recherche Agronomique of Avignon (France) with bees that were a mixture of *Apis mellifera ligustica* and *Apis mellifera mellifera* typically used for beekeeping in south-east France. *Nosema* infection and exposure to imidacloprid were performed on 1-day-old bees held in cages (10.5 × 7.5 × 11.5 cm) and in the dark at 28°C and 70% relative humidity. They were fed *ad libitum* with candy (30% honey, 70% powdered sugar) and water. To simulate as much as possible colony rearing conditions, caged bees were also supplied with pollen to provide proteins required for their normal development and exposed to a Beeboost® (Pherotech, Delta, BC, Canada) releasing one queen-equivalent of queen mandibular pheromone per day.

In order to test the interactions between *Nosema* and imidacloprid on mortality and immunity, four experimental groups were created: control group, groups infected with *Nosema*, groups chronically exposed to imidacloprid and groups both infected with *Nosema* and chronically exposed to imidacloprid.

The chronic treatments were performed over 10 days. Indeed, mortality due to artificial rearing might be observed in longer periods. Three cages of 30 bees and two cages of 120

\* Méthodologie employée pour l'élevage et infection d'abeilles de l'article n°1 ( *Nosema* spp. infection alters pheromone production).



bees per experimental group and colony were, respectively, used for the mortality and immune assays. The experiments were repeated using bees from three colonies. Both mortality and immune assays were performed at the same time to avoid any bias due to the weather or season on bee physiology.

#### *Nosema* infection\*

Spores were isolated from colonies, according to the protocol developed by Higes and colleagues (2007). The spore concentration of the suspension was determined using a haemocytometer, and the solution was used for honey bee infection. To ensure that each bee of *Nosema*-infected groups was infected with the same dose of *Nosema* 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 *Nosema*. Similar spore numbers are known to cause an infection in worker bees (Malone and Gatehouse, 1998; Higes *et al.*, 2007). Control and imidacloprid-treated bees were fed with a sucrose solution.

At days 5 and 10, bees from each cage were collected to determine the level of *Nosema* infection using a haemocytometer. The species identification revealed that our bees were infected with both species of *Nosema*, *N. apis* and *N. ceranae* as it is the case in other regions (Paxton *et al.*, 2007) (see Supporting information for the procedure).

#### Imidacloprid treatment

The neonicotinoid imidacloprid [1-(6-chloro-3-pyridylmethyl)-N-nitro-imidazolidin-2-ylidene amine] was present in concentration reaching 5 µg kg<sup>-1</sup> in honey and pollen in various studies (Bogdanov, 2006), which represents a concentration of around 7 µg kg<sup>-1</sup> of sugar syrup. Accordingly, low, average and high concentrations corresponding to 0.7, 7 and 70 µg kg<sup>-1</sup> of imidacloprid were used for the mortality assay. Preliminary results obtained on young bees showed that an imidacloprid concentration of 7 µg kg<sup>-1</sup> corresponds to a sub-lethal dose in an acute intoxication assay (data not shown).

A stock solution of imidacloprid (Cluzeau, France) was diluted to the required concentration with dimethyl sulfoxide (DMSO), water and finally sucrose feeding to obtain final concentrations of 50% (w/v) sucrose, 0.1% DMSO and imidacloprid at the appropriate concentration (0.7, 7 and 70 µg kg<sup>-1</sup>). The imidacloprid solutions were freshly prepared each day. Solutions containing sucrose and DMSO were used as controls. Bees were chronically exposed to imidacloprid by ingesting imidacloprid-containing sugar syrup (50% sucrose solution, w/v) 10 h per day. This method allowed chronic treatments with minimal disturbance. The feeders were replaced each day at the same time of the day and to estimate the energetic demands the daily sucrose consumption was measured for each cage. The amount of sucrose consumed was expressed per day (10 h period) and per bee, by dividing the amount consumed in a cage by the number of remaining bees in this cage. The rest of the time, they were fed with candy and water *ad libitum*.

#### Immune parameters

Immune parameters were measured in 5- and 10-day-old bees. To determine the THC, haemolymph was extracted with micro capillaries (10 µl) from the second abdominal tergite and diluted 2:10 in ice cold ringer saline. Total haemocyte count per microlitre of haemolymph was performed using a phase contrast microscope (>200) with haemocytometer. Phenoloxidase activity was measured on abdomen devoid of its digestive tract instead of haemolymph. The specific PO activity was lower in the abdomen compared with haemolymph but the variability in the activity was also lower in the abdomen (Fig. S1), probably due to a high variance in the volume of haemolymph between individuals. Glucose oxidase is synthesized in the HPGs (Ohashi *et al.*, 1999). As the size of the HPGs reaches a maximum in c. 10-day-old bees (Crailsheim *et al.*, 1992), GOX activity was measured at day 10 on whole heads. For each enzyme, the activity was normalized to the protein concentration of each sample. In order to correlate the GOX activity with the size of the HPG, we also dissected HPG from workers of each experimental group and their size was classified into five defined stages of development (stage 1: totally undeveloped, stage 5: fully developed).

#### Statistical analysis

In the mortality assay, daily counts of the number of dead bees of corresponding colony replicates were added together. Then, the daily cumulative numbers of dead bees were log-transformed. Analysis of mortality rates was performed using a generalized linear model function. The effects of treatments on *Nosema* infection, THC, protein concentration, enzymatic activity, HPG development and feeding behavior was determined using analysis of the variance (two- and three-way ANOVA and repeated measures two-way ANOVA for the last measurement). Bonferroni post-hoc unpaired *t*-tests were performed for pairwise comparisons between the different treatments. Statistical analyses were performed using Sigmastat 3.10 and Statistica 8.0.

#### Acknowledgements

We thank A. Maisonnasse, D. Beslay and others lab members for assistance with bees; M.R. Schmid and M. Brehélin for advise on experiments; A. Kretzschmar for help with statistics and M.L. Winston, C.M. McDonnell, lab members and two anonymous referees for comments that improved the manuscript. Fundings were provided by HFSP (RGP0042/2007) and FEOGA grants. C. Alaux was supported by an INRA young researcher position (INRA SPE department) and C. Dussaubat by a CONICYT/French Embassy of Chili grant.

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\* Méthodologie employée pour l'élevage et infection d'abeilles de l'article n°1 (*Nosema* spp. infection alters pheromone production).



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#### Supporting information

Additional Supporting Information may be found in the online version of this article:

**Supplementary text 1.** *Nosema* species identification.

**Supplementary text 2.** Enzymatic activity measurements.

**Fig. S1.** Specific PO activities in different body parts of honeybees. Phenoloxidase activity was measured in haemolymph, thorax, abdomen and abdomen devoid of the digestive tract. Means  $\pm$  SE are shown.

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## **Annexe 2**

### **Recherche complémentaire**

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#### *Article en co-auteur*

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

Journal of Invertebrate Pathology

2011. 106, 380-385

Alaux, C., Folschweiller, M., McDonnell, C., Beslay, D., Cousin, M., **Dussaubat, C.\***, Brunet, J-L.,  
Le Conte, Y.

**\*Contribution à cet article** : collaboration avec la méthode d'extraction et purification de spores de *Nosema* à utiliser dans les infections expérimentales. Commentaires au manuscrit.

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

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### ARTICLE INFO

#### Article history:

Received 27 September 2010

Accepted 7 December 2010

Available online 13 December 2010

#### Keywords:

*Nosema ceranae*

Honey bee queen

*Apis mellifera*

Vitellogenin

Queen mandibular pheromones

Antioxidant capacity

### 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 *A. 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).

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### 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: *Nosema apis* (Zander, 1909) and *Nosema 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 *A. 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,

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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). 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 1 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 eight 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 3–7. 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 min and washed with water. The stained tissue was observed under microscope at 400× 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 electrophoresis 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% sodium chloride, 0.1% glucose, pH 7.4). The homogenates were then centrifuged at 15,000g 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 sulfonate]) 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  $\mu$ l of methanol and 100  $\mu$ l of decanoic acid (250 ng/ $\mu$ l; internal standard) for 2 min on ice. 20  $\mu$ l of the supernatant was collected and concentrated under nitrogen stream and then derivatized with 5  $\mu$ l of bistrimethylsilyltrifluoroacetamide. The solution was homogenized and left at room temperature for 40 min. The derivatized sample was then diluted in 100  $\mu$ l of iso-hexane and 1  $\mu$ l 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  $^{\circ}$ C, then increased to 200  $^{\circ}$ C at 40  $^{\circ}$ C min $^{-1}$  and to 250  $^{\circ}$ C at 10  $^{\circ}$ C min $^{-1}$  and held at 250  $^{\circ}$ 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 ( $\pm 6 \times 10^6$ ). 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 used 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

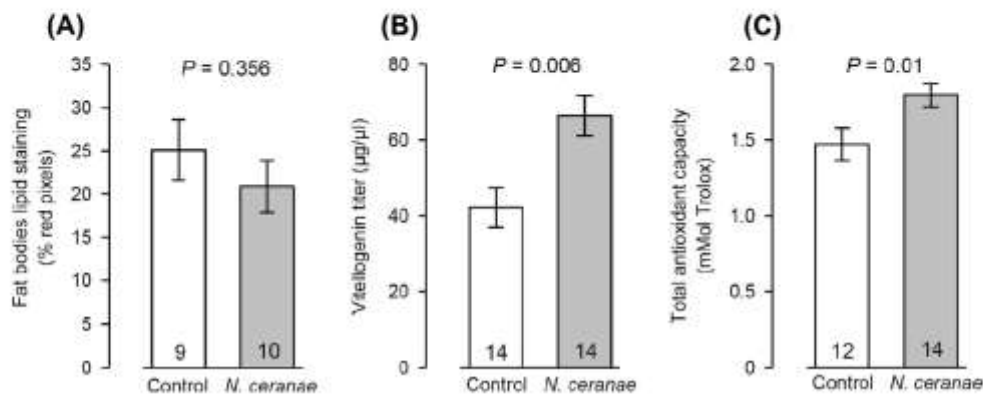


Fig. 1. Pathological effects of *N. ceranae* on different queen physiological parameters (mean  $\pm$  SE). The levels of lipid store in the fat bodies (A), Vg titer in hemolymph (B) and total antioxidant capacities in the whole abdomen (C) between control and infected queens were compared using Mann–Whitney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



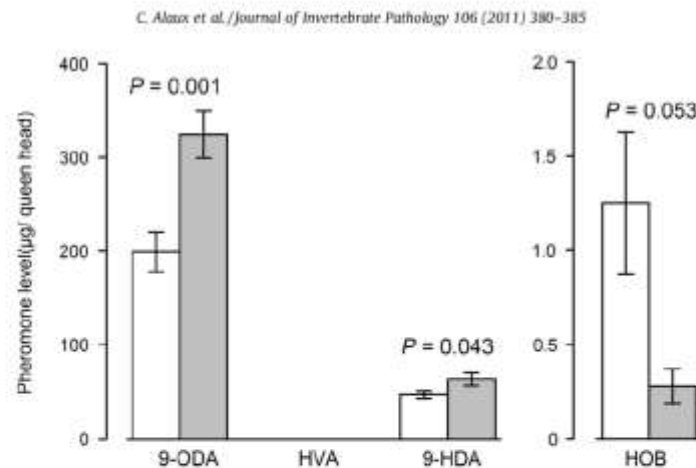


Fig. 2. Pathological effects of *N. ceranae* on QMP levels in queen heads (mean  $\pm$  SE). The levels of 9-ODA, HVA 9-HDA and HOB ( $\mu\text{g}/\text{head}$ ) between control ( $n = 9$ , white bar) and infected queens ( $n = 10$ , gray bar) were compared using Mann-Whitney U tests.

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	<b>-0.604</b>			
9-ODA	0.161	0.089		
HOB	-0.134	-0.290	-0.385	
9-HDA	-0.168	0.265	0.066	-0.215

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, 2009; Tong et al., 2010) 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). 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.

### Acknowledgments

We thank A. Maisonnasse and D. Crauser for their assistance with queen rearing and members of the lab and anonymous referees for comments that improve the manuscript. This work was funded by a BEEDOC grant (FP7, RTD REG/E.A(2009)D/561221). C. Alaux was supported by an INRA young researcher position (INRA SPE department), C. McDonnell by the BEEDOC grant and C. Dussauby by a CONICYT/French Embassy of Chile grant.

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## **Annexe 3**

### **Supporting information**

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#### *Article n°3*

### **Gut pathology and responses to the Microsporidium *Nosema ceranae* in the honey bee *Apis mellifera***

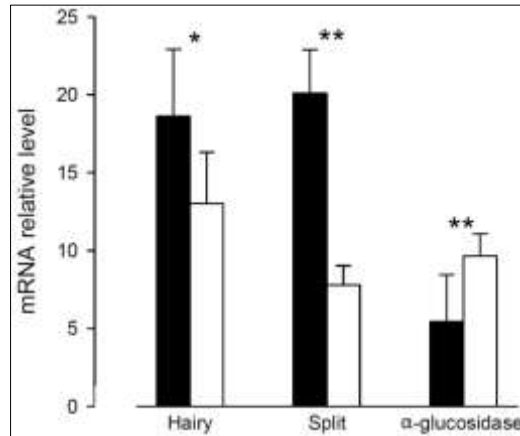
Plos One

2012. 7, e37017.

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**Figure S1.** Validation of microarray results with qPCRs. Expression level of 3 genes chosen among the set of genes differentially transcribed between control (black bars) and *N. ceranae*-infected bees (white bars). Data are normalized to expression levels of *eIF3-58*. Mean $\pm$ SE are shown for 6 pools of 4 bees per treatment (24 bees total/treatment). Significant differences were determined using Mann-Whitney U tests (*Hairy*:  $p=0.041$ , *Slit*:  $p=0.002$ ,  $\alpha$ -glucosidase:  $p=0.004$ ). \* and \*\* denote significant differences at  $p<0.05$  and  $p<0.01$ , respectively.



Annexe 3.2 - Supporting information – Table S1

Article n°3: Gut pathology and responses to the Microsporidium *Nosema ceranae* in the honey bee *Apis mellifera*

**Table S1** : List of honey bee genes differentially expressed after *N. ceranae* parasitism. The *M*-value-log2 fold change and the *q*-value are given for each gene. Corresponding honey bee genes and *Drosophila* orthologs are shown.

Honey bee genes	Drosophila orthologs	M-value	q-value	Honey bee genes	Drosophila orthologs	M-value	q-value
GB16848	/	-0.93	0.035	GB01218	Adherent junction protein p120	-0.71	0.026
GB13267	/	-1.41	0.015	GB19062	CG4238	-0.70	0.029
GB16836	CG13248	-1.31	0.039	GB19990	dally-like	-0.70	0.084
GB18106	CG8552	-1.25	0.038	GB18785	bound in neurons	-0.70	0.071
GB10457	Furin 1	-1.17	0.038	GB10970	/	-0.70	0.052
GB18284	α-Mannosidase I	-1.13	0.035	GB00349	/	-0.70	0.025
GB18435	CG1312	-1.09	0.046	GB16457	γ-shaped	-0.69	0.075
GB29547	CG9409	-1.08	0.035	GB12564	Synapsin	-0.69	0.046
GB4857	Hairy	-1.08	0.030	GB10929	CG42658	-0.69	0.052
GB16811	gutaviv	-1.06	0.041	GB18299	/	-0.69	0.071
GB19294	Keren	-1.03	0.038	GB12936	CG32137	-0.68	0.075
GB13664	Ecdysone-induced protein 75B	-1.03	0.022	GB15886	milton	-0.68	0.086
GB14914	hanta-mania	-1.03	0.080	GB12810	CG12969	-0.68	0.031
GB14128	CG32264	-1.03	0.046	GB17329 (Milk-1)	CG18389	-0.68	0.052
GB11122	PAPS synthetase	-1.02	0.038	GB12708	CG582	-0.67	0.046
GB16799	CG11140	-1.01	0.034	GB13345	/	-0.67	0.071
GB16112	ragosa	-1.01	0.022	GB1731	Muscle-specific protein 300	-0.67	0.055
GB18582	CG1541	-1.01	0.029	GB18394 (foraging)	foraging	-0.67	0.070
GB13544	connetto	-0.99	0.038	GB15295	Pituitary homeobox 1 homolog	-0.67	0.073
GB18723	CG92052	-0.97	0.022	GB17389	shofan	-0.67	0.067
GB20055	Na-pump alpha subunit	-0.97	0.020	GB14311	/	-0.66	0.060
GB14893	Trehalase	-0.96	0.030	GB19994	CG12809	-0.66	0.043
GB16292	CG9214	-0.94	0.020	GB18613	pointed	-0.66	0.057
GB12207	Epidermal growth factor receptor	-0.93	0.038	GB13224	Multi drug resistance 4B	-0.65	0.039
GB16146	/	-0.65	0.037				
GB16363	CG4092	-0.65	0.063				
GB14769	CG42674	-0.65	0.063				
GB11221	CG11221	-0.65	0.084				
GB10930	CG12086	-0.64	0.052				
GB18409	CG18154	-0.64	0.073				
GB10958	CG13523	-0.64	0.066				
GB10529	/	-0.63	0.089				
GB18065	suffrutescens	-0.63	0.072				
GB10648	crossveinless 2	-0.63	0.054				
GB17128	CG17879	-0.62	0.085				
GB18507	CG1600	-0.62	0.086				
GB17701	Sulfated	-0.62	0.054				
GB12589	CG3921	-0.61	0.048				
GB14817	schizo	-0.61	0.052				
GB10012	/	-0.61	0.052				
GB14432	CG12006	-0.61	0.075				
GB11634	Hormone receptor-like m 3B	-0.61	0.065				
GB11341	/	-0.60	0.099				
GB10445	Rap80L	-0.60	0.071				
GB14947	Dystroglycan	-0.60	0.048				
GB19606	Muscle LIM protein at 60A	-0.60	0.048				
GB16723	Pivot	-0.60	0.075				
GB11625	Semaphorin 5c	-0.60	0.061				
GB18394	win	-0.60	0.048				
GB20961	/	-0.60	0.099				
GB11270	CG1218	-0.59	0.067				
GB13893	CG0220	-0.59	0.099				
GB19687	/	-0.58	0.075				
GB17247	Olig family	-0.58	0.062				
GB10061	retinal degeneration 8	-0.58	0.052				
GB11129	de-fanged	-0.58	0.084				
GB14473	polypeptide-GalNAc transferase 5	-0.58	0.083				
GB10904	purvis	-0.58	0.065				
GB19612	CG14335	-0.57	0.067				
GB10129	/	-0.57	0.089				
GB20517	/	-0.56	0.087				
GB17759	abstracted	-0.56	0.071				
GB14729	/	-0.55	0.098				
GB14180	CG13375	-0.55	0.075				
GB13893	Dystrophin	-0.55	0.075				
GB14506	gruoch	-0.54	0.065				
GB18175	/	-0.54	0.095				
GB12765	inspied 2	-0.54	0.083				
GB16401	lethalis	-0.53	0.073				
GB12185	lethal [1]01289	-0.52	0.064				
GB15332	CG14383	-0.52	0.096				
GB18408	/	-0.56	0.088				
GB17356	/	-0.51	0.071				
GB15099	CG14403	-0.51	0.076				
GB18077	CG10044	-0.50	0.080				
GB13220	Zband alternatively spliced PDZ-motif protein 22	-0.50	0.094				
GB10724	/	0.48	0.097				
GB11683	CG1446	0.48	0.099				
GB10809	CG1636	0.48	0.099				
GB11926	CG13941	0.48	0.097				
GB14205	CG11267	0.49	0.099				
GB13292	CG15629	0.50	0.096				
GB12095	/	0.51	0.090				
GB16445	ATP synthase, subunit d	0.52	0.077				
GB11239	/	0.52	0.090				
GB18955	Glutathione peroxidase	0.52	0.099				
GB14901	CG17996	0.53	0.097				
GB15335	Adenylate kinase-5	0.53	0.084				
GB14559	/	0.53	0.069				
GB15565	/	0.53	0.073				
GB11759	mitochondrial ribosomal protein S14	0.53	0.099				
GB14302	/	0.53	0.067				
GB10365	Calpain C	0.53	0.071				
GB14572	/	0.54	0.099				
GB17060	Protein LSM12 homolog A	0.54	0.089				
GB18522	Enoase	0.54	0.097				
GB11978	inigan	0.54	0.067				
GB18376	/	0.54	0.078				
GB11150	CG17856	0.55	0.098				
GB10186	/	0.55	0.085				
GB14185	CG15109	0.55	0.099				
GB15826	CG15808	0.55	0.073				
GB15202	CG1349	0.55	0.090				

Table S1 (continuation)

Honey bee genes	Drosophila orthologs	M-value	q-value	Honey bee genes	Drosophila orthologs	M-value	q-value
GB16434	CG1368	0.55	0.054	GB14047	/	0.80	0.022
GB12896	/	0.55	0.098	GB11650	/	0.80	0.025
GB17648	CG1365	0.56	0.039	GB12831	CG1528	0.75	0.065
GB16685	CG3040	0.56	0.067	GB19754	/	0.81	0.029
GB12849	CG11069	0.56	0.073	GB12337	/	0.81	0.022
GB12087	CG1377	0.56	0.054	GB12932	CG4197	0.81	0.035
GB10007	Inositol 1,4,5-trisphosphate kinase 1	0.56	0.067	GB13895	CG1441	0.81	0.021
GB13888	CG30035	0.56	0.055	GB15830	/	0.83	0.054
GB10195	CG34417	0.56	0.085	GB17722	/	0.83	0.067
GB10723	CG4325	0.56	0.054	GB12125	CG4469	0.84	0.022
GB19658	CG1382	0.57	0.094	GB10080	/	0.84	0.030
GB16802	CG5446	0.57	0.084	GB18816	CG17664	0.85	0.073
GB10005	CG5676	0.57	0.084	GB15176	CG17029	0.86	0.023
GB16050	/	0.57	0.069	GB19701	/	0.86	0.018
GB18931	CG8225	0.57	0.074	GB19954	CG5056	0.86	0.028
GB17789	Histone H4 replicant	0.57	0.072	GB11572	Inositol-3-phosphate synthase	0.86	0.042
GB18735	Niemann-Pick Type C-2	0.58	0.057	GB16582	/	0.90	0.024
GB12779	shar	0.58	0.090	GB16218	Juglithin A	0.90	0.025
GB19230	/	0.58	0.058	GB18760	CG1322	0.90	0.023
GB12875	CG8668	0.58	0.082	GB18709	CG15449	0.90	0.071
GB18197	CG2164	0.58	0.071	GB13166	/	0.91	0.036
GB17584	/	0.58	0.085	GB17389	/	0.91	0.026
GB19718	Sarcoglycan beta	0.58	0.067	GB17185	/	0.91	0.018
GB13257	Transporter	0.58	0.093	GB16688	/	0.91	0.048
GB17992	/	0.58	0.045	GB11064	/	0.94	0.052
GB13851	CG1246	0.59	0.093	GB11439	/	0.97	0.018
GB13327	CG8665	0.59	0.065	GB17823	CG12263	0.98	0.025
GB10125	/	0.59	0.052	GB12782	/	0.98	0.025
GB18082	/	0.60	0.070	GB13005	CG18003	0.99	0.018
GB11698	CG1299	0.60	0.048	GB10990	/	1.00	0.015
GB16697	/	0.60	0.070	GB13228	CG1402	1.01	0.022
GB11710	BMG-bee protein 1A	0.61	0.072	GB10321	/	1.03	0.099
GB17478	CG1777	0.61	0.067	GB17697	CG4188	1.03	0.018
GB18001	Tetraspanin 97E	0.61	0.059	GB11493	CG6414	1.04	0.018
GB15673	CG14196	0.61	0.058	GB15421	/	1.00	0.022
GB10052	CG30157	0.61	0.052	GB15990 (FAMP-like protein)	/	1.12	0.025
GB18694	/	0.61	0.077	GB13135	/	1.12	0.015
GB18683	Armadillo 8	0.62	0.064	GB12705	CG10101	1.14	0.043
GB17041	Rhythmically expressed gene 2	0.62	0.097	GB10992	CG6293	1.24	0.015
GB11787	CG1047	0.62	0.047	GB13230	/	1.27	0.015
GB17752	CG13100	0.62	0.088	GB17633	heat-VI	1.38	0.015
GB19932	pickpocket 23	0.63	0.048	GB12176	CG1326	1.73	0.022
GB16911	Excitatory amino acid transporter 1	0.63	0.075				
GB10795	CG8839	0.63	0.063				
GB11444	CG13937	0.62	0.045				
GB14578	/	0.64	0.051				
GB14528	CG2680	0.64	0.070				
GB15793 (CHP6A64)	/	0.64	0.030				
GB10409	/	0.60	0.048				
GB10466 (CHP6DC1)	/	0.65	0.030				
GB10515	CG6927	0.67	0.061				
GB15494	/	0.68	0.043				
GB12374	CG9149	0.68	0.085				
GB19828	/	0.67	0.052				
GB12128	CG8420	0.67	0.099				
GB12977	p16-ARC	0.67	0.073				
GB10227	Catalase	0.68	0.070				
GB16598	/	0.68	0.030				
GB10417	Cuticular protein 85Aa	0.68	0.038				
GB13291	CG30051	0.68	0.062				
GB15318	Slowpoke binding protein	0.69	0.035				
GB15299 (fatty acid binding protein)	CG6782	0.69	0.028				
GB13332	Phosphotriesterase-related protein	0.69	0.025				
GB19995	/	0.69	0.052				
GB10002	CG10527	0.69	0.025				
GB11370	CG13664	0.69	0.025				
GB15517	CG10337	0.69	0.083				
GB13036	CG9269	0.70	0.090				
GB16452	Gram-negative bacteria binding protein 2	0.70	0.025				
GB15546	UDP-glycosyltransferase 35b	0.70	0.069				
GB12470	CG11534	0.71	0.025				
GB13255	Tetraspanin 68E	0.72	0.052				
GB11465	Multiple inositol polyphosphate phosphatase 1	0.72	0.050				
GB16587	/	0.73	0.088				
GB19017 (alpha- glucosidase)	CG14925	0.73	0.022				
GB15843	/	0.74	0.069				
GB1299 (iodoant binding protein 12)	/	0.74	0.052				
GB14094 (corticosterone releasing hormone binding protein)	CG15537	0.74	0.029				
GB10087	/	0.74	0.058				
GB18099	CG8271	0.75	0.065				
GB11304	CG16654	0.75	0.023				
GB15768	Flavin-containing monooxygenase 1	0.75	0.067				
GB10791	/	0.75	0.022				
GB10545 (trepanin C type II)	Trepanin C at 73f	0.76	0.026				
GB16385	/	0.76	0.046				
GB14306	/	0.77	0.022				
GB18089	CG17044	0.78	0.028				
GB18478	CG42389	0.78	0.025				
GB10018	Sarcoplasmic calcium binding protein 2	0.78	0.056				
GB15970	/	0.78	0.024				
GB14348	/	0.75	0.039				
GB15002	CG41366	0.80	0.046				

**Table S2** : List of genes associated with each Gene Ontology category (see Table 1 from same article). GB accession number, fly orthologs and *M*- and *q*-values are shown.

Downregulated					Downregulated				
GO term	Bee name	Fly name	M-value	q-value	GO term	Bee name	Fly name	M-value	q-value
plasma membrane	GB13229	Zband alternatively spliced PDZ-motif protein	-0.50	0.09	neurotrophin system development	GB14857	hairy	-1.08	0.03
	GB19884	heartless	-0.86	0.02		GB10329	Myosin heavy chain	-0.88	0.02
	GB11625	Semaphorin-5c	-0.60	0.06		GB17989	shotgun	-0.67	0.07
	GB10342	tweetie	-0.74	0.05		GB16088	Cyclin-dependent kinase 4	-0.73	0.02
	GB12463	armadillo	-0.86	0.05		GB16181	crossveinless c	-0.81	0.02
	GB15780	Protein kinase 61C	-0.90	0.07		GB20055	Na pump alpha subunit	-0.96	0.02
	GB10437	Furin 1	-1.17	0.02		GB12207	Epidermal growth factor receptor	-0.94	0.02
	GB11452	CG33208	-0.61	0.07		GB12463	armadillo	-0.86	0.05
	GB12185	lethal (2)01289	-0.52	0.08		GB18065	sulfateless	-0.63	0.07
	GB18384	vein	-0.60	0.05		GB19929	slit	-0.89	0.04
	GB17989	shotgun	-0.67	0.07		GB19838	jing	-0.85	0.02
	GB13853	Dystroglycin	-0.55	0.07	GB18613	pointed	-0.66	0.06	
	GB12765	frizzled 2	-0.54	0.08	axon guidance	GB11999	dally-like	-0.70	0.08
	GB14967	Dystroglycan	-0.60	0.05		GB17989	shotgun	-0.67	0.07
	GB15061	retinal degeneration B	-0.58	0.05		GB11625	Semaphorin-5c	-0.60	0.06
	GB12207	Epidermal growth factor receptor	-0.94	0.02		GB11407	β Spectrin	-0.77	0.02
	GB14803	Trehalase	-0.96	0.03		GB19937	SoxNeuro	-0.85	0.05
	GB19698	Na <sup>+</sup> -driven anion exchanger 1	-0.74	0.03		GB19929	slit	-0.89	0.04
	GB11407	β Spectrin	-0.77	0.02		GB16014	Semaphorin-2A	-0.80	0.02
	GB14273	neuralized	-0.77	0.05		GB11452	CG33208	-0.61	0.07
	GB10186	CG1862	-0.78	0.06		GB17210	smooth	-0.74	0.07
	GB10071	CG18408	-0.51	0.08		GB17989	shotgun	-0.67	0.07
	GB14314	scavenger receptor acting in neural tissue and majority of rhodopsin is absent	-1.03	0.08		GB16012	rugose	-1.01	0.02
	neuron differentiation	GB13120	Glycoprotein 3-α-L-fucosyltransferase A	-0.87	0.05	GB12765	frizzled 2	-0.54	0.08
		GB12564	Synapsin	-0.69	0.05	GB18435	CG1212	-1.09	0.05
		GB20055	Na pump alpha subunit	-0.96	0.02	GB12207	Epidermal growth factor receptor	-0.94	0.02
		GB17403	Ca <sup>2+</sup> -channel-protein-β-subunit	-0.77	0.06	GB16401	basket	-0.80	0.03
		GB18394	foraging	-0.67	0.07	GB11407	β Spectrin	-0.77	0.02
		GB18435	CG1212	-1.09	0.05	GB10186	CG1862	-0.78	0.06
		GB11426	CG14992	-0.65	0.04	GB19838	jing	-0.85	0.02
		GB19294	Keren	-1.03	0.02	GB18613	pointed	-0.66	0.06
		GB13224	Multi drug-resistance 49	-0.65	0.04	GB10751	tramtrack	-0.71	0.05
		GB11999	dally-like	-0.70	0.08	transmembrane receptor protein tyrosine kinase signaling pathway	GB18384	vein	-0.60
GB14857		hairy	-1.08	0.03	GB19884		heartless	-0.86	0.02
GB11625		Semaphorin-5c	-0.60	0.06	GB16457		u-shaped	-0.69	0.07
GB13456		groucho	-0.54	0.06	GB10181		crossveinless c	-0.81	0.02
GB15295		Pituitary homeobox 1 homolog	-0.67	0.07	GB12207		Epidermal growth factor receptor	-0.94	0.02
GB12463		armadillo	-0.86	0.05	GB19294		Keren	-1.03	0.02
GB10504		pumilio	-0.57	0.05	GB15780		Protein kinase 61C	-0.90	0.07
GB19937		SoxNeuro	-0.85	0.05	GB18065		sulfateless	-0.63	0.07
GB19929	slit	-0.89	0.04	GB18613	pointed		-0.66	0.06	
GB16014	Semaphorin-2A	-0.80	0.02	neuronic cellular process	GB11625		Semaphorin-5c	-0.60	0.06
GB11452	CG33208	-0.61	0.07		GB20055		Na pump alpha subunit	-0.96	0.02
GB17210	smooth	-0.74	0.07		GB14273	neuralized	-0.77	0.05	
GB17989	shotgun	-0.67	0.07		GB10751	tramtrack	-0.71	0.05	
GB16012	rugose	-1.01	0.02		enzyme linked receptor protein signaling pathway	GB11999	dally-like	-0.70	0.08
GB12765	frizzled 2	-0.54	0.08	GB18384		vein	-0.60	0.05	
GB18435	CG1212	-1.09	0.05	GB19884		heartless	-0.86	0.02	
GB12207	Epidermal growth factor receptor	-0.94	0.02	GB16457		u-shaped	-0.69	0.07	
GB16401	basket	-0.80	0.03	GB16181		crossveinless c	-0.81	0.02	
GB11407	β Spectrin	-0.77	0.02	GB12207		Epidermal growth factor receptor	-0.94	0.02	
GB10186	CG1862	-0.78	0.06	GB19294		Keren	-1.03	0.02	
GB19838	jing	-0.85	0.02	GB15780		Protein kinase 61C	-0.90	0.07	
GB18613	pointed	-0.66	0.06	GB18065		sulfateless	-0.63	0.07	
GB10751	tramtrack	-0.71	0.05	GB18613		pointed	-0.66	0.06	
neuron development	GB11999	dally-like	-0.70	0.08		positive regulation of cell communication	GB11999	dally-like	-0.70
	GB11625	Semaphorin-5c	-0.60	0.06	GB18384		vein	-0.60	0.05
	GB13456	groucho	-0.54	0.06	GB12463		armadillo	-0.86	0.05
	GB15295	Pituitary homeobox 1 homolog	-0.67	0.07	GB11407		β Spectrin	-0.77	0.02
	GB10504	pumilio	-0.57	0.05	GB19294		Keren	-1.03	0.02
	GB19937	SoxNeuro	-0.85	0.05	GB14273	neuralized	-0.77	0.05	
	GB19929	slit	-0.89	0.04	intracellular signaling cascade	GB16799	CG31140	-1.01	0.03
	GB16014	Semaphorin-2A	-0.80	0.02		GB19901	puckered	-0.92	0.02
	GB11452	CG33208	-0.61	0.07		GB17258	CG9098	-0.71	0.03
	GB17210	smooth	-0.74	0.07		GB14180	CG3375	-0.55	0.08
	GB17989	shotgun	-0.67	0.07		GB20120	CG5712	-0.73	0.05
GB16012	rugose	-1.01	0.02	GB16688		Cyclin-dependent kinase 4	-0.73	0.02	
GB12765	frizzled 2	-0.54	0.08	GB10445		Raggap1	-0.60	0.07	
GB18435	CG1212	-1.09	0.05	GB16811		gustavus	-1.06	0.04	
GB12207	Epidermal growth factor receptor	-0.94	0.02	GB11276		CG3216	-0.59	0.07	
GB16401	basket	-0.80	0.03	GB11950		Rai guanine nucleotide exchange factor 2	-0.86	0.03	
GB11407	β Spectrin	-0.77	0.02			GB16401	basket	-0.80	0.03
GB10186	CG1862	-0.78	0.06		GB19294	Keren	-1.03	0.02	
GB19838	jing	-0.85	0.02		GB16444	CG10757	-0.78	0.03	
					GB18613	pointed	-0.66	0.06	



Table S2 (continuation)

Downregulated					Upregulated					
GO term	See name	Fly name	M-value	q-value	GO term	See name	Fly name	M-value	q-value	
protein amino acid phosphorylation	GB19884	heartless	-0.86	0.02	response to oxidative stress	GB10907	inositol 1,4,5-triphosphate kinase 1	0.56	0.07	
	GB15780	Protein kinase 61C	-0.90	0.07		GB15202	CG1349	0.55	0.09	
	GB16688	Cyclin-dependent kinase 4	-0.73	0.02		GB10227	Catalase	0.68	0.05	
	GB10752	Seminal centre kinase III	-0.74	0.07		GB12875	CG6666	0.58	0.06	
	GB14600	smell impaired 35A	-0.88	0.05		GB18955	Glutathione peroxidase	0.52	0.10	
	GB18394	foraging	-0.67	0.07	oxidation reduction	GB13505	CG18009	0.99	0.02	
	GB13245	Chromosomal serine/threonine-protein kinase JIL-1	-0.93	0.02		GB16218	sluggish A	0.90	0.02	
	GB11278	CG1218	-0.59	0.07		GB12327	CG8665	0.59	0.07	
	GB12207	epidermal growth factor receptor	-0.94	0.02		GB11683	CG3446	0.48	0.10	
	GB11426	CG14992	-0.65	0.04		GB10227	Catalase	0.68	0.05	
	GB12884	CG17090	-0.78	0.09		GB18760	CG7322	0.90	0.03	
	GB16401	basket	-0.80	0.03		GB13292	CG15629	0.30	0.10	
	GB14793	CG11221	-0.65	0.08		GB11150	CG17856	0.55	0.10	
	morphogenesis of an epithelium	GB19901	puckered	-0.92		0.02	GB15768	Flavin-containing monooxygenase 1	0.75	0.07
		GB17889	shotgun	-0.67		0.07	GB12875	CG6666	0.58	0.06
		GB16457	u-shaped	-0.69	0.07	GB18955	Glutathione peroxidase	0.52	0.10	
		GB14967	Dystroglycan	-0.60	0.05	Sugar transporter, contains six	GB12932	CG4797	0.81	0.04
GB16181		crossveinless c	-0.81	0.02	GB16424		CG3168	0.55	0.05	
GB14141		cheerio	-0.87	0.02	GB13688		CG30035	0.56	0.05	
GB12207		epidermal growth factor receptor	-0.94	0.02	GB17752		CG11100	0.67	0.08	
GB12453		armadillo	-0.86	0.05						
GB19294		keren	-1.03	0.02						
GB16401		basket	-0.80	0.03						
GB18065		suffocateless	-0.63	0.07						
GB18613		pointed	-0.66	0.06						



**Table S3:** List of genes integrated within the gene network downregulated by *N. ceranae* (see Fig. 4 from same article). GB accession number, fly orthologs and *M*- and *q*-values are shown.

Bee name	Fly name	Fly symbol	M-value	q-value
GB14857	hairy	hairy	-1,08	0,030
GB16811	gustavus	gus	-1,06	0,041
GB19294	Keren	Krn	-1,03	0,018
GB16012	rugose	rg	-1,01	0,022
GB12207	Epidermal growth factor receptor	egfr	-0,95	0,018
GB13245	JIL-1	JIL-1	-0,93	0,025
GB19901	puckered	puc	-0,92	0,023
GB19884	heartless	HTL	-0,90	0,019
GB19929	slit	slt	-0,89	0,045
GB12463	armadillo	arm	-0,86	0,048
GB11950	Rai guanine nucleotide exchange factor	Rgl	-0,86	0,035
GB19937	SoxNeuro	SoxN	-0,85	0,054
GB19838	jing	jing	-0,85	0,022
GB16569	diminutive	dm	-0,85	0,023
GB16401	basket	bsk	-0,80	0,029
GB11407	$\beta$ Spectrin	beta-Spec	-0,77	0,022
GB10792	Germinal centre kinase III	GckIII	-0,74	0,069
GB16688	Cyclin-dependent kinase 4	Cdk4	-0,73	0,025
GB10751	tramtrack	ttk	-0,71	0,046
GB30128	Adherens junction protein p120	p120ctn	-0,71	0,026
GB11999	dally-like	dlp	-0,70	0,084
GB16457	u-shaped	ush	-0,69	0,075
GB12936	CG32137	CG32137	-0,68	0,075
GB15886	milton	mlt	-0,68	0,086
GB12810	CG32369	CG32369	-0,68	0,031
GB18394	foraging	For	-0,67	0,070
GB17989	shotgun	shg	-0,67	0,067
GB18613	pointed	pnt	-0,66	0,057
GB18065	sulfateless	sfl	-0,63	0,072
GB14817	schizo	siz	-0,61	0,052
GB14967	Dystroglycan	Dg	-0,60	0,048
GB18384	vein	vn	-0,60	0,048
GB13456	groucho	gro	-0,54	0,065
GB12765	frizzled 2	fz2	-0,54	0,083

**Table S4:** List of *N. ceranae* probes significantly expressed. The *M*-value-log2 fold change and the *q*-value are given for each probe. Pfam and BLAST data on *Encephalitozoon cuniculi* genome originated from Cornman *et al.* (2009) [66].

Probe ID	Gene ID	PFAM	BLAST	M-value	q-value
NC000015_2575F5000019980	NcORF-00337	S-adenosylmethionine synthetase, C-ter (2.90E-081)	<i>S. cerevisiae</i> : S-adenosylmethionine synthetase, catalyzes transfer of the adenosyl group of ATP to the sulfur atom of methionine; one of two differentially regulated isozymes (Sam1p and Sam2p); Sam2p (7.00E-120) E. cuniculi: UBIQUITIN-ACTIVATING ENZYME E1 E. cuniculi: DNA-DIRECTED RNA-POLYMERASE II SECOND LARGEST SUBUNIT	1,48	0,07
NC000175_7705F5000005203	NcORF-01589	ThiF family (8.00E-043)	E. cuniculi: UBIQUITIN-ACTIVATING ENZYME E1	1,47	0,07
NC000075_12577F5000000782	NcORF-01025	RNA polymerase Rpb2, domain (8.50E-193)	E. cuniculi: DNA-DIRECTED RNA-POLYMERASE II SECOND LARGEST SUBUNIT	1,48	0,05
NC000010_28743F5000009872	NcORF-00245	Ribosomal Protein L2, C-terminal doma (2.30E-033)	E. cuniculi: 60S ribosomal protein L8	1,49	0,06
NC000003_44917F5000014282	NcORF-00087	Elongation factor Tu GTP binding domain (2.20E-063)	E. cuniculi: TRANSLATION ELONGATION FACTOR 2	1,52	0,04
NC000080_12260F5000000294	NcORF-01068	Ubiquitin-conjugating enzyme (1.50E-055)	E. cuniculi: UBIQUITIN CONJUGATING ENZYME E2-16kDa	1,58	0,04
NC000021_21707F50000007269	NcORF-00438	MCM2/3/5 family (1.20E-132)	E. cuniculi: DNA REPLICATION LICENSING FACTOR MCM4	1,57	0,04
NC000168_7822F5000005171	NcORF-01566	MCM2/3/5 family (7.20E-190)	E. cuniculi: PROTEASOME REGULATORY SUBUNIT E. cuniculi: DNA REPLICATION LICENSING FACTOR MCM2	1,57	0,06
NC000064_15148F5000004177	NcORF-00941	MCM2/3/5 family (7.20E-190)	E. cuniculi: DNA REPLICATION LICENSING FACTOR MCM2	1,58	0,08
NC000127_3606F500000597	NcORF-01383	ATPase family associated with various (8.50E-075)	E. cuniculi: HSP 101 RELATED PROTEIN	1,58	0,09
NC000083_11868F5000006564	NcORF-01092	ATPase family associated with various (1.30E-085)	E. cuniculi: ATPase	1,59	0,07
NC000084_11825F5000004096	NcORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuniculi: TUBULIN BETA CHAIN	1,61	0,02
NC000047_17039F50000011030	NcORF-00773	Uncharacterised ACR, Yagf family COG1 (2.10E-077)	E. cuniculi: similarity to HYPOTHETICAL TRANSMEMBRANE PROTEIN YAGF_SCHPD	1,62	0,08
NC000164_8004F5000005577	NcORF-01550	ADP-ribosylation factor family (1.20E-017)	E. cuniculi: ADP-RIBOSYLATION FACTOR-LIKE GTP-BINDING PROTEIN	1,62	0,06
NC000045_17399F5000014711	NcORF-00747	ATPase family associated with various (2.70E-004)	E. cuniculi: 26S PROTEASOME REGULATORY SUBUNIT 10	1,64	0,07
NC000020_22137F5000015805	NcORF-00423	TCP-1/cpn60 chaperonin family (2.30E-037)	E. cuniculi: T-COMPLEX PROTEIN 1 ZETA SUBUNIT	1,64	0,02
NC000029_19847F5000017673	NcORF-00560	Proteasome subunit A N-terminal signal (3.30E-007)	E. cuniculi: 26S PROTEASOME SUBUNIT ALPHA-4	1,64	0,03
NC000021_21707F5000005276	NcORF-00433	Rad51 (3.20E-197)	E. cuniculi: DNA REPAIR PROTEIN RAD51 HOMOLOG	1,66	0,03
NC000295_4886F5000000563	NcORF-01897	Elongation factor Tu GTP binding domain (4.20E-064)	E. cuniculi: TRANSLATION ELONGATION FACTOR 1 ALPHA	1,66	0,06
NC000084_11825F5000004271	NcORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuniculi: TUBULIN BETA CHAIN	1,71	0,02
NC000005_3680F50000023668	NcORF-00138	Zinc finger, C2HC4 type (RING finger) (8.80E-004)	E. cuniculi: similarity to HYPOTHETICAL INTEGRAL MEMBRANE PROTEIN YG55_CAEEL	1,72	0,06
NC000193_7106F50000003756	NcORF-01655	Metallo-beta-lactamase superfamily (7.50E-015)	E. cuniculi: similarity to HYPOTHETICAL PROTEIN Y162_METJA	1,74	0,02
NC000020_22137F5000008265	NcORF-00418	Armadillo/beta-catenin-like repeat (1.20E-003)	E. cuniculi: IMPORTIN ALPHA SUBUNIT (KARYOPHIBIN)	1,76	0,03
NC000016_24848F500000564	NcORF-00344	TCP-1/cpn60 chaperonin family (1.80E-006)	E. cuniculi: T-COMPLEX PROTEIN 1 EPSILON SUBUNIT	1,77	0,01
NC000063_15153F5000005996	NcORF-00936	Radical SAM superfamily (4.90E-015)	E. cuniculi: similarity to HYPOTHETICAL PROTEIN YB3E_METJA	1,78	0,07
NC000264_5403F5000002991	NcORF-01831	Dynamain family (6.60E-075)	E. cuniculi: DYNAMAIN-LIKE VACUOLAR PROTEIN SORTING PROTEIN	1,79	0,02
NC000001_85607F5000019213	NcORF-00015	ATPase family associated with various (5.20E-003)	E. cuniculi: 26S PROTEASOME REGULATORY SUBUNIT 7	1,80	0,10
NC000010_28743F5000006296	NcORF-00242	SNF2 family N-terminal domain (5.60E-101)	E. cuniculi: helicase DNA-binding protein	1,80	0,01
NC000175_7705F5000004971	NcORF-01589	ThiF family (8.00E-043)	E. cuniculi: UBIQUITIN-ACTIVATING ENZYME E1	1,81	0,05
NC000247_5488F5000001279	NcORF-01800	RNA polymerase Rpb1, domain (9.70E-166)	E. cuniculi: DNA-DIRECTED RNA-POLYMERASE II	1,82	0,02
NC000164_8004F5000005592	NcORF-01550	ADP-ribosylation factor family (1.20E-017)	E. cuniculi: ADP-RIBOSYLATION FACTOR-LIKE GTP-BINDING PROTEIN	1,83	0,08
NC000127_3606F5000007802	NcORF-01383	ATPase family associated with various (8.50E-075)	E. cuniculi: HSP 101 RELATED PROTEIN	1,83	0,02
NC000024_20906F5000018453	NcORF-00497	Core histone H2A/H2B/H3/H4 (1.80E-010)	E. cuniculi: histone H4	1,84	0,03
NC000075_12577F5000001837	NcORF-01025	RNA polymerase Rpb2, domain (8.50E-193)	E. cuniculi: DNA-DIRECTED RNA-POLYMERASE II SECOND LARGEST SUBUNIT	1,84	0,04
NC000150_8497F5000007563	NcORF-01486	MCM2/3/5 family (3.40E-140)	E. cuniculi: DNA REPLICATION LICENSING FACTOR OF THE MCM FAMILY (MCM6)	1,84	0,07
NC000010_28743F5000005646	NcORF-00242	SNF2 family N-terminal domain (5.60E-101)	E. cuniculi: helicase DNA-binding protein	1,85	0,06
NC000048_16997F5000008530	NcORF-00785		E. cuniculi: similarity to HYPOTHETICAL WD-REPEAT PROTEIN YNS7_yeast	1,85	0,02
NC000040_18116F5000001333	NcORF-00688	IRNA synthetases class II (D, K and N) (5.70E-076)	E. cuniculi: asparagine-tRNA-glycine	1,89	0,01
NC000211_8337F5000002667	NcORF-01704	SRP54-type protein, GTPase domain (7.70E-011)	E. cuniculi: SIGNAL RECOGNITION PARTICLE 54kDa SUBUNIT (SRP54)	1,90	0,01
NC000045_17399F5000014491	NcORF-00747	ATPase family associated with various (2.70E-004)	E. cuniculi: 26S PROTEASOME REGULATORY SUBUNIT 10	1,90	0,01
NC000083_11868F5000005503	NcORF-01092	ATPase family associated with various (1.30E-085)	E. cuniculi: ATPase	1,90	0,03
NC000001_85607F5000018745	NcORF-00015	ATPase family associated with various (5.20E-003)	E. cuniculi: 26S PROTEASOME REGULATORY SUBUNIT 7	1,91	0,02
NC000295_4886F5000000983	NcORF-01897	Elongation factor Tu GTP binding domain (4.20E-064)	E. cuniculi: TRANSLATION ELONGATION FACTOR 1 ALPHA	1,92	0,04
NC000154_8366F5000006004	NcORF-01506	Ribosomal protein S5, C-terminal doma (1.40E-028)	E. cuniculi: 40S RIBOSOMAL PROTEIN S2	1,93	0,01
NC000103_10738F5000008747	NcORF-01220	Plug domain of SecE1p (9.00E-018)	E. cuniculi: ER protein-translation complex subunit alpha	1,93	0,02
NC000015_2575F50000019960	NcORF-00337	S-adenosylmethionine synthetase, C-ter (2.90E-081)	<i>S. cerevisiae</i> : S-adenosylmethionine synthetase, catalyzes transfer of the adenosyl group of ATP to the sulfur atom of methionine; one of two differentially regulated isozymes (Sam1p and Sam2p); Sam2p (7.00E-120) E. cuniculi: UBIQUITIN-ACTIVATING ENZYME E1 E. cuniculi: DNA-DIRECTED RNA-POLYMERASE II SECOND LARGEST SUBUNIT	1,94	0,00
NC000014_26276F5000009854	NcORF-00321	TCP-1/cpn60 chaperonin family (8.40E-105)	E. cuniculi: T-COMPLEX PROTEIN 1 ETA SUBUNIT	1,96	0,00
NC000010_28743F5000005661	NcORF-00242	SNF2 family N-terminal domain (5.60E-101)	E. cuniculi: helicase DNA-binding protein	1,97	0,02
NC000005_2693F50000002149	NcORF-02189	ATPase family associated with various (4.30E-004)	E. cuniculi: 26S PROTEASOME REGULATORY SUBUNIT 4	1,98	0,01
NC000091_11393F5000008066	NcORF-01137	RNA recognition motif, (a. k. a. RRM, RR) (3.20E-005)	E. cuniculi: similarity to 20kDa NUCLEAR CAP-BINDING PROTEIN	1,98	0,04
NC000016_24848F5000004512	NcORF-00349	LNS2 (Lipin/Ned1/Smp2) (5.90E-093)	E. cuniculi: 60S RIBOSOMAL PROTEIN L19	2,00	0,08
NC000356_3856F5000001859	NcORF-02018	LNS2 (Lipin/Ned1/Smp2) (5.90E-093)	E. cuniculi: similarity to yeast gene INVOLVED IN PLASMID MAINTENANCE	2,00	0,02
NC000038_18383F5000002529	NcORF-00667	RNA polymerase Rpb2, domain (8.50E-134)	E. cuniculi: DNA-DIRECTED RNA-POLYMERASE I, SECOND LARGEST SUBUNIT	2,01	0,00
NC000272_5233F5000002746	NcORF-01850	MutS domain V (1.10E-070)	E. cuniculi: DNA MISMATCH REPAIR PROTEIN OF THE MUTS FAMILY	2,03	0,00
NC000021_21707F50000007289	NcORF-00438	MCM2/3/5 family (1.20E-132)	E. cuniculi: DNA REPLICATION LICENSING FACTOR MCM4	2,04	0,01
NC000008_29729F5000015153	NcORF-00216	Ribosomal protein L3 (1.30E-096)	E. cuniculi: 60S ribosomal protein L3	2,04	0,00
NC000175_7705F5000005648	NcORF-01589	ThiF family (8.00E-043)	E. cuniculi: UBIQUITIN-ACTIVATING ENZYME E1	2,04	0,00
NC000127_3606F5000007578	NcORF-01383	ATPase family associated with various (8.50E-075)	E. cuniculi: HSP 101 RELATED PROTEIN	2,07	0,06



Annexe 3.5 - Supporting information – Table S4

Article n°3: Gut pathology and responses to the Microsporidium *Nosema ceranae* in the honey bee *Apis mellifera*

Table S4 (continuation)

Probe ID	Gene ID	PFAM	BLAST	h-value	q-value
NC000016_24848F5000000828	NCORF-00344	TCF-1/cpn60 chaperonin family (1.80E-006)	E. cuticulari: T COMPLEX PROTEIN 1 EPSILON SUBUNIT	2.10	0.02
NC000083_11868F50000005133	NCORF-01092	ATPase family associated with various (1.30E-085)	E. cuticulari: ATPase	2.10	0.00
NC000010_28743F50000006521	NCORF-00342	SMF2 family N-terminal domain (5.60E-101)	E. cuticulari: helicase DNA-binding protein	2.12	0.01
NC000045_17399F50000014501	NCORF-00747	ATPase family associated with various (2.70E-064)	E. cuticulari: 26S PROTEASOME REGULATORY SUBUNIT 3D	2.14	0.00
NC000287_50227F5000001957	NCORF-01879	TatD related DNase (8.00E-058)	E. cuticulari: similarity to putative DEOXYRIBONUCLEASE OF THE TATD FAMILY	2.15	0.01
NC000016_24848F50000012167	NCORF-00558	Transcription factor TFIIIB repeat (1.30E-016)	E. cuticulari: TRANSCRIPTION INITIATION FACTOR TFIIIB	2.15	0.00
NC000465_2934F5000001117	NCORF-02141	ATP synthase alpha/beta family, nucleot (9.80E-011)	E. cuticulari: VACUOLAR ATP SYNTHASE SUBUNIT B	2.15	0.02
NC000086_11778F5000010935	NCORF-01114	DNA polymerase family 8 (3.50E-063)	E. cuticulari: DNA polymerase delta catalytic subunit	2.17	0.00
NC000016_24848F50000004532	NCORF-00349		E. cuticulari: 60S RIBOSOMAL PROTEIN L19	2.19	0.01
NC000262_5409F5000004867	NCORF-01825	Tubulin/FtsZ family, GTPase domain (4.20E-102)	E. cuticulari: TUBULIN ALPHA CHAIN	2.23	0.00
NC000083_11868F50000005488	NCORF-01092	ATPase family associated with various (1.30E-085)	E. cuticulari: ATPase	2.24	0.00
NC000083_11868F50000004953	NCORF-01092	ATPase family associated with various (1.30E-085)	E. cuticulari: ATPase	2.25	0.02
NC000003_44937F50000014377	NCORF-00087	Elongation factor Tu GTP binding domain (2.70E-063)	E. cuticulari: TRANSLATION ELONGATION FACTOR 2	2.27	0.00
NC000025_20388F5000010538	NCORF-00508	SMF2 family N-terminal domain (1.30E-101)	E. cuticulari: transcriptional activator	2.28	0.01
NC000126_9E32F50000005367	NCORF-01576	AMP-binding enzyme (1.40E-060)	E. cuticulari: LONG CHAIN FATTY ACID CoLIGASE	2.30	0.00
NC000075_12577F5000001082	NCORF-01025	RNA polymerase Rpb2, domain (8.30E-193)	E. cuticulari: DNA-DIRECTED RNA POLYMERASE II SECOND LARGEST SUBUNIT	2.31	0.00
NC000029_19847F5000010865	NCORF-00556	Ribosomal L37ae protein family (8.30E-035)	E. cuticulari: 60S RIBOSOMAL PROTEIN L37A (L43)	2.33	0.01
NC000169_7776F5000001927	NCORF-01570	Glyceraldehyde 3-phosphate dehydrogenas (9.00E-083)	E. cuticulari: GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE	2.36	0.01
NC000072_13066F50000001553	NCORF-01021	Glycoproteinase family (1.10E-061)	E. cuticulari: O-galactosylprotein endopeptidase	2.38	0.02
NC000465_2934F5000001097	NCORF-02141	ATP synthase alpha/beta family, nucleot (9.80E-011)	E. cuticulari: VACUOLAR ATP SYNTHASE SUBUNIT B	2.38	0.00
NC000291_4833F5000002245	NCORF-01589	Ribosomal L5P family C-terminus (1.90E-043)	E. cuticulari: 60S RIBOSOMAL PROTEIN L11	2.37	0.00
NC000064_15146F5000010298	NCORF-00945	Transketolase, thiamine diphosphate b (1.70E-084)	E. cuticulari: TRANSKETOLASE	2.37	0.00
NC000084_11825F50000003461	NCORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuticulari: TUBULIN BETA CHAIN	2.38	0.00
NC000026_20217F50000004285	NCORF-00514	RNA helicase IUPF2 interacting domain (3.70E-008)	E. cuticulari: INVOLVED IN mRNA DECAV CONTROL (DNA2/NAM7 HELICASE FAMILY)	2.38	0.00
NC000175_7705F50000005558	NCORF-01589	Thif family (8.00E-043)	E. cuticulari: UBIQUITIN-ACTIVATING ENZYME E1	2.39	0.00
NC000084_11825F50000003971	NCORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuticulari: TUBULIN BETA CHAIN	2.40	0.00
NC000021_21837F50000008524	NCORF-00474	Histone deacetylase domain (5.70E-100)	E. cuticulari: HISTONE DEACETYLASE 1	2.42	0.02
NC000084_11825F50000003401	NCORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuticulari: TUBULIN BETA CHAIN	2.42	0.00
NC000505_2693F50000002134	NCORF-02189	ATPase family associated with various (4.30E-004)	E. cuticulari: 26S PROTEASOME REGULATORY SUBUNIT 4	2.43	0.00
NC000009_2949F50000011546	NCORF-00226	RNA polymerase Rpb1, domain (2.30E-080)	E. cuticulari: DNA-DIRECTED RNA POLYMERASE I	2.44	0.01
NC000001_65607F50000059978	NCORF-00041	RNA polymerase Rpb2, domain (9.30E-159)	E. cuticulari: DNA-DIRECTED RNA POLYMERASE II SUBUNIT 2 (130kDa SUBUNIT)	2.44	0.01
NC000007_29952F50000000555	NCORF-00188	ATPase family associated with various cells (8.20E-003)	E. cuticulari: 26S PROTEASOME REGULATORY SUBUNIT 5A (TAT-BINDING PROTEIN 1)	2.46	0.00
NC000010_28743F50000009602	NCORF-00245	Ribosomal Proteins L2, C-terminal doma (2.30E-033)	E. cuticulari: 60S ribosomal protein L8	2.46	0.00
NC000262_5409F50000004617	NCORF-01825	Tubulin/FtsZ family, GTPase domain (4.20E-102)	E. cuticulari: TUBULIN ALPHA CHAIN	2.46	0.01
NC000047_5648F50000000893	NCORF-01800	RNA polymerase Rpb1, domain (9.70E-166)	E. cuticulari: DNA-DIRECTED RNA POLYMERASE II	2.46	0.01
NC000122_9911F50000004357	NCORF-01254	ATP synthase alpha/beta family, nucleot (1.60E-017)	E. cuticulari: VACUOLAR ATP SYNTHASE CATALYTIC SUBUNIT A (67 kDa)	2.47	0.00
NC000193_7106F50000003776	NCORF-01655	Metallo-beta-lactamase superfamily (7.50E-015)	E. cuticulari: similarity to HYPOTHETICAL PROTEIN Y162_METEA	2.47	0.01
NC000439_3061F5000001183	NCORF-02114	Hsp70 protein (6.20E-208)	E. cuticulari: HEAT SHOCK RELATED 70kDa PROTEIN (HSP 70 FAMILY)	2.51	0.00
NC000084_11825F50000004131	NCORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuticulari: TUBULIN BETA CHAIN	2.53	0.00
NC000068_14616F50000001729	NCORF-00978	WD domain, G-beta repeat (3.40E-002)	E. cuticulari: GUANINE NUCLEOTIDE BINDING PROTEIN BETA SUBUNIT	2.54	0.00
NC000016_24848F50000000814	NCORF-00344	TCF-1/cpn60 chaperonin family (1.80E-006)	E. cuticulari: T COMPLEX PROTEIN 1 EPSILON SUBUNIT	2.54	0.00
NC000021_21707F500000005291	NCORF-00435	Rad51 (3.20E-157)	E. cuticulari: DNA REPAIR PROTEIN RAD51 HOMOLOG	2.57	0.00
NC000003_44937F50000015303	NCORF-00087	Elongation factor Tu GTP binding domain (2.70E-063)	E. cuticulari: TRANSLATION ELONGATION FACTOR 2	2.57	0.01
NC000008_35708F50000028859	NCORF-00182	Chitin synthase (4.60E-242)	E. cuticulari: chitin synthase I	2.57	0.01
NC000023_21337F50000013549	NCORF-00479	ATPase family associated with various (3.50E-003)	E. cuticulari: SEC18-LIKE VESICULAR FUSION PROTEIN	2.57	0.00
NC000010_28743F50000001231	NCORF-00242	SMF2 family N-terminal domain (5.60E-101)	E. cuticulari: helicase DNA-binding protein	2.59	0.00
NC000021_21344F50000012157	NCORF-00458	Core histone H2A/H2B/H3/H4 (2.80E-022)	E. cuticulari: HISTONE H3	2.60	0.00
NC000022_21344F5000014194	NCORF-00460	Calcineurin-like phosphoesterase (1.90E-040)	E. cuticulari: SER/THR PROTEIN PHOSPHATASE 2-A	2.63	0.00
NC000084_11825F50000004351	NCORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuticulari: TUBULIN BETA CHAIN	2.63	0.00
NC000247_5648F50000000848	NCORF-01800	RNA polymerase Rpb1, domain (9.70E-166)	E. cuticulari: DNA-DIRECTED RNA POLYMERASE II	2.64	0.00
NC000022_21344F5000014334	NCORF-00460	Calcineurin-like phosphoesterase (1.90E-040)	E. cuticulari: SER/THR PROTEIN PHOSPHATASE 2-A	2.64	0.00
NC000127_9606F50000008792	NCORF-01383	ATPase family associated with various (8.30E-075)	E. cuticulari: HSP 101 RELATED PROTEIN	2.68	0.00
NC000083_11868F50000005583	NCORF-01092	ATPase family associated with various (1.30E-085)	E. cuticulari: ATPase	2.72	0.00
NC000415_3289F500000002017	NCORF-02085	Hsp70 protein (1.50E-058)	E. cuticulari: similarity to HSP70-RELATED PROTEIN	2.75	0.00
NC000083_11868F50000005978	NCORF-01092	ATPase family associated with various (1.30E-085)	E. cuticulari: ATPase	2.76	0.00
NC000295_4886F50000000958	NCORF-01897	Elongation factor Tu GTP binding domain (4.20E-064)	E. cuticulari: TRANSLATION ELONGATION FACTOR 1 ALPHA	2.80	0.00
NC000084_11825F50000003341	NCORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuticulari: TUBULIN BETA CHAIN	2.93	0.00
NC000048_16997F50000008505	NCORF-00785		E. cuticulari: similarity to HYPOTHETICAL WD-REPEAT PROTEIN WMS7_ yeast	2.93	0.00
NC000010_28743F50000006196	NCORF-00342	SMF2 family N-terminal domain (5.60E-101)	E. cuticulari: helicase DNA-binding protein	2.96	0.00
NC000084_11825F50000004156	NCORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuticulari: TUBULIN BETA CHAIN	3.01	0.00
NC000005_36805F50000006199	NCORF-00142	Peptidase family M1 (1.70E-005)	E. cuticulari: glutamyl aminopeptidase	3.03	0.00
NC000070_1353F50000004035	NCORF-00996	TCF-1/cpn60 chaperonin family (2.30E-131)	E. cuticulari: T COMPLEX PROTEIN 1 ALPHA SUBUNIT	3.04	0.00
NC000023_21337F50000013564	NCORF-00479	ATPase family associated with various (3.50E-003)	E. cuticulari: SEC18-LIKE VESICULAR FUSION PROTEIN	3.07	0.00
NC000024_20906F5000010910	NCORF-00493	TCF-1/cpn60 chaperonin family (3.00E-105)	E. cuticulari: T complex protein 1 subunit beta	3.07	0.00
NC000084_11825F50000003546	NCORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuticulari: TUBULIN BETA CHAIN	3.07	0.00
NC000127_9606F50000008774	NCORF-01383	ATPase family associated with various (8.50E-075)	E. cuticulari: HSP 101 RELATED PROTEIN	3.08	0.00
NC000247_5648F50000002524	NCORF-01800	RNA polymerase Rpb1, domain (9.70E-166)	E. cuticulari: DNA-DIRECTED RNA POLYMERASE II	3.12	0.00
NC000084_11825F50000003506	NCORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuticulari: TUBULIN BETA CHAIN	3.18	0.00
NC000007_29952F50000000860	NCORF-00188	ATPase family associated with various cells (8.20E-003)	E. cuticulari: 26S PROTEASOME REGULATORY SUBUNIT 6A (TAT-BINDING PROTEIN 1)	3.18	0.00
NC000521_2607F50000002315	NCORF-02208	Ribosomal protein S11 (3.40E-022)	E. cuticulari: 40S RIBOSOMAL PROTEIN S14	3.21	0.00
NC000029_19847F50000010850	NCORF-00556	Ribosomal L37ae protein family (8.30E-035)	E. cuticulari: 60S RIBOSOMAL PROTEIN L37A (L45)	3.23	0.00
NC000025_19847F50000010765	NCORF-00556	Ribosomal L37ae protein family (8.30E-035)	E. cuticulari: 60S RIBOSOMAL PROTEIN L37A (L45)	3.26	0.00
NC000175_7705F50000005578	NCORF-01589	Thif family (8.00E-043)	E. cuticulari: UBIQUITIN-ACTIVATING ENZYME E1	3.27	0.00
NC000169_7776F50000002077	NCORF-01570	Glyceraldehyde 3-phosphate dehydrogenas (9.00E-083)	E. cuticulari: GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE	3.33	0.00
NC000001_65607F50000019180	NCORF-00015	ATPase family associated with various (5.20E-003)	E. cuticulari: 26S PROTEASOME REGULATORY SUBUNIT 7	3.35	0.00
NC000039_18336F50000016397	NCORF-00687	Bromodomain (1.60E-022)	E. cuticulari: TRANSCRIPTION INITIATION FACTOR TFIIID 113kDa SUBUNIT	3.35	0.00

Table S4 (continuation)

Probe ID	Gene ID	PFAM	BLAST	M-value	q-value
NC000017_24761F5000009277	NcORF-00374	Hydroxymethylglutaryl-coenzyme A synt (3.80E-053)	E. cuniculi: 3-HYDROXY-3-METHYLGLUTARYL-CoA SYNTHASE 2	3,37	0,00
NC000084_11825F5000004281	NcORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuniculi: TUBULIN BETA CHAIN	3,37	0,00
NC000001_65607F5000019120	NcORF-00015	ATPase family associated with various (5.20E-003)	E. cuniculi: 26S PROTEASOME REGULATORY SUBUNIT 7	3,38	0,00
NC000007_29952F5000001165	NcORF-00188	ATPase family associated with various cellu (8.20E-003)	E. cuniculi: 26S PROTEASOME REGULATORY SUBUNIT 6A (TAT-BINDING PROTEIN 1)	3,41	0,00
NC000075_12577F5000001857	NcORF-01025	RNA polymerase Rpb2, domain (8.50E-193)	E. cuniculi: DNA-DIRECTED RNA POLYMERASE II SECOND LARGEST SUBUNIT	3,41	0,00
NC000007_29952F5000000585	NcORF-00188	ATPase family associated with various cellu (8.20E-003)	E. cuniculi: 26S PROTEASOME REGULATORY SUBUNIT 6A (TAT-BINDING PROTEIN 1)	3,42	0,00
NC000001_65607F50000058217	NcORF-00041	RNA polymerase Rpb2, domain (9.30E-159)	E. cuniculi: DNA-DIRECTED RNA POLYMERASE III SUBUNIT 2 (130kDa SUBUNIT)	3,43	0,00
NC000023_21337F50000008504	NcORF-00474	Histone deacetylase domain (5.70E-100)	E. cuniculi: HISTONE DEACETYLASE 1	3,44	0,00
NC000084_11825F50000004106	NcORF-01097	Tubulin/FtsZ family, GTPase domain (2.20E-098)	E. cuniculi: TUBULIN BETA CHAIN	3,48	0,00
NC000169_7776F50000001942	NcORF-01570	Glyceraldehyde 3-phosphate dehydrogenas (9.00E-083)	E. cuniculi: GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE	3,48	0,00
NC000001_65607F50000029176	NcORF-00021	Ribosomal protein L11, RNA binding do (4.00E-005)	E. cuniculi: 60S RIBOSOMAL PROTEIN L12	3,54	0,00
NC000010_28743F50000005506	NcORF-00242	SNF2 family N-terminal domain (5.60E-101)	E. cuniculi: helicase DNA-binding protein	3,57	0,00
NC000037_18421F5000001312	NcORF-00658	Peptidase family M1 (2.50E-150)	E. cuniculi: glutamyl aminopeptidase	3,60	0,00
NC000024_20966F50000010890	NcORF-00493	TCP-1/Cpn60 chaperonin family (3.00E-105)	E. cuniculi: T complex protein 1 subunit beta	3,65	0,00
NC000026_20217F50000004310	NcORF-00514	RNA helicase (UPF2 interacting domain (3.70E-008)	E. cuniculi: INVOLVED IN mRNA DECAV CONTROL (DNA2/NAM7 HELICASE FAMILY)	3,72	0,00
NC000072_13066F5000012151	NcORF-01017	Ribonucleotide reductase, barrel doma (1.00E-267)	E. cuniculi: RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE	3,76	0,00
NC000071_13320F5000002731	NcORF-01005	MCM2/3/5 family (2.90E-209)	E. cuniculi: DNA REPLICATION LICENSING FACTOR OF THE MCM FAMILY MCM5	3,77	0,00
NC000247_5648F50000002499	NcORF-01800	RNA polymerase Rpb1, domain (9.70E-166)	E. cuniculi: DNA-DIRECTED RNA POLYMERASE II	3,81	0,00
NC000023_21337F5000013114	NcORF-00479	ATPase family associated with various (3.50E-003)	E. cuniculi: SEC18-LIKE VESICULAR FUSION PROTEIN	3,82	0,00
NC000022_21344F5000014319	NcORF-00460	Calcineurin-like phosphoesterase (1.90E-040)	E. cuniculi: SER/THR PROTEIN PHOSPHATASE 2-A	3,85	0,00
NC000022_6362F50000004490	NcORF-01743	Actin (5.40E-202)	E. cuniculi: actin	4,02	0,00
NC000063_15153F5000010031	NcORF-00936	Radical SAM superfamily (4.90E-015)	E. cuniculi: similarity to HYPOTHETICAL PROTEIN YB36_METIA	4,08	0,00
NC000001_65607F5000001719	NcORF-00002	AN1-like Zinc finger (3.70E-006)	E. cuniculi: hypothetical protein ECU04_0270	4,14	0,00
NC000003_44937F5000014307	NcORF-00087	Elongation factor Tu GTP binding domain (2.20E-063)	E. cuniculi: TRANSLATION ELONGATION FACTOR 2	4,17	0,00
NC000037_18421F5000001327	NcORF-00658	Peptidase family M1 (2.50E-150)	E. cuniculi: glutamyl aminopeptidase	4,22	0,00
NC000122_9917F50000004432	NcORF-01354	ATP synthase alpha/beta family, nucleot (1.60E-017)	E. cuniculi: VACUOLAR ATP SYNTHASE CATALYTIC SUBUNIT A (67 kDa)	4,28	0,00
NC000247_5648F50000000873	NcORF-01800	RNA polymerase Rpb1, domain (9.70E-166)	E. cuniculi: DNA-DIRECTED RNA POLYMERASE II	4,30	0,00
NC000122_9917F50000004412	NcORF-01354	ATP synthase alpha/beta family, nucleot (1.60E-017)	E. cuniculi: VACUOLAR ATP SYNTHASE CATALYTIC SUBUNIT A (67 kDa)	4,33	0,00
NC000023_21337F5000013589	NcORF-00479	ATPase family associated with various (3.50E-003)	E. cuniculi: SEC18-LIKE VESICULAR FUSION PROTEIN	4,37	0,00
NC000075_12577F5000001057	NcORF-01025	RNA polymerase Rpb2, domain (8.50E-193)	E. cuniculi: DNA-DIRECTED RNA POLYMERASE II SECOND LARGEST SUBUNIT	4,42	0,00
NC000038_18383F50000000999	NcORF-00667	RNA polymerase Rpb2, domain (6.50E-134)	E. cuniculi: DNA-DIRECTED RNA POLYMERASE I, SECOND LARGEST SUBUNIT	4,42	0,00
NC000071_13320F5000002746	NcORF-01005	MCM2/3/5 family (2.90E-209)	E. cuniculi: DNA REPLICATION LICENSING FACTOR OF THE MCM FAMILY MCM5	4,43	0,00
NC000003_44937F5000015338	NcORF-00087	Elongation factor Tu GTP binding domain (2.20E-063)	E. cuniculi: TRANSLATION ELONGATION FACTOR 2	4,49	0,00
NC000047_17039F5000013569	NcORF-00774	Hydroxymethylglutaryl-coenzyme A reductas (1.30E-061)	E. cuniculi: 3-HYDROXY-3-METHYLGLUTARYL CoA REDUCTASE	4,54	0,00
NC000122_9917F50000004192	NcORF-01354	ATP synthase alpha/beta family, nucleot (1.60E-017)	E. cuniculi: VACUOLAR ATP SYNTHASE CATALYTIC SUBUNIT A (67 kDa)	4,56	0,00
NC000063_15153F5000010021	NcORF-00936	Radical SAM superfamily (4.90E-015)	E. cuniculi: similarity to HYPOTHETICAL PROTEIN YB36_METIA	4,65	0,00
NC000022_21344F5000012182	NcORF-00458	Core histone H2A/H2B/H3/H4 (2.80E-022)	E. cuniculi: HISTONE H3	4,69	0,00
NC000127_9606F50000007827	NcORF-01383	ATPase family associated with various (8.50E-075)	E. cuniculi: HSP 101 RELATED PROTEIN	4,78	0,00
NC000122_9917F50000004207	NcORF-01354	ATP synthase alpha/beta family, nucleot (1.60E-017)	E. cuniculi: VACUOLAR ATP SYNTHASE CATALYTIC SUBUNIT A (67 kDa)	4,82	0,00
NC000022_21344F5000012202	NcORF-00458	Core histone H2A/H2B/H3/H4 (2.80E-022)	E. cuniculi: HISTONE H3	4,82	0,00

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**Références - Article n°1 : *Nosema* spp. infection alters pheromone production in honey bees (*Apis mellifera*)**

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## Références - Article n°2: Flight behavior and pheromone changes associated to *Nosema ceranae* infection of honeybee workers (*Apis mellifera*) in field conditions

Soumis à *Journal of Invertebrate Pathology*

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## Références - Article n°3: Gut pathology and responses to the microsporidium *Nosema ceranae* in the honey bee *Apis mellifera*

*PLoS ONE*, 2012, 7(5): e37017

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## Références - Article n°4 : Comparative study of *Nosema ceranae* (*Microsporidia*) isolates from two different geographic origins

*Journal of Veterinary Microbiology*, doi:10.1016/j.vetmic.2012.09.012

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