NOSEMA CERANAE IN WESTERN HONEY BEES (*APIS MELLIFERA*): BIOLOGY AND MANAGEMENT

by

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Submitted in partial fulfilment of the requirements
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at

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DALHOUSIE UNIVERSITY

DEPARTMENT OF BIOLOGY

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Signature of Author

For honey bees,

and their keepers

And for the bee's knees

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ABSTRACT

Western honey bees (*Apis mellifera*; hereafter honey bees) provide vital pollination services to global agriculture and biodiversity. However in recent years they have experienced severe population declines in many regions of the northern hemisphere. Although causes of these honey bee declines are not well understood, multiple pressures such as changes in land-use and climate, management issues, and introduced parasites are believed to be responsible. First described in honey bees in 2006 during a period of high colony mortalities, the microsporidian gut parasite *Nosema ceranae* became of great concern. In this dissertation I investigated the distribution, management, virulence, and inter-specific interactions of this introduced species. First, I described and clarified the multiple pressures believed to influence honey bee health, including *N. ceranae*, especially in relation to the mysterious phenomenon Colony Collapse Disorder. I then surveyed colonies in Maritime Canada for *N. ceranae* and the historic honey bee microsporidian *Nosema apis*. Although both species were present at a regional scale, intensive sampling in Nova Scotia revealed that *N. ceranae* was highly prevalent compared to the historic congener. Next, I investigated two potential management options for the parasite. Chemotherapy using the fungicide fumagillin reduced *N. ceranae* spore intensity but had no effect on colony survival, and indoor over-wintering did not reduce spore intensity but was associated with increased colony survivorship in spring. Using a comparative approach, I observed that *N. ceranae* infection significantly reduced honey bee longevity in the laboratory but did not influence overall colony health or strength in the field. Last, a laboratory study demonstrated reduced spore production during *N. ceranae* and *N. apis* co-infection, possibly due to inter-specific competition that has resulted in the displacement of the historic *Nosema* species by *N. ceranae* in many global regions. This dissertation provides crucial information on biology and management of *N. ceranae* that can be used towards the development of an integrated pest management strategy, and for future studies investigating factors that may influence the parasite's distribution, virulence, and inter-specific interactions.

LIST OF ABBREVIATIONS & SYMBOLS USED

Acadia	Acadia University
ANOVA	Analysis of Variance
bp	Base pair
CANPOLIN	Canadian Pollination Initiative
CAP	Coordinated Agricultural Project
CCD	Colony Collapse Disorder
cf.	Confer (compare)
COLOSS	Prevention of honey bee colony losses network
Ct	Threshold cycle
d	Day
Dal	Dalhousie University
df	Degrees of freedom
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
e.g.	Exempli gratia (for example)
et al.	Et alii, et aliae, et alia (and others)
F	F-value
HSD	Honestly significant difference
i.e.	Id est (that is)
km	Kilometre
IPM	Integrated pest management
ME	Minimum evolution
mg	Milligram
ML	Maximum likelihood
ml	Millilitre
mM	Millimolar
MP	Maximum parsimony
n	Sample size
ng	Nanogram

NSERC	Natural Science and Engineering Research Council of Canada
P	P-value
PCR	Polymerase chain reaction
PhD	Doctor of Philosophy
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
s	Second
SD	Standard deviation
SS	Single stranded
TAE	Tris base, acetic acid, ethylenediaminetetraacetic acid
U	Enzyme unit
UK	United Kingdom
USA	United States of America
w/v	weight / volume
Z	Z value
ZBF	Zentrum für Bienenforschung
µl	Microlitre
χ^2	Chi-square
%	Percent
~	Approximately
$<$	Greater than
$>$	Less than
±	Plus or minus
≤	Greater than or equal
≥	Greater than or equal
®	Registered trademark
°C	Degree Celsius

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No doubt this thesis is the culmination of years of learning and discovery that started well before my good friend Lance Lo and I made the great 5000 km trek from Alberta to Nova Scotia so that I could study honey bees. I generally and sincerely thank all those who indirectly allowed me to undertake this thesis research, ranging from friends and family growing up, to colleagues and bosses, to school teachers and professors, to sports coaches. For the remainder of this section I focus on individuals that have significantly helped me accomplish this thesis as a whole. Individuals or organizations that contributed to specific chapters are included as co-authors or are in the acknowledgements; I owe you all a great deal.

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CHAPTER 1 INTRODUCTION

Part of the work presented in Chapter 1 appeared as my Preliminary Exam essay and report.

Western honey bees (*Apis mellifera*; hereafter honey bees) are among the most vital and versatile pollinators on the planet, contributing to production of 39 of the world's 57 most important monoculture crops (Klein et al., 2007). Currently the beekeeping industry faces significant hurdles to maintain honey bee colonies that are economically viable for crop pollination. Many stressors limit growth of colony numbers around the world, such as parasites, changes in land-use and climate, insufficient genetic diversity, and socio-economic factors (vanEngelsdorp and Meixner, 2010). A lack of understanding about how these factors affect honey bee health has resulted in an inability to mitigate losses. Because of the importance of honey bees to global food security, and because of parallel population declines of other pollinators around the world (e.g., Biesmeijer et al., 2006; National Research Council, 2007), there is an urgent need to study honey bee health.

The parasite *Nosema ceranae* attracted considerable attention beginning in 2006 after it was first detected in honey bees during a period of high colony losses in much of North America and Europe (Higes et al., 2006). Since then, significant research efforts have focused on studying biology and management of *N. ceranae*, resulting in over 200 peer-reviewed scientific publications; previous to its discovery in honey bees only one paper was available (i.e., Fries et al., 1996). For the remainder of this chapter I provide a brief overview of *Nosema* in honey bees at the time my graduate studies commenced in 2006. This was the time when I, along with my supervisors and committee, first developed the direction of my doctoral studies on *N. ceranae*. I then outline the objectives of my work and provide a short description of my thesis structure. Because the majority of research on *Nosema* in honey bees has focused on *N. ceranae* rather than *Nosema apis* since the time when I began my graduate studies, all information and discussions provided in this chapter concerning the latter species should be considered current unless otherwise stated.

1.1 General biology of *Nosema* in honey bees

The genus *Nosema* falls within the Kingdom Fungi, Phylum Microspora (i.e., microsporidia). Microsporidians are a group of obligate, intracellular parasites that lack mitochondria and flagella and have an environmentally resistant spore stage (Keeling and Fast, 2002). Over 1200 species of microsporidians parasitize animals (Didier and Weiss, 2006). Within Microspora, the genus *Nosema* is particularly well-studied in a number of insect hosts because of its economic importance (e.g., Bhat et al., 2009; Lewis et al., 2009), and contains two species that infect honey bees. *N. apis*, widely considered to be the historic microsporidian parasite of honey bees (Fries, 1993; Klee et al., 2007), was first detected early in the 20th century (Zander, 1909). Its lifecycle is described in detail by Fries (1993). Briefly, the parasite follows a faecal-oral transmission route. Spores ingested with food, water, or wax enter the alimentary canal, germinate, and penetrate epithelial cells lining the midgut; here the parasite reproduces. Newly created infective spores then germinate into neighbouring cells, or invade the gut lumen and are expelled with frass (i.e., faeces) when infected host cells burst. Fully developed infections in the midgut can reach ~50 million spores, and result in reduced honey bee survivorship (Bailey, 1981; Wang and Moeller, 1970). *Nosema ceranae*, the second microsporidian known to parasitize honey bees, is believed to be the historic microsporidian of Asian honey bees (*Apis cerana*) (Fries et al*.,* 1996); it was first detected in honey bees in 2005 (Higes et al., 2006). *N. ceranae* is also a gut parasite (Fries et al., 1996; Higes et al., 2006); however, virtually nothing was known about its biology when my doctoral research began.

1.2 Distribution of *Nosema* in honey bees

N. apis is a nearly ubiquitous parasite of honey bees (Fries, 1993; Klee et al., 2007). However, a coarse-scale survey revealed that *N. ceranae* possibly experienced a host switch from Asian honey bees in the late 20th century, and subsequently experienced a

large range expansion to many regions of the world (Klee et al., 2007). This suggested that both parasites successfully fulfill host encounter and compatibility filters that determine their exposure to (i.e., spatial and temporal parasite-host overlap, and ability of parasite to locate and enter a host), and development within, honey bees (Poulin, 2007). At the time my graduate studies commenced, few details of the fine-scale (e.g., regional, apiary) distribution patterns of either *Nosema* parasite in honey bees were available.

1.3 MANAGEMENT OF *NOSEMA* IN HONEY BEES

Nosema spores can be detected and quantified within honey bees using light microscopy; however, due to morphological similarities between congeners, molecular assays are required to confidently identify species. Out of more than 200 compounds investigated (Fries, 1993), the most effective and only registered form of oral chemotherapy for *N. apis* in Canada and the United States is the antibiotic fumagillin, marketed as Fumagilin-B® (Medivet Pharmaceuticals Ltd., High River, Canada; Kozak et al., 2013). Fumagillin interferes with DNA replication during intracellular development of the non-spore stage, and therefore does not affect resilient spores that are important for parasite transmission (Katznelson and Jamieson, 1952; Hartwig and Przelecka, 1971). Contaminated beekeeping equipment can be sterilised by fumigation using acetic acid (Fries, 1993) or heat treatment by exposing frames to 49°C for at least 24 hours (Cantwell and Shimanuki, 1969). These techniques are considered viable options for controlling *N. apis* because they act on the spore stage. Because of its recent detection, protocols for *N. ceranae* control using Fumagilin-B® and other techniques were lacking when I began my studies, especially regarding the development of a strategy of Integrated Pest Management (IPM) for *Nosema* control.

IPM is an invaluable tool to control pests for agriculture because it has the potential to reduce quantities of pesticides in the environment and slow development of chemically resistant pests by diversifying sources of mortality (Mota-Sanchez, 2002). To implement, IPM requires a thorough understanding of a pest's biology, as well as the biology of other important organisms of the system. It can be divided into four main components: 1.)

continual monitoring, to accurately identify and quantify pests; 2.) prevention, using methods that present very little risk to humans or the environment to keep pest populations at an acceptable level; 3.) control, using highly targeted chemicals once a pest population has exceeded an acceptable level; and 4.) development of an action threshold, a point at which control must be undertaken to prevent economic damage (EPA, 2009). A programme of IPM was urgently needed to reduce detriments of both *N. apis* and *N. ceranae*, but a lack of knowledge in early 2007 regarding *Nosema* distribution and virulence, and potential agents for control hampered efforts.

1.4 VIRULENCE OF *NOSEMA* IN HONEY BEES

N. apis is a problem mainly in temperate climates, where long winters increase horizontal transmission by promoting defecation inside hives because honey bees are not able to perform external cleansing flights (Moeller, 1972). Parasitism in adults disrupts hypopharyngeal gland production of larval food (Wang and Moeller, 1969; 1971), as well as reduces honey bee lifespan by inducing physiological aging (Wang and Moeller, 1970). At the colony level, *N. apis* infection increases winter mortality, or slows build-up of surviving colonies in spring (Fries, 1993). Conversely, no data were available for *N. ceranae* virulence in early 2007; only anecdotal evidence associated it with reduced colony survivorship because timing of its first detection and widespread distribution in honey bees coincided with high levels of colony mortality (Higes et al., 2006; Klee et al., 2007).

1.5 CONGENER INTERACTIONS OF *NOSEMA* IN HONEY BEES

Klee *et al.* (2007) observed a decline in prevalence of *N. apis* during a period of increased prevalence of *N. ceranae*. This suggests that displacement could be occurring from competition for limited resources because it is rare to observe parasitism by multiple microsporidian species within sympatric individuals of the same insect species (Solter *et al*., 2002). Although there was little information on the interactions between *Nosema*

species in honey bees when I started graduate work, *N. apis* infection was known to be associated with other parasites, including Black queen cell virus, Bee virus Y, and Filamentous virus (Bailey et al., 1983). Understanding interactions between *N. ceranae* and other organisms, especially *N. apis*, may help to explain distribution and virulence of the novel parasite.

1.6 Objectives and Thesis Structure

When I began my doctoral studies, the lack of knowledge of *N. ceranae* in honey bees during a period of increased colony mortality suggested that a better understanding of this parasite-host system was urgently needed, particularly regarding a scheme of IPM. Although virtually all aspects of *N. ceranae*'s biology and management were open to study because so little was known when I started, I chose to investigate what mattered to researchers and beekeepers most: distribution, management, virulence, and inter-specific interactions of *N. ceranae* in honey bees, so as to contribute to a strategy of IPM.

The specific objectives of my doctoral research were to: 1.) highlight the diversity of factors contributing to colony mortality globally; 2.) better understand the distribution of *Nosema* in Maritime Canada; and investigate *N. ceranae* 3.) management, 4.) virulence, and 5.) interactions with *N. apis*, in honey bees. Without doubt, the fortuitous detection of both *N. apis* and *N. ceranae* in Maritime Canada in the early stages of my graduate studies strongly influenced the path I took. The remaining sections of my thesis fulfill my research objectives, and are outlined briefly in the remainder of this chapter. I chose to prepare manuscripts intended for publication throughout my graduate studies because my research philosophy includes rapid dissemination of knowledge to all relevant stakeholders. As such, the majority of my thesis includes papers that are published in, or are in preparation for, peer-reviewed scientific journals. Although I am not the sole author of these papers, I was majority contributor for all chapters herein. Similar to most work environments, collaboration with colleagues is vital in academia. I am indebted to many colleagues during my studies for their expertise, guidance, and support. When justified, I rewarded these individuals with co-authorship.

Chapter 2 highlights the diversity of stressors responsible for reduced honey bee survivorship (Objective 1). I was inspired by the misunderstanding of many researchers and the public about the various causes of honey bee mortality, including a mysterious phenomenon known as Colony Collapse Disorder (CCD), as well as by a paper by Ratnieks and Carreck (2010) that I felt did not properly characterize CCD within the context of other honey bee colony mortalities.

Before more theoretical investigations could begin, basic aspects of *N. ceranae* biology needed to be documented. Chapters 3, 4, and 6 include *Nosema* species quantification and species identification data from various beekeeping operations in Maritime Canada, and document both parasites' distributions (Objective 2). Additional research to which I contributed but did not include in my thesis also added to characterizing *Nosema* distribution patterns in Atlantic Canada (the Maritime Provinces, and Newfoundland and Labrador) (e.g., Burgher-MacLellan et al., 2010; Williams et al., 2010; Shutler et al., submitted).

Chapters 4, 5, and 6 investigated management of *N. ceranae* (Objective 3). The first two examined the effect of fumagillin. It was not known how effective this fungicide would be against this parasite because it did not control the closely-related *Nosema bombi* in bumble bees (Whittington and Winston, 2003). The latter chapter examined the effect of indoor versus outdoor over-wintering of honey bee colonies on *Nosema* parasitism because a large portion of honey bee colonies in Maritime Canada are wintered in this manner. Because this practice can restrict honey bee defecation to inside hives, there was concern that indoor over-wintering could promote *Nosema* parasitism through increased horizontal transmission.

Chapters 5, 6, and 7 studied possible effects of *Nosema* parasitism on honey bees (Objective 4) in complementary laboratory and field experimental arenas. Whereas a laboratory provides a unique opportunity to execute specialized experiments under

relatively controlled conditions, the field arena is more representative of conditions with which beekeepers must contend.

Lastly, Chapter 7 investigated interactions between *Nosema* congeners (Objective 5). It was suggested by Klee *et al.* (2007) that *N. ceranae* may be competitively displacing *N. apis* in many regions of the world; however, this was based on surveys lacking historical samples. No experiments had tested this hypothesis and no one had investigated inter-specific interactions between *Nosema* species in honey bees.

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CHAPTER 2 COLONY COLLAPSE DISORDER IN CONTEXT

The work presented in Chapter 2 also appears in:

Williams, G.R., Tarpy, D.R., vanEngelsdorp, D., Chauzat, M.-P., Cox-Foster, D.L., Delaplane, K.S., Neumann, P., Pettis, J.S., Rogers, R.E.L., Shutler, D., 2010. Colony Collapse Disorder in context. BioEssays 32, 845-846.

2.1 Commentary

Although most of humanity relies upon foods that do not require animal pollination (Ghazoul et al., 2005), production of 39 of the world's 57 most important monoculture crops still benefits from this ecosystem service (Klein et al., 2007). Western honey bees (*Apis mellifera*) are undoubtedly the single most valuable animal pollinators to agriculture because they can be easily maintained and transported to pollinator-dependent crops. Yet, despite an almost 50% increase in world honey bee stocks over the last century, beekeepers have not kept pace with the >300% increase in pollinator-dependent crops (Aizen and Harder, 2009). This has led to great uncertainty surrounding the recent largescale die-offs of honey bees around the world, and has sparked enormous interest from both scientists and the general public. Although sharp regional declines in honey bee populations have occurred in the past, such as the so-called unexplainable ''Isle of Wight'' disease in the early 1900s (Underwood and vanEngelsdorp, 2007), the magnitude and velocity of these recent declines are likely unprecedented. Often in the media, and sometimes in the scientific literature (e.g. Ratnieks and Carreck, 2010), these losses are inappropriately equated with ''Colony Collapse Disorder'' or CCD, which is characterized by the rapid disappearance of adult bees from colonies containing brood and food stores but lacking damaging levels of parasitic *Varroa destructor* mites or *Nosema* microsporidians (vanEngelsdorp et al., 2009). Although, we agree that CCD is indeed a significant cause for concern, we believe that it is imperative to appropriately place CCD within the greater context of other honey bee morbidities occurring

worldwide. In many cases, these morbidities can be explained by known parasites or beekeeper management issues. One example is the devastation caused by beekeepers' inability to control *V. destructor*, which not only feeds on host haemolymph and weakens host immunity, but also vectors a variety of viruses (Rosenkranz et al., 2009). In other cases, however, these morbidities are genuinely unexplainable, including those attributed to CCD *sensu stricto* (vanEngelsdorp et al., 2009). In recent winters, colony mortality in Europe has averaged ~20% (ranging from 1.8 to 53% among countries), with starvation and parasites believed to be the main contributors (COLOSS, 2009). Colony mortality during the 2006/2007, 2007/2008, and 2008/2009 winters in the US, the only country where CCD has been documented *sensu stricto*, was 32% (vanEngelsdorp et al., 2007), 36% (vanEngelsdorp et al., 2008), and 29% (vanEngelsdorp et al., 2010), respectively. During the winter of 2008/2009, ~10% of the 2.3 million managed honey bee colonies in the US died with ''CCD-like symptoms'', and US beekeepers self-diagnosed CCD as only the 8th most important contributor to colony mortality, behind starvation, queen-related issues, and parasites (vanEngelsdorp et al. 2010). The point is, honey bees die from many things. We must be careful to not synonymize CCD with all honey bee losses.

There is a growing consensus that colony mortality is the product of multiple factors, both known and unknown, acting singly or in combination (Rogers and Williams, 2007; Neumann and Carreck, 2010). Considering the reliance that modern agriculture places on honey bees for pollination, coordinated efforts, such as those of CANPOLIN (Canadian Pollination Initiative, www.uoguelph.ca/canpolin), COLOSS (Prevention of Honeybee Colony Losses, www.coloss.org), and the US Department of Agriculture's Areawide and Managed Pollinator CAP (Coordinated Agricultural Project) (Pettis and Delaplane, 2010), are urgently needed to understand and mitigate these losses. The first step in these efforts should be to objectively discriminate among types of colony mortality occurring worldwide. This will permit a more informed and appropriate allocation of research efforts into CCD specifically and other causes of mortality in general.

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CHAPTER 3 FIRST DETECTION OF *NOSEMA CERANAE*, A MICROSPORIDIAN PARASITE OF EUROPEAN HONEY BEES (*APIS MELLIFERA*), IN CANADA AND CENTRAL U.S.A.

The work presented in Chapter 3 also appears in:

Williams, G.R., Shafer, A.B.A., Rogers, R.E.L., Shutler, D., Stewart, D.T., 2008. First detection of *Nosema ceranae*, a microsporidian parasite of European honey bees (*Apis mellifera*), in Canada and central U.S.A. Journal of Invertebrate Pathology 97, 189-192.

3.1 Abstract

Nosema ceranae is an emerging microsporidian parasite of European honey bees, *Apis mellifera*, but its distribution is not well known. Six *Nosema*-positive samples (determined from light microscopy of spores) of adult worker bees from Canada (two each from Nova Scotia, New Brunswick, and Prince Edward Island) and two from USA (Minnesota) were tested to determine *Nosema* species using previously-developed PCR primers of the 16S rRNA gene. We detected for the first time *N. ceranae* in Canada and central USA. One haplotype of *N. ceranae* was identified; its virulence may differ from that of other haplotypes.

3.2 Introduction

Nosema apis (Zander, 1909) is a microsporidian parasite of European honey bees, *Apis mellifera*. *Nosema* affects adults only, infecting epithelial cells lining the midgut after spores are ingested (Bailey, 1955). *N. apis* can induce queen supersedure (Webster et al., 2004), reduce pollen collection (Anderson and Giacon, 1992), and shorten bee life span (Wang and Moeller, 1970).

Previously, nosemosis in European honey bees was attributed exclusively to *N. apis* (Ellis and Munn, 2005), with the recent exception of disease in regions of Asia and Europe that were ascribed to the closely-related *Nosema ceranae* (Higes et al., 2006; Huang et al., 2007). However, it appears that *N. ceranae* is an emerging pathogen that has increased its distribution in the past decade by jumping from Asian honey bees, *Apis cerana*, to European honey bees (Klee et al., 2007). *N. ceranae* has now been detected on four continents (Asia, Europe, North America, and South America) and it may be displacing *N. apis* in European honey bees (Klee et al., 2007). Originally isolated from Asian honey bees (*Apis cerana*) (Fries et al., 1996), *N. ceranae* is highly pathogenic when experimentally inoculated into European honey bees (Higes et al., 2007), and is associated with reduced honey production and increased winter mortality (Higes et al., 2006). Here, we describe the first detection of *N. ceranae* in European honey bees in Canada and central USA.

3.3 METHODS

We collected adult worker bees from Minnesota, USA (in July 2006, 1 beekeeper and 10 colonies), and from the three Canadian Maritime Provinces (New Brunswick in September 2006, 3 beekeepers and 9 colonies; Prince Edward Island in September 2006, 10 beekeepers and 35 colonies; Nova Scotia in April 2007, 2 beekeepers and 2 colonies). Bees were kept at -20°C until suspensions could be created by adding 10 ml of distilled water to the abdomens of 10 bees (from a single colony) that were crushed using a mortar and pestle (Rogers et al., 2003). Ratios of *Nosema*-positive hives in Minnesota, New Brunswick, Prince Edward Island, and Nova Scotia, determined from light microscopy, were 4/10, 3/9, 4/35, and 2/2, respectively. Two *Nosema*-positive suspensions were chosen from Minnesota, USA and each Canadian Maritime Province for molecular analyses. Each selected suspension represented a separate beekeeping operation, with the exception of bees collected in Minnesota, which originated from a single beekeeper.

For each of the eight selected suspensions, an aliquot of 10 µl was added to 10 µl of proteinase K (20 mg/ml), vortexed, and warmed to 37°C. After 10 min, 100 µl of Chelex

100/Instagene matrix (Bio-Rad) was added, and the solution was boiled for 10 min, and then incubated at 56°C overnight (Walsh et al., 1991). The solution was centrifuged for 3 min at 13,000 g. The resulting supernatant was used as the DNA template in the subsequent polymerase chain reaction (PCR), which was performed on a TGradient thermal cycler (Biometra). The ~25-µl reaction mix consisted of 10.9 µl ultrapure H_2O, 5 µl 5x PCR buffer (Promega), 2.5 µl $MgCl_2$ (25 mM), 0.5 µl dNTPs (10 mM each), 2.5 µl primers (10 mM each), 1.25 U GoTaq polymerase (Promega), and 1 µl of template DNA. Primers NOS-FOR and NOS-REV (Higes et al., 2006) used to amplify the 16S rRNA gene were optimized using an annealing temperature gradient and then run under the following parameters: an initial 2-min denaturing period at 94°C, followed by 40 cycles of 45 s denaturing at 94°C, 45 s annealing at 58°C, and a 1-min extension period at 72°C. The cycling period was followed by an additional 72°C extension period of 3 min. PCR products were visualized and purified from a 1% agarose gel in 1x TAE buffer using a gel extraction kit (Qiagen). The PCR contained a negative control, which was verified by confirming the absence of a band in the gel. Double-stranded sequencing was performed at Florida State University (Tallahassee) using a gene analyzer with capillary electrophoresis (Applied Biosystems).

The 16S rRNA fragment sequences were aligned using BioEdit v.7.0.5.3 (Hall, 1999) and confirmed by eye. The generated consensus sequences, created from both forward and reverse sequences, were compared to those of related species obtained from GenBank (Figure 3.1). All newly generated sequences were deposited in GenBank (Accession Nos. EF584418-EF584425).

The phylogenetic position of each *Nosema*-positive sample was determined using minimum evolution (ME), maximum parsimony (MP), and maximum likelihood (ML) methods, with 16S rRNA sequences from *Vairimorpha imperfecta* (Canning et al., 1999) used as an outgroup reference. Genetic distances among sequences for ME analysis were calculated using the Tamura-Nei model as selected by Modeltest v.3.06 (Posada and Crandall, 1998). The accompanying tree was constructed using the software package MEGA 3 (Kumar et al., 2004). The software package PAUP V.4.0b10 (Swofford, 2002)

was used for both MP and ML analyses. For MP analysis, we implemented a full heuristic search with all characters unweighted and unordered, and we used the TrN+I model of nucleotide substitution for the ML analysis. Support for all the phylogenetic analyses was measured using 1000 bootstrap replicates.

3.4 RESULTS & DISCUSSION

Five out of eight samples produced high probability matches on GenBank to *N. ceranae* (99-100%), and the remaining three produced high probability matches to *N. apis* (100%). The amplified fragment lengths were 252 and 240 bp for *N. ceranae* and *N. apis*, respectively. Phylogenetic clustering using ME, MP, and ML analyses further confirmed our samples as either *N. ceranae* or *N. apis* (Figure 3.1), and were supported by bootstrap values ≥50% (although most bootstrap values at the species level were >96%). When *V. imperfecta* was excluded from the ML analysis, the split between *N. ceranae* and *N. apis* was supported by a bootstrap value of 100%. This suggests that the low bootstrap support for the ML analysis was influenced by the relatively large degree of divergence between the genera *Nosema* and *Vairimorpha*.

Phylogenetic clustering of sequence data was further supported by genetic distances calculated using Tamura-Nei's model; average within-group distance for *N. ceranae* and *N. apis* was 0.2% and 0% (standard deviations 0.2% and 0%), respectively, and the average distance between groups was 6.4% (standard deviation 1.7%). In addition, the sequence of our North American *N. ceranae* SS rRNA differs from those reported from China (Fries et al., 1996) and Spain (Higes et al., 2006) by the insertion of either an adenine or guanine at the 19th bp position and by a transition mutation at the 21st bp position; however, the North American haplotype is shared with those reported from Austria (i.e., EF458655-58).

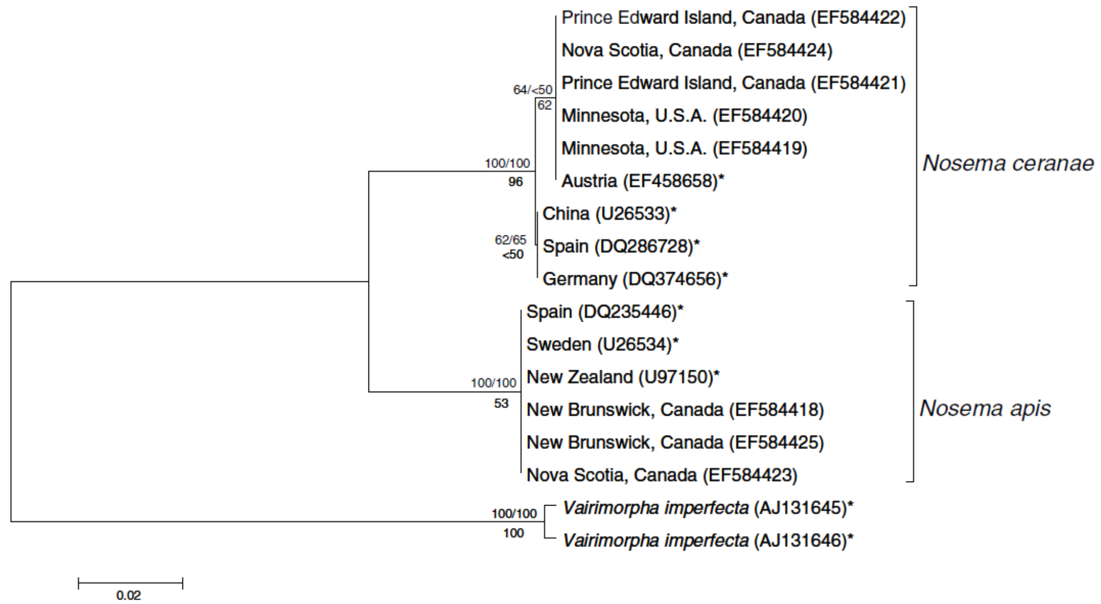

Figure 3.1 Phylogenetic tree illustrating the position of *Nosema* collected from European honey bees (*Apis mellifera*) in the Canadian Maritime Provinces (Nova Scotia, New Brunswick, and Prince Edward Island) and Minnesota USA, with previous *Nosema* GenBank submissions (denoted by *). *Vairimorpha imperfecta* is used as the outgroup reference. GenBank accession numbers are in parentheses. Bootstrap support values above the branch (minimum evolution/maximum parsimony) are from 1000 replicates. Support for the maximum likelihood analysis, below the branch, has also been obtained from 1000 bootstrapped replicates.

The detection of *N. ceranae* in European honey bees from the Maritime Provinces of Canada and Minnesota, USA expands the known distribution of this emerging parasite. As in other geographic regions of North America, *N. apis* was suspected as the single cause of nosemosis in European honey bee colonies, although Klee et al. (2007) just reported that *N. ceranae* was detected in eastern USA (District of Columbia) in 2004. Thus, *N. ceranae* is likely a relatively recent arrival to Canada compared to other regions of the world, such as southern and eastern Europe, Asia, and the rest of the Americas, where the more virulent *N. ceranae* has possibly displaced *N. apis* (Klee et al., 2007). Further work is needed to determine the temporal pattern of *N. ceranae* range expansion into beekeeping operations that were previously thought to contain only *N. apis*.

The presence of only a single *N. ceranae* 16S rRNA haplotype in North America suggests that parasites here originated from a single source, likely Europe. Different haplotypes of *N. bombi* may vary in virulence in bumblebees (Tay et al., 2005). Thus, virulence of these different *N. ceranae* haplotypes in European honey bees should be examined, as a more virulent haplotype may infect honey bees in North America.

Although we did not detect *N. ceranae* in New Brunswick, it is likely present (but perhaps at a low prevalence) because it was found in neighbouring Nova Scotia and Prince Edward Island (the latter is only ~13 km offshore). Moreover, New Brunswick is the only Maritime Province with an effectively open border to inter-province honey bee importation, and it has a history of being the first in Canada to receive exotic bee parasites, such as the mite *Varroa destructor* in 1989 (Clay, 1996). New Brunswick also borders Maine, which accepts thousands of honey bee colonies each spring from all parts of the USA for low-bush blueberry (*Vaccinium angustifolium*) pollination. This could potentially expose Canadian colonies to infected bees on the American side of the border, and vice versa. Intensive surveys are needed to determine the distribution and prevalence of *N. ceranae* in North America.

Recent survey and experimental work suggests that *N. ceranae* is a serious threat to the global beekeeping industry (Higes et al., 2006, 2007). Little is known about the pathogenicity of this parasite to European honey bees, and it is possible that this parasite is one of many factors contributing to high bee mortalities recently being reported (Oldroyd, 2007). Currently, Fumagilin-B® (Medivet Pharmaceuticals Ltd.) is the only registered product in Canada available to beekeepers for the control of *N. apis*; it was ineffective against a closely related species, *Nosema bombi*, in the bumble bee *Bombus occidentalis* (Whittington and Winston, 2003). Studies are needed to determine the efficacy of Fumagilin-B® against *N. ceranae*.

3.5 ACKNOWLEDGEMENTS

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CHAPTER 4 DOES FUMAGILLIN CONTROL THE RECENTLY DETECTED INVASIVE PARASITE *NOSEMA CERANAE* IN WESTERN HONEY BEES (*APIS MELLIFERA*)?

The work presented in Chapter 4 also appears in:

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4.1 ABSTRACT

Western honey bee (*Apis mellifera*) colonies in Nova Scotia, Canada were sampled in spring and late summer 2007 to evaluate efficacy of fumagillin dicyclohexylammonium (hereafter, fumagillin) against *Nosema ceranae*. Colonies treated with fumagillin in September 2006 (n = 94) had significantly lower *Nosema* intensity in spring 2007 than did colonies that received no treatment (n = 51), but by late summer 2007 no difference existed between groups. Molecular sequencing of 15 infected colonies identified *N. ceranae* in 93.3% of cases, suggesting that fumagillin is successful at temporarily reducing this recent invasive parasite in western honey bees.

4.2 INTRODUCTION

Nosemosis of western honey bees (*Apis mellifera*) is caused by two different microsporidians, *Nosema apis* and *Nosema ceranae*. Infection occurs in adult midgut epithelial cells after spores are ingested during trophallaxis or cleaning of contaminated comb (Bailey, 1981; Webster, 1993). Pathology associated with *N. apis*, the historical *Nosema* parasite of western honey bees, is well-described, and includes dysentery, reduced honey yield, increased winter mortality, and poor spring build-up of surviving colonies (Fries, 1993). First detected in western honey bees in 2005 (Huang et al., 2007), *N. ceranae* likely jumped from the Asian honey bee (*Apis cerana*) over 10 years ago

(Klee et al., 2007; Paxton et al., 2007; Chen et al., 2008), so its pathology is not as well understood. In Spain, *N. ceranae* was associated with reduced honey production and increased colony mortality (Higes et al., 2006a), and was highly pathogenic when inoculated experimentally (Higes et al., 2007; Paxton et al., 2007).

To combat *N. apis*, apiculturists recommend the use of the antibiotic fumagillin dicyclohexylammonium (hereafter, fumagillin), which disrupts this parasite's DNA replication (Katznelson and Jamieson, 1952; Hartwig and Przelecka, 1971; Webster, 1994). In Canada, Fumagilin-B® (Medivet Pharmaceuticals Ltd.) is the only commercially registered product containing fumagillin available to beekeepers for *Nosema* treatment. Chemotherapy typically occurs during fall syrup-feeding of hives (Gochnauer and Furgala, 1969), before peak infection during winter and early spring (Pickard and El-Shemy, 1989). Fall and spring chemotherapy is often recommended for severe infections, but this may not reduce *N. apis* below damaging levels (Wyborn and McCutcheon, 1987). It is not known if fumagillin is effective against *N. ceranae*, in part, because fumagillin was ineffective against the closely related *Nosema bombi* in the bumble bee *Bombus occidentalis* (Whittington and Winston, 2003). Because *N. ceranae* may be more virulent than *N. apis*, and because the former has only recently spread from Eurasia to become a global concern, data on the efficacy of fumagillin against this parasite are of significant interest. Here we present evidence that fumagillin is effective at managing *N. ceranae* in western honey bees.

4.3 METHODS

Eight different beekeeping operations from 5 counties in Nova Scotia, Canada, volunteered their colonies for this study; 94 (5 beekeeping operations) and 51 (3 beekeeping operations) colonies had been treated or not treated with Fumagilin-B®, respectively, in September 2006 according to label instructions (Table 4.1). We collected bees in both spring (20 April-4 May) and late summer (20-26 August) 2007 (n = 15-21 colonies per operation) from each of these 145 colonies. Workers were collected from the hive entrance using a portable vacuum device, and kept at ~20°C until spore

suspensions for each colony from each sampling period were created by adding 15 ml of distilled water to crushed abdomens of 15 randomly selected individuals (Cantwell, 1970; Rogers and Williams, 2007a). Estimation of *Nosema* intensity per colony (mean spores per bee) was accomplished using light microscopy and a hemacytometer (Cantwell, 1970; Rogers and Williams, 2007a). For each spore suspension, averages of 2 estimates of intensity were used.

We performed molecular analyses of the 16S rRNA gene (Higes et al., 2006a) on a random subset (n = 15 infected colonies from 7 operations in the spring, 1-3 per operation) of the 145 colonies to identify species of *Nosema* that were present. PCR conditions and sequencing methods are described in Williams et al. (2008). Blastn searches compared sequence data to those of related species on GenBank. Sample representatives were deposited in GenBank (Accession Nos. EU545140, EU545141).

We first used repeated-measures ANOVA to analyze effects of fumagillin treatment, using R 2.0.1. (R Development Core Team). Infection intensity data were square root-transformed to improve fit to normality, but perfect fit could not be achieved due to the high frequency of uninfected colonies (Table 4.1). Nonetheless, our analyses are likely to be robust because of our large sample sizes.

Table 4.1 Median intensity (number of spores/bee) and prevalence (percent of colonies) of *Nosema* in spring (20 April-4 May) and late summer (20-26 August) 2007 in western honey bee (*Apis mellifera*) colonies (n) from 8 beekeeping operations in Nova Scotia, Canada that had been treated or untreated with Fumagilin-B® in September 2006.

Operation	*n*	Spring		Late summer	
		Median intensity	Prevalence (%)	Median intensity	Prevalence (%)
Untreated					
1	15	10,725,000	100	1,425,000	80
2	19	2,725,000	74	1,625,000	89
3	17	1,475,000	82	1,875,000	71
Treated					
4	16	0	31	0	38
5	21	0	29	0	33
6	17	0	6	2,625,000	88
7	20	700,000	70	2,925,000	90
8	20	2,375,000	90	2,687,500	95

4.4 RESULTS & DISCUSSION

Fourteen of 15 (93.3%) colonies had high probability (100%) matches on GenBank to *N. ceranae*, and one colony had a high probability (100%) match to *N. apis*. Results are comparable to molecular analyses we performed on a subset (*n* = 7 infected colonies, 3 belonging to 3 operations previously sampled from the 145 colonies above and 4 belonging to 2 operations not previously sampled) of 345 colonies sampled in spring 2007, where 6 of 7 (85.7%) infected colonies had high probability matches (100%) on GenBank to *N. ceranae*, and one had a high probability match (100%) to *N. apis* (Williams, unpublished data). As has been reported from other geographic regions (Klee et al., 2007; Paxton et al., 2007; Chen et al., 2008), our data suggest that *N. ceranae* is displacing *N. apis*. Because the historical parasite *N. apis* is still present in Canada (Williams et al., 2008), as well as northern and western Europe and Australasia (Klee et al., 2007), *N. ceranae* is likely a relatively recent arrival to these regions compared to

regions, such as the United States (Chen et al., 2008), where only *N. ceranae* has recently been detected.

Infection intensities were significantly different between treatment groups (repeated-measures ANOVA $F_{1,143} = 24.6$, $P < 0.001$). Because of the significant treatment effect, we tested for differences within sampling periods with ANOVA. *Nosema* intensity in the spring was significantly lower in colonies treated with Fumagilin-B® the previous fall than colonies that had not been treated (Figure 4.1, $F_{1,143} = 39.3$, $P < 0.001$), but by late summer no difference existed between groups (Figure 4.1, $F_{1,143} = 0.1$, $P = 0.82$). Given that 93.3% (14/15) of the colonies on which we did molecular work were infected with *N. ceranae*, our surveys suggest that fumagillin treatment in the fall successfully reduced intensity of this invasive parasite in the subsequent spring. Because only a single colony infected with *N. apis* was part of our statistical analyses, we were unable to test whether fumagillin was more effective against one of the *Nosema* species.

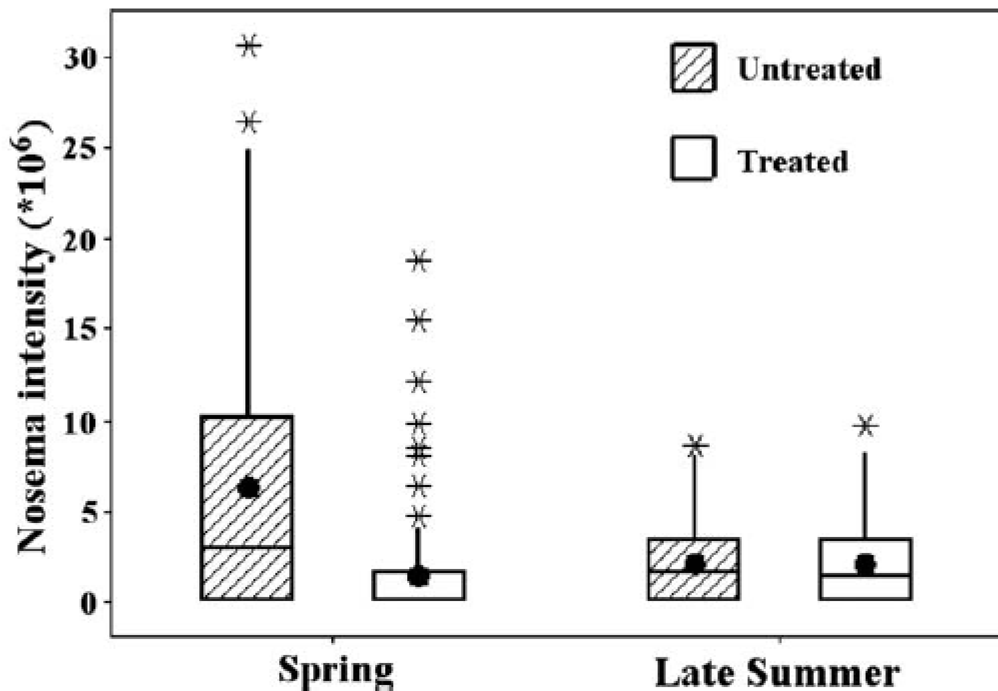

Figure 4.1 Comparison of western honey bee (*Apis mellifera*) colonies in Nova Scotia, Canada treated (*n* = 94, from 5 beekeepers) and untreated (*n* = 51, from 3 beekeepers) with Fumagilin-B® in September 2006 in spring (20 April-4 May) and late summer (20-26 August) 2007 (*n* = 15-21 colonies per operation). Boxplots show interquartile range (box), median (black line within interquartile range), data range (vertical lines), and outliers (asterisks). Black dots represent means.

Our results could be due to differences in beekeeping management practices, rather than because of differences in fumagillin treatment. We believe this is unlikely for several reasons. First, we observed extreme differences in *Nosema* intensities (Figure 4.1) that we judge would be difficult to ascribe to shared differences in beekeeping management for the eight operations we sampled. Second, geographic locations of the fumagillin-treated and untreated bee operations overlap, so that local differences in, for example, microclimates, are unlikely to be responsible for the significant differences in infection intensities. Third, bees from all of the operations are transported long distances (100s of km) through the same regions of Nova Scotia, and thus are all likely to have broadly similar exposures to *Nosema* (and many other pathogens). Fourth, our findings are supported by additional unpublished observations (Higes et al., 2006b). Nonetheless, future cage and field trials should be conducted to evaluate the efficacy of fumagillin against *N. ceranae*. Future studies should also investigate if fumagillin is favoring displacement of *N. apis* by *N. ceranae* because it is more effective against the former.

Differences between treated and untreated colonies disappeared approximately 1 year after treatment, suggesting that infected colonies naturally recover during the summer (Pickard and El-Shemy, 1989), that fumagillin loses its efficacy (Furgala, 1962), or that fumagillin becomes depleted from colony honey stores.

N. ceranae has been blamed for colony collapse of western honey bees in Spain (Martin-Hernandez et al., 2007), whereas Israeli acute paralysis virus has been associated with colony collapse in the United States (Cox-Foster et al., 2007). However, many colony collapses likely result from synergistic interactions among multiple pathogens and other stressors (Rogers and Williams, 2007b). Moreover, as is the case for *N. bombi* (Tay et

al., 2005), virulence may vary among *N. ceranae* haplotypes. Virulence in Spain may be higher than in other regions of the world, such as in eastern Canada and other regions of North America, that appear to be colonized by a different European haplotype (Williams et al., 2008). Investigating virulence and efficacy of fumagillin against these different haplotypes should be a priority to protect bees whose pollination services to agriculture are valued at over $14 billion annually in the United States alone (Morse and Calderone, 2000).

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CHAPTER 5 THE MICROSPORIDIAN *NOSEMA CERANAE*, THE ANTIBIOTIC FUMAGILIN-B®, AND WESTERN HONEY BEE (*APIS MELLIFERA*) COLONY STRENGTH

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5.1 ABSTRACT

Western honey bees (*Apis mellifera*) are under threat from a number of emerging pathogens, including the microsporidian *Nosema ceranae* historically of Asian honey bees (*Apis cerana*). Because of its recent detection, very little is known about the biology, pathology, and control of *N. ceranae* in western honey bees. Here we investigated effects of the antibiotic Fumagilin-B®, which is commonly used to control the historical *Nosema* parasite of western honey bees *Nosema apis*, on *N. ceranae*, as well as effects of *N. ceranae* on colony strength (i.e. number of bees and amount of capped and uncapped brood, honey, and pollen) and colony mortality. Similar to our previous study, fall Fumagilin-B® treatment lowered, albeit weakly, *N. ceranae* intensity the following spring. However, *N. ceranae* was not associated with variation in colony strength measures or with higher colony winter mortality.

5.2 INTRODUCTION

In recent years the apiculture industry has been besieged by a spate of emerging diseases. The microsporidian *Nosema ceranae* parasitizes both the Asian (*Apis cerana*) and western (*Apis mellifera*) honey bee, although it is more closely related to *Nosema bombi* that parasitizes bumble bees than it is to *Nosema apis* that parasitizes western honey bees (Shafer et al., 2009). *N. ceranae* jumped from the Asian to the western honey bee some

time before 1998 (Paxton et al., 2007; Chen et al., 2008). It is now ubiquitous in western honey bees (e.g. Higes et al., 2006; Klee et al., 2007; Chen et al., 2008; Williams et al., 2008a), and has displaced the historical *N. apis* parasite of western honey bees in many regions (Klee et al., 2007; Chen et al., 2008; Williams et al., 2008b).

Very little is known about *N. ceranae* biology and pathology in western honey bees (Fries, 2010). Like *N. apis*, it primarily infects midgut epithelia; however, *N. ceranae* appears to be more pathogenic when inoculated experimentally (Higes et al., 2007; Paxton et al., 2007). It is possible that unique strains of *N. ceranae* (Williams et al., 2008a) vary in virulence, so there may be geographic differences in effects on beekeeping industries. In Spain, *N. ceranae* maintains year-round high prevalence (proportion of bees infected) (Martín-Hernández et al., 2007), in contrast to the early summer peaks of infection that characterize *N. apis* (Pickard and El-Shemy, 1989). Moreover, *N. ceranae* can cause sudden collapse of seemingly asymptomatic colonies (Higes et al., 2008). In the United States, the cause of Colony Collapse Disorder, a phenomenon that occurs in colonies when adult bees rapidly disappear from a colony (i.e. absence of dead bees in the colony) lacking damaging levels of *V. destructor* and *Nosema* populations, leaving behind unattended brood and intact food stores (vanEngelsdorp et al., 2009), remains unexplained. It is likely that multiple stressors acting alone or in concert with each other weaken bees and allow for opportunistic pathogens to invade and kill the colony (vanEngelsdorp et al., 2009). In Canada, large-scale unexplained colony collapses have not been observed, despite above-average colony losses in recent years. Suspected contributors include weather, the parasitic mite *Varroa destructor*, and *N. ceranae*, but few supporting data exist. Regardless of these differences, data on pathology of *N. ceranae*, and efficacy of control measures used against it, are of extreme interest.

Numerous control methods exist for *N. apis* in western honey bees, including heat treatment (Cantwell and Shimanuki, 1969), fumigation (Bailey, 1957), and replacement of aged equipment (Fries, 1988). Most common in Canada and the United States is oral administration of the antibiotic fumagillin dicyclohexylammonium (hereafter, fumagillin) with sugar syrup during fall feeding (Gochnauer and Furgala, 1969), but an additional

spring application is often recommended for severe infections. With the exception of Spain, fumagillin is no longer registered for use in Europe because honey cannot contain antibiotic residues on that continent (Europa, 2009). Medicated colonies have lower *N. apis* intensities the following spring, although no differences in survival were observed with different fumagillin concentrations (Szabo and Heikel, 1987). It is not known what control methods used against *N. apis* in western honey bees are appropriate for *N. ceranae*, especially because fumagillin was ineffective against closely related *N. bombi* in the bumble bee *Bombus occidentalis* (Whittington andWinston, 2003). In eastern Canada, fumagillin applied at the manufacturer's recommended fall dosage for *N. apis* (administered as Fumagilin-B®; one treatment of 190 mg fumagillin per colony) controlled *N. ceranae* over the wintering period (Williams et al., 2008b); however, we did not account for potential variation among beekeeping operations that differ, for example, in location of wintered colonies, in condition of colonies in fall, or in use of additional parasite management treatments. In Spain, fumagillin (administered as Fumadil-B®; four weekly treatments for a total of 120 mg fumagillin per colony) also controlled *N. ceranae* within a 1-yr period, and significantly reduced the risk of depopulation (Higes et al., 2008).

Using colonies over-wintered in a single apiary, we investigated effects of Fumagilin-B® on *N. ceranae*, and effects of *N. ceranae* on western honey bee colony strength (i.e., size of adult worker population, and amounts of capped and uncapped brood, honey, and pollen) and colony mortality. In parallel, we repeated our earlier study (Williams et al., 2008b) using colonies over-wintered by seven different beekeepers.

5.3 Methods

Two experiments were conducted between August 2007 and May 2008 in Nova Scotia, Canada.

5.3.1 Experiment 1

We collected worker honey bees from hive entrances of 57 two-brood-chambered commercial colonies in three apiaries in Hants County on 4 September 2007 using a portable vacuum device. Bees were kept at -20°C until spore suspensions could be made for each colony by adding 30 ml of distilled water to crushed abdomens of 30 randomly-selected individuals. We estimated *Nosema* intensity per colony using light microscopy and a haemocytometer. For each sample we counted the number of spores in 80 haemocytometer squares (i.e., 5 groups of 16 squares) (Cantwell, 1970; Rogers and Williams, 2007).

Between 6 and 12 September 2007, we visually estimated (in number of frames out of 10) coverage with bees, capped and uncapped brood, honey, and pollen (we refer to these as colony strength measures) for every other frame (due to time constraints) in the upper and lower brood boxes of each colony. Presence of eggs and disease, such as American and European foulbroods, chalkbrood, and deformed-winged workers, were also recorded.

Colonies were moved to a single winter apiary at the end of September 2007. Based on colony assessments, 47 queenright colonies were chosen for the experiment on 10 October 2007; 26 colonies received 8 l each of fumagillin-medicated 2:1 (determined by weight) sugar syrup (one treatment of 190 mg fumagillin per colony) and 21 colonies received 8 l of un-medicated 2:1 sugar syrup. Colonies were placed on 4-way commercial beekeeping pallets grouped as medicated or un-medicated colonies to minimize bees visiting colonies of the opposing treatment group. All colonies received equal treatments of Check-Mite® in mid-September 2007 and Terramycin® in mid-October 2007 to control *V. destructor* and American foulbrood, respectively. In addition, sticky boards inserted on 11 October 2007 were used to monitor 24-hour natural drop of *V. destructor*.

Bees were re-sampled from hive entrances for *Nosema* quantification (see above) on 21 April 2008 and for colony strength between 28 April and 6 May 2008. Presence of eggs

and disease (see above) were recorded, and sticky boards were inserted on 27 April 2008 to monitor *V. destructor*. Colony mortality was determined by the beekeeper; dead colonies were defined as those without a queen or those with <2 frames of bees during spring 2008 inspections.

All statistical analyses were performed using R. 2.7.2. (R Development Core Team), unless otherwise stated. Repeated measures ANOVAs were used to compare hive strength variables, *Nosema* intensity, and *V. destructor* intensity between control and fumagillin-treated groups. A discriminant function analysis was used to compare a composite of the colony strength variables using SAS 9.2. Where appropriate, data were square-root transformed to improve fit to normality. Perfect fit could not always be achieved; nonetheless, our analyses are likely to be robust because of our large sample sizes. We used logistic regression to analyse the relationship between fall 2007 *Nosema* spore intensity and winter mortality. We tested for differences in colony mortality between treatment groups using χ^2 tests.

5.3.2 Experiment 2

Worker honey bees were collected from the hive entrances of 130 commercial colonies from 7 different beekeeping operations in Kings, Hants, Colchester, Cumberland, and Guysborough counties between 20 and 26 August 2007 using a portable vacuum device. Number of colonies sampled per operation ranged between 8 and 37. Bees were kept at −20°C until *Nosema* could be assessed (see above). Between mid-September 2007 and early October 2007, approximately half of the colonies sampled in each apiary received 8 l of fumagillin-medicated 2:1 sugar syrup (one treatment of 190 mg fumagillin per colony), depending on beekeeping operation; remaining colonies received 8 l of un-medicated 2:1 sugar syrup. All colonies were managed for *V. destructor* and American foulbrood by their respective beekeepers. Worker bees were re-sampled for *Nosema* quantification from hive entrances of surviving (defined above) colonies between 9 and 16 April 2008.

As before, *Nosema* data were square-root transformed, and repeated measures and split-plot ANOVAs were used to analyse effects of beekeeper and fumagillin treatment on *N. ceranae*. We used logistic regression to analyse the relationship between fall 2007 *N. ceranae* and winter mortality, and χ^2 tests to analyse differences in colony mortality between treatment groups.

5.3.3 *Nosema* species identification

Molecular analyses were performed using duplex PCR (see Williams et al., 2008b) and primers 321APIS-FOR and 321APIS-REV for *N. apis* and 218MITOC-FOR and 218MITOC-REV for *N. ceranae* (Martín-Hernández et al., 2007) on all Experiment 1 colony suspensions created in spring 2008 that contained *Nosema* spores, in addition to a few without spores. Analyses were not performed on Experiment 2 samples because a previous survey of the same beekeepers identified *N. ceranae* in >90% of colonies tested (Williams et al., 2008b).

5.4 RESULTS

5.4.1 Experiment 1

Nosema ceranae was not significantly lower in fumagillin-medicated than in control colonies that survived to spring 2008 (control colonies' mean ± SD in millions for 2007, 2008: 1.3 ± 1.5, 2.2 ± 5.4, fumagillin-treated colonies' mean ± SD in millions for 2007, 2008: 0.9 ± 1.1, 0.3 ± 0.7, repeated measures ANOVA $F_{1,37} = 3.5$, $P = 0.07$). There were no significant differences between treatment groups for estimates of bees ($F_{1,37} = 0.1$, $P = 0.76$), capped ($F_{1,37} = 0.8$, $P = 0.37$) and uncapped ($F_{1,37} = 1.16$, $P = 0.29$) brood, honey ($F_{1,37} = 1.9$, $P = 0.17$), or pollen ($F_{1,37} = 0.2$, $P = 0.68$) (Figures 5.1a-e). Moreover, discriminant function analyses of these variables failed to distinguish between control and fumagillin-treated colonies in either 2007 (32/47 colonies correctly discriminated, $F_{5,41} = 1.5$, $P = 0.23$) or 2008 (27/40 colonies correctly discriminated, $F_{5,34} = 0.9$, $P =$

0.48), and there were no significant differences for *V. destructor* (control colonies' mean ± SD for 2007, 2008: 21.9 ± 23.6, 0.4 ± 0.6, fumagillin-treated colonies' mean ± SD: 17.3 ± 17.1, 0.4 ± 0.6, repeated measures ANOVA $F_{1,35} = 0.3$, $P = 0.60$). Fall 2007 *Nosema* spore counts and winter mortality were not related when all colonies (logistic regression: $Z = 0.6$, $P = 0.52$), control colonies ($Z = 0.3$, $P = 0.71$), or fumagillin-treated colonies ($Z = 0.6$, $P = 0.58$) were grouped together. Similarly, there was no relationship between fumagillin treatment and winter mortality ($\chi^2_1 < 0.1$, $P = 0.93$), with 3/21 control and 4/26 fumagillin treated colonies dying.

Additional diseases were infrequent; one fumagillin-treated colony contained 67 and 30 cells of chalkbrood mummies in fall and spring, respectively, and two fumagillin treated colonies contained workers with deformed wings. American and European foulbroods were not observed.

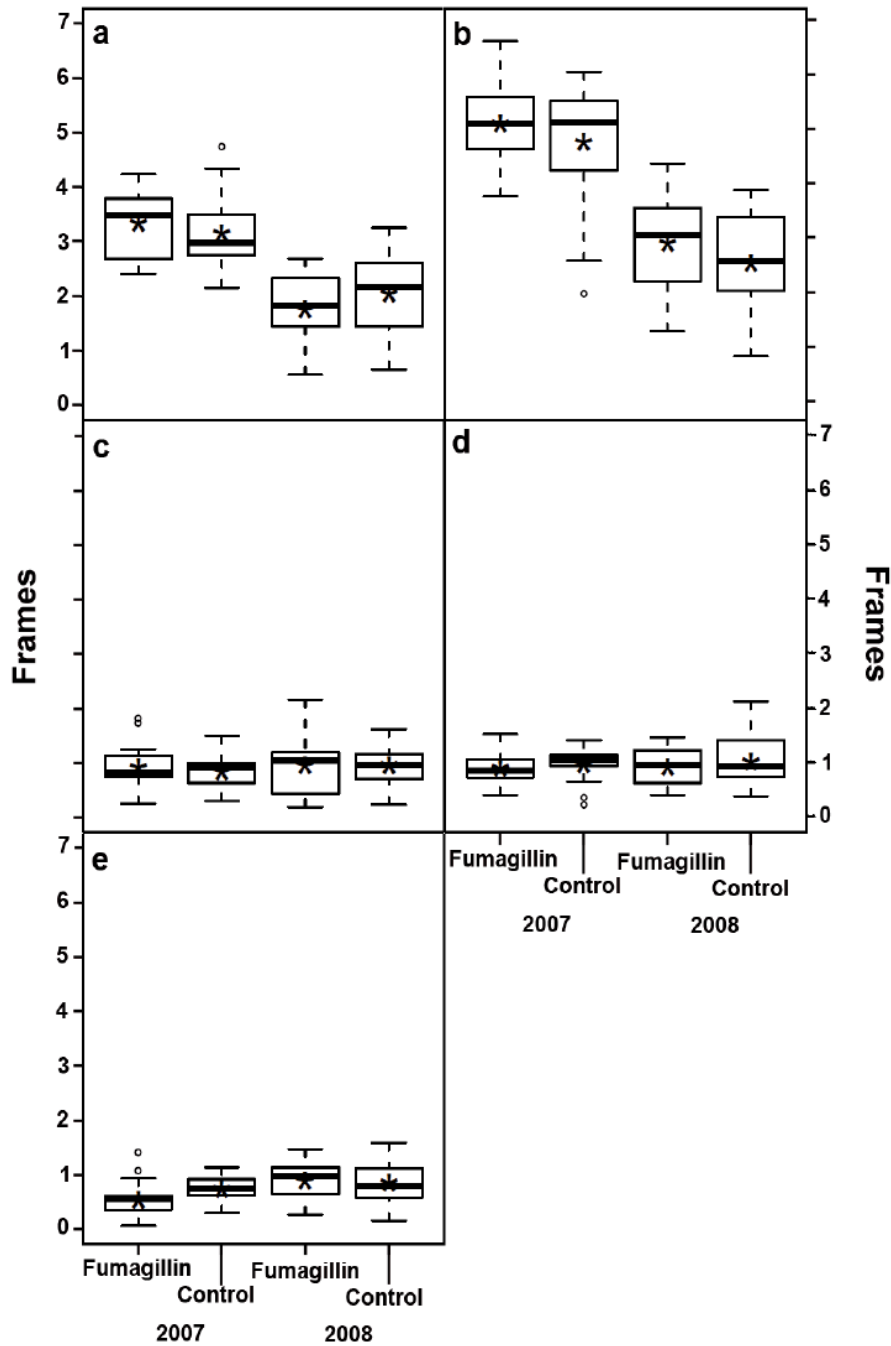

Figure 5.1 Comparisons between control and fumagillin-treated honey bee colonies in fall 2007 and spring 2008 for number of frames out of 10 of: (a) adult bees; (b) honey; (c) pollen; (d) capped brood; and (e) uncapped brood. Boxplots show interquartile range (box), median (black line within interquartile range), data range (dashed vertical lines), and outliers (open dots). Asterisks represent means. Estimates were made for every other frame in each colony.

5.4.2 Experiment 2

Nosema was significantly lower in fumagillin-medicated colonies (repeated measures ANOVA $F_{1,103} = 4.0$, $P = 0.05$) and remained so after we controlled for beekeeper (split-plot ANOVA for beekeeper and treatment: $F_{1,97} = 5.0$, $P = 0.03$); however, *Nosema* was only significantly lower in treated colonies for 2/7 beekeepers (Repeated measures ANOVAs; Table 5.1). Similar to Experiment 1 results, there were no relationships between fall 2007 *Nosema* spore counts and winter mortality when all colonies (logistic regression $Z = 0.4$, $P = 0.66$), control colonies ($Z = 2.5$, $P = 0.21$), or treated colonies ($Z = -0.6$, $P = 0.53$) were grouped together. Similarly, there was no relationship between fumagillin treatment and winter mortality ($\chi^2_1 = 0.3$, $P = 0.56$), with 11/53 control and 14/52 fumagillin-treated colonies dying.

Table 5.1 Summary statistics of *Nosema* spore counts (in millions) for control and fumagillin-treated honey bee colonies from seven beekeepers in Nova Scotia during fall 2007 and spring 2008, and corresponding repeated measures ANOVA results (significant differences in bold).

Beekeeper	Fall 2007				Spring 2008				df	*F*	*P*
	Control		Fumagillin-treated		Control		Fumagillin-treated				
	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
1	0.3	0.5	0.9	1.2	0.1	0.3	<0.1	0.1	1.24	0.3	0.58
2	2.5	0.2	3.2	1.0	5.4	11.2	1.3	2.0	1.14	0.2	0.64
3	2.2	2.4	2.1	1.3	5.8	5.4	3.2	2.3	1.15	0.8	0.40
4	2.7	1.6	1.3	0.9	5.5	2.0	0.9	0.9	1.8	24.6	**<0.01**
5	3.5	2.7	3.3	2.1	8.3	7.7	6.4	10.0	1.12	0.1	0.78
6	1.5	1.4	0.7	0.7	5.9	4.8	0.1	0.2	1.5	13.1	**0.02**
7	1.6	1.2	1.9	2.3	0.5	1.2	<0.1	<0.1	1.13	0.7	0.43

5.4.3 *Nosema* species identification

Fifteen of 20 samples molecularly analyzed had *N. ceranae*, and one had both *N. ceranae* and *N. apis*. The remaining 4 samples failed to amplify any PCR product, possibly due to DNA degradation.

5.5 DISCUSSION

Overall, Fumagilin-B® reduced *N. ceranae* intensities the following spring, but results were highly variable both within and among beekeepers, and the degree of control was weak (i.e, $P = 0.07$ and 0.03 for experiments 1 and 2, respectively). Among a large number of potential explanations for this variability, a colony's initial strength and food intake (i.e., sugar syrup and thus dosages of fumagillin) in fall may partially explain differences in effectiveness of fumagillin. Similarly, variability could arise from variation in stress from previous summer commercial pollination activities, forage availability, and winter weather, as well as beekeeper management; however, very little is known about factors influencing *N. ceranae* growth and development. Our data highlight the importance of large sample sizes and of monitoring conditions under which bees are maintained in evaluating *N. ceranae* and its management in commercial colonies.

Despite lower *N. ceranae* intensity in fumagillin-treated colonies, there were no differences in indices of colony strength or winter mortality between treatment groups the following spring, suggesting that both fumagillin and *N. ceranae* had little effect on colonies under these experimental conditions. There are a number of possible explanations for this lack of pathology.

First, haplotypes of *N. ceranae* may differ in virulence, and the *N. ceranae* haplotype in Nova Scotia, as well as other regions of Canada and the United States (Williams et al., 2008a), may be less virulent than those found in other regions of the world, such as in Spain where it is implicated in colony die-offs (Higes et al., 2008).

Second, damaging thresholds for *N. ceranae* have not been concretely quantified, and only limited data set the threshold at >1,000,000 *N. apis* spores per bee. *N. ceranae* damage thresholds may not have been met in this study, although in fall 2007 we observed mean spore loads of >1,000,000 in 6 of 7 beekeepers' colonies. More research is needed to understand damage thresholds for both *N. apis* and *N. ceranae* under a number of environmental conditions, and for a diversity of haplotypes, because Nova

Scotia colonies appear to be unaffected in spring when mean spore counts of foraging bees were >10 million the previous fall (Williams et al., 2010).

Third, weather during our experiments was normal, with beekeepers reporting plentiful pollen supplies and nectar flow during late summer (J. Moran, unpubl. data); therefore, colonies may have been healthy and not susceptible to *N. ceranae*.

Fourth, the experiment was terminated after spring assessments because beekeepers were moving their colonies to pollinate crops. Effects of *N. ceranae* could have occurred after we stopped collecting data; colonies may have undergone a "false recovery" as reported by Higes et al. (2008). We consider this unlikely because experimental colonies were acquired from a commercial operation that had not treated colonies with fumagillin the previous fall; therefore, colonies would not have had low spore counts in spring 2007 characteristic of the "asymptomatic" phase (Higes et al., 2008).

Fifth, it is possible that proportion of infected foragers, rather than spore counts determined from composite samples of 30 foraging bees, are a better indicator of colony disease from *N. ceranae* infection (Higes et al., 2008). This was also true for *N. apis* and honey yield, although both individual and composite estimates were strongly correlated with each other (Fries et al., 1984).

Lastly, because *N. ceranae* intensity did not differ significantly between controls and fumagillin-treated colonies in Experiment 1, we might not expect to have differences in our indices of colony strength.

Very little is known about consequences of *N. ceranae* to western honey bees because of its recent detection (Huang et al., 2007). Understanding factors influencing colony-level pathology of *N. ceranae* is crucial, particularly due to conflicting data suggesting that the parasite is more pathogenic in Europe than in North America (Higes et al., 2008; vanEngelsdorp et al., 2009). Although fumagillin applied at manufacturer-recommended rates appears to control *N. ceranae* to a certain extent, more work is needed to determine

ideal dosages of fumagillin for *N. ceranae*-infected colonies, such as amount of active ingredient required and number of applications per treatment. Recent attention has focussed on how multiple factors affect colony health, including parasites and pesticides (vanEngelsdorp et al., 2009). Until influences of all important stressors, both singly and in combination, are known, it may be difficult to understand differences in strength and mortality among western honey bee colonies.

5.6 ACKNOWLEDGEMENTS

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CHAPTER 6 EFFECTS AT NEARCTIC NORTH-TEMPERATE LATITUDES OF INDOOR VERSUS OUTDOOR OVERWINTERING ON THE MICROSPORIDIUM *NOSEMA CERANAE* AND WESTERN HONEY BEES (*APIS MELLIFERA*).

The work presented in Chapter 6 also appears in:

Williams, G.R., Shutler, D., Rogers, R.E.L., 2010. Effects at Nearctic north-temperate latitudes of indoor versus outdoor overwintering on the microsporidium *Nosema ceranae* and western honey bees (*Apis mellifera*). Journal of Invertebrate Pathology 104, 4-7.

6.1 ABSTRACT

In northern temperate climates, western honey bee (*Apis mellifera*) colonies can be wintered outdoors exposed to ambient conditions, or indoors in a controlled setting. Because very little is known about how this affects the recently-detected microsporidium *Nosema ceranae*, we investigated effects of indoor versus outdoor overwintering on spring *N. ceranae* intensity (spores per bee), and on winter and spring colony mortality. For colonies medicated with Fumagilin-B® to control *N. ceranae*, overwintering treatment did not affect *N. ceranae* intensity, despite outdoor-wintered colonies having significantly greater mortality. These findings suggest that *N. ceranae* may not always pose the most significant threat to western honey bees, and that indoor-wintering may ensure that a greater number of colonies are available for honey production and pollination services during the summer.

6.2 INTRODUCTION

Winter poses a significant challenge to beekeepers in Nearctic northern temperate climates, where western honey bee *Apis mellifera* colonies face months of sub-zero temperatures during which they have to survive on honey and pollen reserves. In these

regions, colonies can be wintered outdoors in sheltered locations with insulated wrapping. Alternatively, they can be wintered indoors in complete darkness in buildings maintained at ~5°C and equipped with air circulation and ventilation systems that exchange heat and carbon dioxide produced by colonies with fresh outdoor air (Currie et al., 1998; Shimanuki et al., 2007). Outdoor wintering affords bees opportunities to leave the colony to defecate (cleansing flights), and to rear brood during mild temperatures; however, it also exposes colonies to long periods of sub-freezing temperatures during which bees must form a tight, ball-shaped cluster to maintain warmth. This cluster restricts movement of bees, making them vulnerable to starvation because they cannot leave to feed (Shimanuki et al., 2007). Conversely, indoor wintering reduces nutritional requirements (Genc and Kaftanoglu, 1997), and allows colonies to be moved outdoors when environmental conditions are ideal; however, it reduces brood-rearing and prevents cleansing flights because of constant cool temperatures and complete darkness. For much of the 20th century, beekeepers in Nearctic northern temperate regions did not overwinter their colonies, but rather imported packages each spring consisting of 0.9-1.4 kg of bees and a mated queen. Because of the recent threat of introducing parasites (e.g. the parasitic mite *Varroa destructor*) from countries exporting packages, as well as high import costs, beekeepers are now encouraged to overwinter their colonies (Currie et al., 1998).

Another potential disease that can be introduced is *Nosema*, caused historically in western honey bees by the microsporidium *Nosema apis*. Infection often results in reduced longevity and degeneration of infected queen ovaries, and ultimately colony death during winter, or slow spring build-up of survivors (Fries, 1993). In 2005, a second microsporidium, *Nosema ceranae*, was discovered parasitizing western honey bees in Taiwan (Huang et al., 2007), and has since been found distributed almost globally (e.g., Klee et al., 2007; Williams et al., 2008a; Higes et al., 2009). *N. ceranae* has likely parasitized western honey bees for decades (Paxton et al., 2007; Chen et al., 2008), but due to its recent detection, very little is known about its biology and management (Fries, 2009). In Europe, it has been associated with colony collapse (Higes et al., 2008) and high pathogenicity in caged bees (Higes et al., 2007; Paxton et al., 2007), whereas in

North America, it appears to be more benign (Williams et al., 2010). Both species can be controlled using the antibiotic Fumagilin-B®; unfortunately, treatment never completely eliminates infection (Higes et al., 2008; Williams et al., 2008b). Because infection levels of *N. apis* typically increase rapidly in spring as brood-rearing starts but while cleansing flights are infrequent (Pickard and El-Shemy, 1989), indoor wintering may reduce *Nosema* levels in spring by allowing beekeepers to move colonies outdoors when cleansing flights are possible. Here, we investigated effects of overwintering environment (indoor versus outdoor) on spring *N. ceranae* intensity and on winter and spring colony mortality.

6.3 METHODS

Worker honey bees were collected from hive entrances of 61 commercial colonies from two beekeeping operations from the province of Nova Scotia and one from the province of New Brunswick between 26 August and 5 September 2009. Samples were stored at -20°C until *Nosema* spore intensity could be estimated. We created suspensions for each colony by finely crushing 30 abdomens in 30 ml distilled water using a mortar and pestle, and then used light microscopy and a hemocytometer to count spores (see Williams et al., 2008b). Approximately half of the colonies sampled in each apiary remained outdoors until spring, with remaining colonies moved indoors between 4 and 15 December 2008 (Table 6.1). Indoor-wintered colonies were moved outdoors between 27 March and 19 April 2009 (Table 6.1), and for two beekeeping operations to the same location as the outdoor-wintered colonies; for beekeeping operation 1, indoor-wintered colonies were moved to a yard ~4 km away. Worker bees from hive entrances of surviving colonies were re-sampled for *Nosema* twice in spring 2009: first, within 4 weeks of moving indoor-wintered colonies outdoors and second, just prior to colonies being moved to blueberries for pollination. New Brunswick samples were collected from under the hive lid in April because very few bees were at the hive entrance due to cool temperatures. Since new bees had not emerged, those collected were likely wintered bees similar to those collected from the hive entrance at the other operations. *V. destructor* intensity was determined for a random subset of colonies (for beekeeping operations 1, 2, and 3, three,

eight, and three colonies were sampled, respectively) in late summer 2008 by collecting ~200 bees from a brood frame. Bees were placed in a stainless steel mesh strainer and agitated in a basin containing windshield washer fluid (for use at -40 °C) and lined with a cotton sheet. After ~3 min, or when no more mites were observed, number of detached mites on the cotton sheet and number of bees washed were determined to calculate number of mites per 100 bees.

All colonies within each operation received the same medications, including in mid-September 2008 Fumagilin-B® (Medivet Pharmaceuticals Ltd., High River, Alberta, Canada) according to manufacturer recommendations for *N. ceranae* (one dose of 190 mg fumagillin per colony), and between spring sampling periods (four weekly doses of 30 mg fumagillin per colony), as well as acaricides for the parasitic mite *V. destructor* and Terramycin® for the bacterium causing American foulbrood, *Paenibacillus larvae*. Colony mortality was determined by the beekeeper; dead colonies were defined as those without a queen or those with <2 frames of bees during spring 2009 inspections.

Molecular analyses were performed on four randomly-selected *Nosema*-infected suspensions from each beekeeping operation from April 2009 using duplex PCR (Williams et al., 2008b) and primers 321APIS-FOR and 321APIS-REV for *N. apis* and 218MITOC- FOR and 218MITOC-REV for *N. ceranae* (Martín-Hernández et al., 2007).

Nosema and *V. destructor* intensity data were square-root transformed to improve fit to normality where appropriate. Split-plot ANOVAs tested for effects of beekeeper and overwintering treatment on *Nosema* intensity at each sampling period, and repeated measures ANOVAs tested for effect of overwintering treatment on *Nosema* intensity at all sampling periods simultaneously. We used logistic regression to test for a relationship between fall 2008 *Nosema* intensity and winter mortality. Differences in colony mortality between overwinter treatment groups were compared using a χ^2 test. All statistical analyses were performed in R version 2.9.0. (R Development Core Team).

Table 6.1 Indoor over-wintering management conditions for participating beekeeping operations in Maritime Canada, and sampling regime for *Nosema* spp. quantification.

Province	Beekeeping Operation	Timing of movements		Indoor Temp. (°C)	Sampling dates		
		Indoors	Outdoors		Fall	Spring I	Spring II
Nova Scotia	1	4 December	14 April	5.6	5 September	25 April	19 May
	2	15 December	28 March	0-5	26 August	10 April	21 May
New Brunswick	3	10 December	28 March	5-7	27 August	27 April	26 May

6.4 RESULTS

Nosema intensity was not significantly different between indoor- and outdoor-overwintered colonies for August, April, or May, regardless of whether we controlled for beekeeper (August, April, and May split-plot ANOVAs for beekeeper and overwinter treatment $F_{1,44} < 0.1$, $P = 0.93$; $F_{1,44} = 0.5$, $P = 0.47$; $F_{1,44} = 2.4$, $P = 0.13$, respectively) or did not (repeated measures ANOVA $F_{1,46} = 0.1$, $P = 0.74$) (Figure 6.1; Table 6.2); however, *Nosema* intensity was significantly higher in outdoor-wintered colonies for one of the three beekeepers (beekeeping operation 1 repeated measures ANOVA $F_{1,15} = 0.5$, $P = 0.04$). There were no significant relationships between fall 2008 *Nosema* spore intensity and winter mortality by April or May when all colonies (logistic regression: April, $Z = -0.9$, $P = 0.38$; May, $Z = -0.1$, $P = 0.91$), indoor-wintered colonies (April, $Z = 0.7$, $P = 0.48$; May, $Z = 0.7$, $P = 0.48$), or outdoor-wintered colonies (April, $Z = -1.6$, $P = 0.12$; May, $Z = -0.7$, $P = 0.48$) were pooled. There was no significant relationship between overwintering treatment and mortality ($\chi^2_1 = 1.5$, $P = 0.22$) in April, with 3 of 29 and 7 of 32 indoor- and outdoor-wintered colonies dying, respectively; however, significantly more outdoor-wintered colonies died (10 of 32) by May than did indoor-wintered colonies (3 of 29; $\chi^2_1 = 4.0$, $P = 0.05$).

Eight of twelve samples molecularly analysed had *N. ceranae*, and one had both *N. ceranae* and *N. apis*. The remaining three samples failed to amplify a PCR product, possibly due to DNA degradation. These data support previous studies (e.g., Williams et al. 2008b) that show a predominance of *N. ceranae* in honey bees in Maritime Canada. *V. destructor* intensity was above the recommended treatment threshold of 3.3 mites per 100 bees for 2 of 14 samples (McRory et al., 2009); both were from beekeeping operation 2. There was no significant difference in *V. destructor* intensity between overwintering treatment groups ($F_{1,12} = 0.8$, $P = 0.39$; mean ± SD for indoor and outdoor: 1.2 ± 1.2 and 2.3 ± 2.3, respectively).

Table 6.2 Summary statistics of mean *Nosema ceranae* intensity (in millions of spores/bee) for indoor (Ind.) - and outdoor (Out.) - wintered western honey bee colonies, as well as colony mortality, from three beekeeping operations in Maritime Canada.

Province	Operation	Total	Ind.	Out.	Mean *N. ceranae* intensity (millions spores/bee) August 2008			April 2009			May 2009			Mortality (colonies) April 2009		May 2009	
					Average	Indoor	Outdoor	Average	Indoor	Outdoor	Average	Indoor	Outdoor	Ind.	Out.	Ind.	Out.
NS	1	21	11	10	21.0	20.0	22.2	3.6	1.8	6.8	2.3	1.6	3.7	0	4	0	4
	2	21	10	10	1.4	1.8	1.0	0.1	0.1	<0.1	<0.1	0	<0.1	2	3	2	5
NB	3	20	8	12	2.7	2.6	2.7	1.1	2.0	0.7	0.1	0.2	<0.1	1	0	1	1

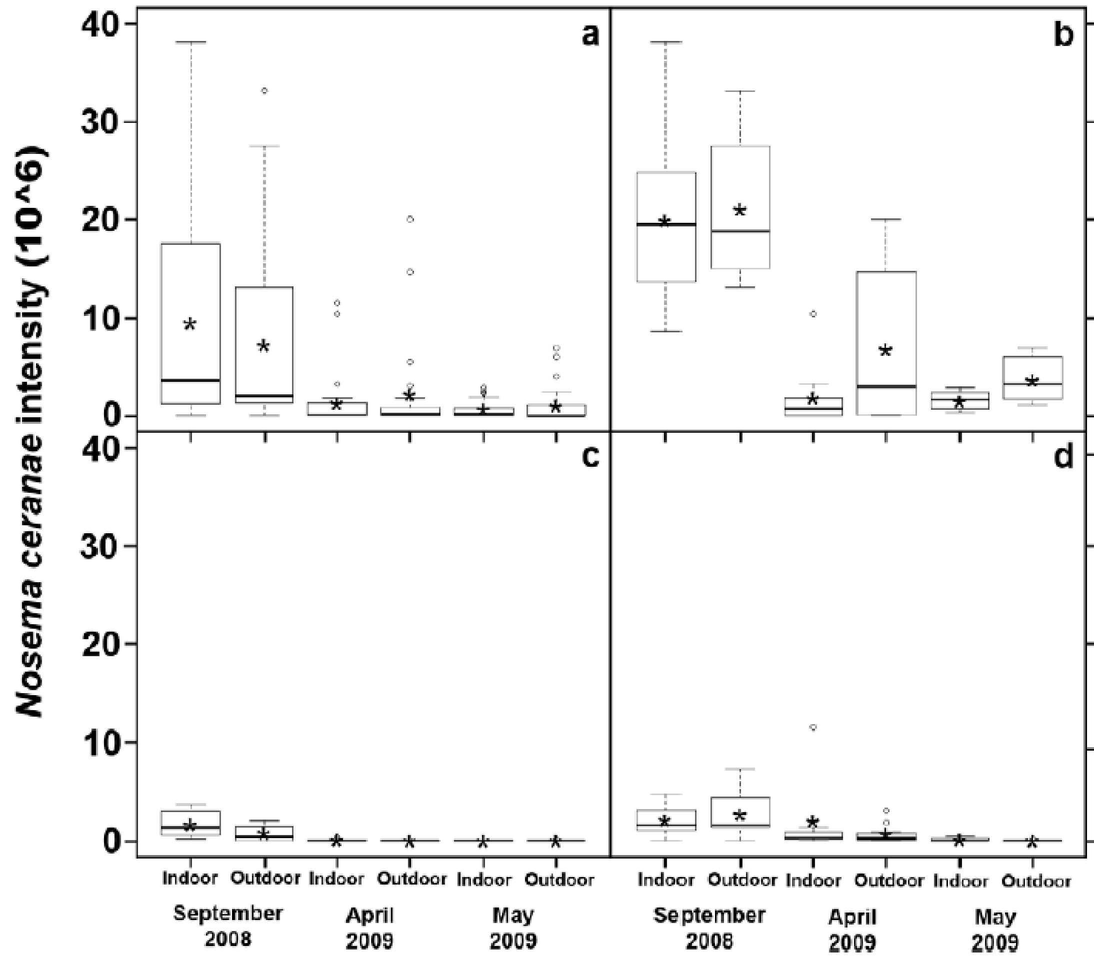

Figure 6.1 Comparisons of *Nosema ceranae* intensity (in millions of spores/bee) between indoor- and outdoor-wintered western honey bee colonies in September 2008, April 2009, and May 2009 for: (a) all beekeeping operations, (b) beekeeping operation 1, (c) 2, and (d) 3. Boxplots show interquartile range (box), median (black line within interquartile range), data range (dashed vertical lines), and outliers (open dots). Asterisks represent means.

6.5 DISCUSSION

Overall, overwintering treatment (indoor versus outdoor) did not affect *N. ceranae* intensity the following spring. It is possible that potential differences in *N. ceranae* intensity between indoor- and outdoor-wintered colonies were removed by application of Fumagilin-B® to all colonies in fall because fall application is effective at reducing *N. ceranae* intensity the following spring (Williams et al., 2008b). Future experiments

should investigate effects of overwintering treatment on *N. ceranae* in the absence of Fumagilin-B® treatment, as well as investigate why *N. ceranae* intensity was higher in outdoor-wintered colonies in spring for beekeeping operation 1 only. Possibly, differences in spring colony locations of indoor- and outdoor-wintered colonies affected results for beekeeping operation 1; however, this is unlikely, at least during April sampling, because samples were collected from colonies within 10 days of being moved outdoors. Fumagilin-B® may on its own be effective at reducing spring *N. ceranae* intensity when fall intensity is below an unknown threshold; however, when intensity is high, such as was the case in colonies belonging to beekeeping operation 1 (Table 6.2), indoor-wintering may aid the antibiotic in reducing *N. ceranae* the following spring. It is also likely that length of time spent indoors influences *N. ceranae*, although outdoor winter conditions were not considered particularly challenging to colonies during the study. Colonies belonging to beekeeping operation 1 remained indoors >3 weeks longer than operations 2 and 3; moving colonies outdoors too early in spring may encourage broodrearing during nectar and pollen dearth, possibly also encouraging *Nosema* development.

Although overwintering treatment had no significant effects on *N. ceranae* intensity, mortality was significantly higher in colonies wintered outdoors. This, coupled with an insignificant relationship between fall *N. ceranae* intensity and colony mortality, supports data that *N. ceranae* may not always pose the most significant threat to western honey bees (Guzmán-Novoa et al., 2010; Williams et al., 2010). We can only speculate, but it is possible that *N. ceranae* present in Maritime Canada (Williams et al., 2008b) differs in virulence from strains found in other regions of the world, or that experimental colonies were not sufficiently stressed, and therefore not particularly susceptible to *N. ceranae* disease. More work is needed to investigate damaging thresholds of *N. ceranae*, as well as factors influencing *N. ceranae* pathogenicity, such as haplotype and overall colony health. Although overwintering treatment did not affect *N. ceranae* intensity, the ultimate measure of colony strength, colony survival, benefitted from indoor wintering likely because winter food reserves lasted until nectar and pollen dearth ended, thus

ensuring a greater number of colonies were available for pollination services and honey production throughout the summer.

6.6 ACKNOWLEDGEMENTS

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CHAPTER 7 INFRA-POPULATION AND -COMMUNITY DYNAMICS OF *NOSEMA* PARASITE CONGENERS, AND CONSEQUENCES FOR HONEY BEE (*APIS MELLIFERA*) HOSTS

The work presented in Chapter 7 also appears in:

Williams, G.R., Shutler, D., Burgher-MacLellan, K.L., Rogers, R.E.L., 2013. Infra-population and -community dynamics of *Nosema* parasite congeners, and consequences for honey bee (*Apis mellifera*) hosts. In preparation for a peer-reviewed journal.

7.1 ABSTRACT

Nosema spp. fungal gut parasites are among myriad possible explanations for contemporary increased mortality of western honey bees (*Apis mellifera*, hereafter honey bee) in many regions of the world. Invasive *Nosema ceranae* is particularly worrisome because it recently switched hosts from Asian honey bees (*Apis cerana*) and now has a nearly global distribution in honey bees, apparently displacing its historic congener *Nosema apis*. We examined parasite fecundity and effects on hosts of *N. apis*, *N. ceranae*, and mixed *Nosema* honey bee infections in controlled laboratory experiments. Both infection intensity and honey bee mortality were significantly greater for *N. ceranae* than for *N. apis* or mixed infections; mixed infection resulted in mortality similar to *N. apis* parasitism and reduced spore intensity, possibly due to inter-specific competition This is the first long-term (> 15 d) laboratory study to demonstrate lethal consequences of *N. apis* and *N. ceranae* and mixed *Nosema* parasitism in honey bees, and suggests that differences in fecundity and intra-host competition, coupled with regional genetic and environmental influences, may explain apparent heterogeneous exclusion of the historic parasite by the invasive species.

7.2 INTRODUCTION

Western honey bees (*Apis mellifera*, hereafter honey bees) are among the most vital and versatile pollinators on the planet, contributing to production of 39 of the world's 57 most important crops (Klein et al., 2007). Unfortunately, today's beekeepers face significant hurdles to maintain healthy colonies that are capable of crop pollination because of dramatic honey bee colony mortalities in many regions of the world. A great deal of attention has focussed on these mortalities because humanity's reliance on pollinator-dependent crops has increased significantly in the last half century (Aizen and Harder, 2009). Honey bee mortality is believed to result from multiple stressors acting alone or in combination, including nutritional deficiencies, management issues, agro-chemicals, and especially introduced parasites (Neumann and Carreck, 2010; vanEngelsdorp and Meixner, 2010; Williams et al., 2010a).

Significant interest has recently focussed on the newly detected microsporidian gut parasite *Nosema ceranae* because unusually high honey bee colony mortality coincided with its apparent host-switch from Asian honey bees (*Apis cerana*) to honey bees (Fries, 1996; Botías et al., 2012), as well as its subsequent widespread dispersal (Higes et al., 2006; Klee et al., 2007; Chen et al., 2008; Huang et al., 2008; Williams et al., 2008b). *N. ceranae* causes histopathological damage (Higes et al., 2007; Dussaubat et al., 2012a, 2012b), nutritional stress (Mayack and Naug, 2009, 2010; Alaux et al., 2010a), and suppresses host immunity (Chaimanee et al., 2012). However, colony-level effects are highly variable. In Spain for example, *N. ceranae* is typically associated with reduced colony survivorship (Higes et al., 2008a), whereas in other parts of Europe (Genersch et al., 2010) and in North America (Guzmán-Novoa et al., 2010; Williams et al., 2010b, 2010c), this has not been the case. Possible explanations for this variation include parasite or host genetics (Williams et al., 2008b; Chaimanee et al., 2010; Dussaubat et al., 2012a; Medici et al., 2012), climate/weather (Gisder et al., 2010; Chen et al., 2012), nutrition (Alaux et al., 2010b), or other factors, such as environmental contaminants or parasites. For example, multiple studies have demonstrated relationships, including possible synergisms, between *N. ceranae* and agro-chemicals (Alaux et al., 2010a;

Aufauvre et al., 2012), as well as viruses (Bromenshenk et al., 2011; Costa et al., 2012). Although biological mechanisms underlying relationships among stressors of honey bees are not well understood, it is likely that exploitative competition for limited resources, as well as host stress resulting from tissue pathology and immune suppression, play important roles (Alaux et al., 2010a; Costa et al., 2012; Dussaubat et al., 2012b), and could lead to numerical (i.e., intensity) or functional (i.e., realised niche) responses by parasites that are either symmetrical (both species experience equal responses) or asymmetrical (Poulin, 2007).

It is rare for multiple microsporidian species to be parasitic within sympatric individuals of the same insect species (Weiser, 1969; Solter et al., 2002). Nonetheless, sympatric honey bee populations, and even individuals, can be co-parasitized by both *N. ceranae* and *Nosema apis* (Klee et al., 2007; Burgher-MacLellan et al., 2010; Traver and Fell, 2011), the latter being the historical microsporidian species of honey bees (Fries, 1993; Williams et al., 2008a, 2010b). Similar to *N. ceranae*, *N. apis* can cause significant tissue damage in the gut that ultimately results in increased winter colony mortality or poor build-up of surviving colonies in spring (Fries, 1993). Within the last decade, *N. ceranae* has been detected on all continents where honey bees are maintained, while the occurrence of *N. apis* has diminished (e.g., Klee et al., 2007; Chen et al., 2008; Williams et al., 2008a, 2010b; Invernizzi et al., 2009; Higes et al., 2009), suggesting a numerical response by *N. apis* to co-infection that has resulted in decreased prevalence and distribution of the parasite. This apparent exclusion appears to be geographically heterogeneous, and is likely governed by previously discussed genetic and environmental factors influencing dispersal and competition for limited resources during density-dependent parasite regulation (e.g., Poulin, 2007 Williams et al., 2008b; Alaux et al., 2010b; Chaimanee et al., 2010; Gisder et al., 2010; Williams et al., 2010d; Chen et al., 2012; Dussaubat et al., 2012a; Medici et al., 2012).

Few studies have investigated both parasites simultaneously under experimental conditions, especially with regard to inter-specific competition. Paxton et al. (2007) observed higher mortality in *N. ceranae*-infected worker honey bees compared to those

parasitized by *N. apis*, and no difference in spore intensity (number of vegetative parasite cells per host) between the two species. Forsgren and Fries (2010) similarly found no difference in spore intensity between *N. ceranae* and *N. apis*. Conversely, they observed no difference in mortality between workers infected by the two *Nosema* species, and using molecular techniques detected no competitive advantage during co-infection by either parasite congener. Lastly, Martín-Hernández et al. (2011) reported higher mortality and increased nutritional demand by workers infected with *N. ceranae* compared to *N. apis*.

Because of the conflicting results regarding differences in virulence between *N. ceranae* and *N. apis*, and because the former has only recently spread from Asia to become a global concern, data on pathology associated with *N. ceranae* infection and interactions between *Nosema* congeners are of significant interest. Here we present an experiment that compared host mortality and nutritional demand, as well as parasite fecundity (quantified by spore intensity and DNA amount) and interspecific interactions, using honey bees artificially infected by *N. apis*, *N. ceranae*, or both. Uniquely, non-European sourced *Nosema* and honey bees were used to investigate possible geographic differences in this parasite-host system, and experimental hosts were observed for over four weeks, the typical length of time that worker honey bees spend performing intra-hive duties (Winston, 1987). Previous work (Paxton et al., 2007; Forsgren and Fries, 2010; Martín-Hernandez et al., 2011) used European-collected parasites and hosts, and terminated experiments between days 7 and 15 post inoculation. Based on laboratory and field investigations previously discussed, we hypothesised that *Nosema*-infected honey bees, in particular those parasitized by *N. ceranae*, would exhibit greater virulence than controls. We also predicted greater *N. ceranae* fecundity compared to *N. apis*, during both single and co-infections, as well as asymmetrical competition whereby *N. apis* experiences a greater reduction of infra-population size than *N. ceranae*. This would help to explain apparent exclusion of *N. apis* by *N. ceranae* in many regions of the world (Klee et al., 2007), including the area where our experimental parasites and hosts were collected (Williams et al., 2008a, 2008b).

7.3 METHODS

7.3.1 Experimental design

Laboratory experiments consisted of four treatment groups housed at Acadia University in Wolfville, Nova Scotia, Canada (Table 7.1). Each treatment group had 3 cages (wooden frame with hardware cloth and plexiglass sides; volume = 2,652 cm^3), each containing 20 Buckfast honey bee workers.

At 0 d (18 July 2010), 20 newly-emerged workers for each cage were orally inoculated (Table 7.1), and then subsequently fed 50% (weight/volume; i.e., 1 g granulated sucrose per 2 ml water) sucrose solution *ad libitum* for the duration of the experiment using a 10-ml syringe with the adaptor removed. The experiment was terminated at 30 d when no living workers remained for one of the treatment groups because they had either died in the cage or had been removed to quantify *Nosema* infection.

Table 7.1 Summary of experimental treatment groups, including quantity of *Nosema* spores inoculated orally to newly emerged workers in 5 µl of 75% (weight/volume) sucrose-water solution at 0 d.

Treatment group	# Spores	
	N. apis	*N. ceranae*
Control	0	0
N. apis	35,000	0
N. ceranae	0	35,000
N. apis & *N. ceranae* (mixed)	17,500	17,500

7.3.2 Obtaining workers for experiments

A single frame containing nearly emerged capped brood was collected on 14 July 2010 from each of two Buckfast honey bee colonies that had been sampled to confirm low *Nosema* spp. and *Varroa destructor* mite levels (Williams unpubl. data). Frames were

placed in individual nylon mesh sacs to keep emerging workers on them, and maintained at typical hive conditions of 34°C, 40% relative humidity, and complete darkness in a growth chamber (Kraus and Velthuis, 1997). Newly emerged individuals were moved to cages provided with feeders containing 50% w/v sucrose solution (described previously) every 4-8 h using a bee brush, and further maintained under aforementioned growth chamber conditions for the duration of the experiment.

7.3.3 Inoculation

N. apis and *N. ceranae* spores were obtained from honey bees from multiple commercial colonies in the provinces of Nova Scotia and Prince Edward Island in the Maritimes region of Canada. Species confirmation was performed molecularly as described below and in Burgher-MacLellan et al. (2010). Each species of *Nosema* was propagated in workers in the laboratory prior to experiments to ensure that experimental workers were inoculated with fresh, viable spores because storage conditions influences germination of *N. apis* and *N. ceranae* spores differently (Fries, 2010).

Once ~1000 newly-emerged individuals were collected from the two source frames, 50 were randomly allocated among each of 12 cages without sucrose solution overnight to promote ingestion of inocula. The following morning, 20 workers from each of the cages that were ~2-3 d old were individually fed 5 µl of 75% w/v sucrose solution that contained crushed *Nosema*-parasitized or unparasitized honey bee abdomens, depending on treatment (Table 7.1), and placed in new cages. Inocula spore densities that we used consistently result in infections in honey bees (e.g., Forsgren and Fries, 2010).

7.3.4 Host mortality & food consumption

Mortality was recorded daily; dead individuals were removed from cages and stored at -80°C. Food consumption was also measured daily to quantify nutritional demand (Mayack and Naug, 2009) by visually recording quantities of sucrose solution depleted from syringes; per worker daily consumption was calculated by using the number of living workers at the end of each 24-h interval. Food was replaced every week to limit

microbial growth and to ensure sucrose solution was provided *ad libitum*. Comparison of food consumption among groups was not performed after 25 d post inoculation, when one cage contained a single living worker.

7.3.5 Parasite fecundity

Nosema spore intensity (spores per bee) was quantified at 7, 14, and 21 d post inoculation using one randomly chosen living worker per cage (*n* = 3 per treatment group). Spores, as well as DNA, were further quantified on all workers that died between 28 and 30 d (*n* = 2, 8, 7, and 9 for control, *N. apis*, *N. ceranae*, and mixed treatments, respectively), immediately prior to experiment termination. Spore intensity in living and dead workers could not be compared on the same days due to the low number of dead workers at 7, 14, and 21 d, and the low number of living workers between 28 and 30 d. All workers were stored immediately at -80°C after collection from cages until *Nosema* quantification was performed.

7.3.6 Congeneric parasite interactions

N. apis and *N. ceranae* DNA quantities were compared within individual living workers from the mixed *N. apis* & *N. ceranae* treatment at 28 d post inoculation (*n* = 3), and also within workers that died between 28 and 30 d post inoculation as described in the previous section.

7.3.7 *Nosema* quantification - microscopy

Suspensions were created with an individual honey bee abdomen crushed with a pellet pestle in 1 ml distilled water. *Nosema* spores were counted in these suspensions using a haemocytometer and light microscopy (Cantwell, 1970; Thermo Fisher Scientific, Waltham, Massachusetts, USA).

7.3.8 *Nosema* quantification - simplex real-time PCR

Nosema DNA (ng) was quantified using methods outlined by Burgher-MacLellan et al. (2010) and Forsgren and Fries (2010). Briefly, DNA was isolated from each honey bee by pre-treating a 250-µl aliquot of a crushed abdomen suspension (described in the previous section) with 10 µl proteinase K (20 mg/ml) (Sigma-Aldrich Canada, Oakville, Ontario, Canada) for 20 min at 37°C. DNA was then purified using a modified protocol (steps 1-3 omitted) from the Ultra Clean Tissue DNA Extraction Kit (Mo Bio Laboratories, Carlsbad, California, USA). DNA was quantified using a Nanodrop 1000 spectrophotometer (Fisher Scientific, Ottawa, Ontario, Canada), and samples stored at -20°C until real-time PCR was performed.

Simplex quantitative real-time PCR (qPCR) was performed using an Mx4000 thermocycler (Stratagene, La Jolla, California, USA). Each separate qPCR reaction consisted of 12.5 µl Maxima SYBR Green/Rox qPCR master mix (Thermo Scientific, Rockford, Illinois, USA), 0.2 µl *N. apis* or *N. ceranae* primer sets (Martín-Hernández et al., 2007), 1 µl template (100 ng extracted *Nosema* DNA) and nuclease-free water to a final volume of 25 µl. Each PCR reaction was performed in triplicate on the same plate and contained negative controls (no template DNA). Triplicate means were reported. PCR amplification parameters included: an initial 10-min denaturing period at 95°C followed by 40 cycles of 30-s denaturing at 95°C, 30-s annealing at 60°C, and 30-s extension at 72°C, and a final 5-min extension period at 72°C. Amplified products were confirmed using melting curve analysis plots where temperature profiles were 1 min at 95°C, 30 s at 55°C, followed by forty 30-s increases of 1°C, and a final holding temperature at 4°C. Each qPCR run included the appropriate quantification standard curves (*i.e.* $R^2 > 0.98$ and primer efficiency > 94%) prepared using serial dilutions ranging between 1.0^{-1} and 1.0^{-7} ng of purified PCR products (*N. apis* and *N. ceranae*) for target DNA. Bee DNA samples were quantified for *Nosema* DNA amount by plotting cycle threshold (Ct) values against nanograms of target DNA.

7.3.9 Statistical analyses

All statistical analyses were performed using R 2.15.2 (R Development Core Team; Vienna, Austria), except for the survival analysis which was performed using Minitab® 16 (Minitab Inc., State College, Pennsylvania, USA). Cumulative mortality was analysed using the Kaplan-Meier Log-Rank survival analysis for 'censored' data because time of death for some workers was not known (i.e., some living workers were killed periodically to quantify spore intensity during the experiment, and some were still living when the experiment was terminated) (Crawley, 2005). Food consumption and *Nosema* intensities were evaluated using ANOVAs or Repeated Measures ANOVAs; Tukey's HSD post hoc test was used for multiple comparisons between treatments. Where appropriate, data were square-root transformed to improve fit to normality.

7.4 RESULTS

7.4.1 Host mortality & food consumption

Mortality at 30 d post-inoculation was 25.0, 70.0, 95.0, and 76.7% for control, *N. apis*, *N. ceranae*, and mixed treatments, respectively (Figure 7.1). By 30 d, all *N. ceranae* treatment workers had either died in the cage or had been removed to quantify *Nosema* infection. Workers in the *N. ceranae* treatment had significantly increased mortality compared to workers from the other treatments (Kaplan-Meier Log-Rank Test, all Ps < 0.002), whereas controls had significantly lower mortality compared to all other treatments (Kaplan-Meier Log-Rank, all Ps < 0.001). Mortality did not differ significantly between workers in the *N. apis* and mixed treatments (Kaplan-Meier Log-Rank, $P = 0.67$) (Figure 7.1).

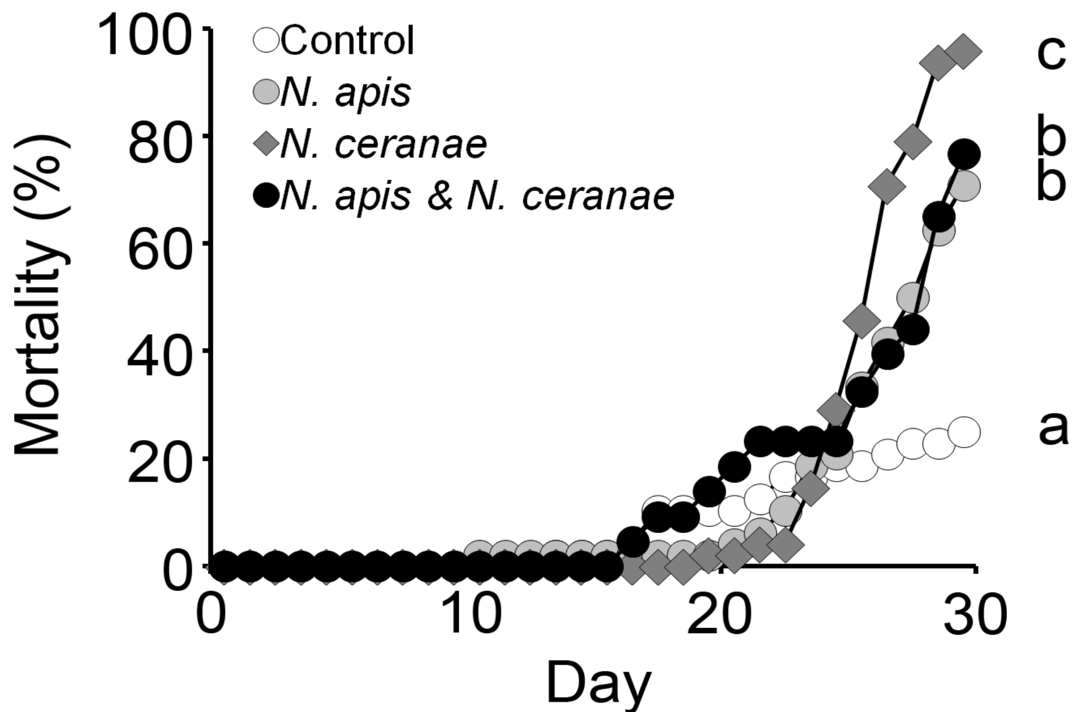

Figure 7.1 Effect of *Nosema* infection on adult worker western honey bee mortality. Mortality is shown as the cumulative percentage of dead individuals per treatment (Table 7.1) per day. The experiment was terminated at 30 d post inoculation when no living workers remained from the *N. ceranae* treatment. Treatments with different letters had significant differences in mortality.

Daily food consumption changed significantly over time (Repeated Measures ANOVA, $F_{20,160} = 0.4$, $P < 0.0001$); however, no difference was observed among treatments (Repeated Measures ANOVA, $F_{3,8} = 0.4$, $P = 0.79$) (Figure 7.2). By Week 4 (22-25 d post inoculation), mean daily consumption per worker was ~3.6, 4.4, 4.6, and 3.9 µl for control, *N. apis*, *N. ceranae*, and mixed treatments, respectively.

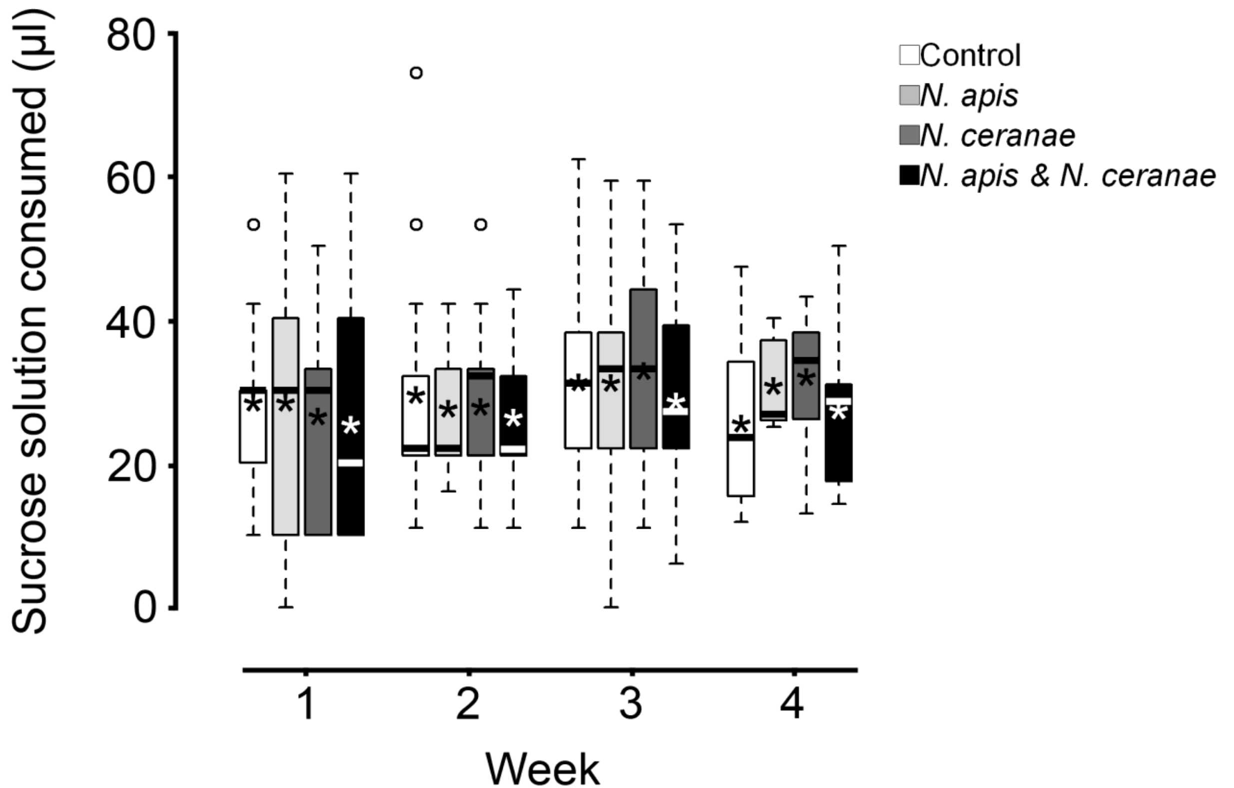

Figure 7.2 Effect of *Nosema* infection on adult worker western honey bee nutritional demand. Sucrose solution consumed is shown as volume of 50% (weight / volume) sucrose-water mixture consumed per bee per week post inoculation of appropriate treatment (Table 7.1) inoculum (Week 4 included only consumption at 22-25 d post inoculation). Boxplots show interquartile range (box), median (black or white line within interquartile range), data range (dashed vertical lines), and outliers (open dots); asterisks (black or white) represent means. No significant differences were observed among treatments for daily consumption per worker.

7.4.2 Parasite fecundity

Nosema intensity increased over time for all *Nosema* treatments (Figure 7.3). Because no infections (other than likely artifacts) were observed in controls, they were excluded from remaining analyses. Despite greater spore intensities for *N. apis* and *N. ceranae* treatments at 7 and 14 d, respectively, no statistical differences were observed (both ANOVAs, $F_{2,6} \leq 0.5$, Ps ≥ 0.62). At 21 d, however, spore intensity was significantly greater in the *N. ceranae* than in the *N. apis* treatment (Tukey's HSD, $P = 0.05$).

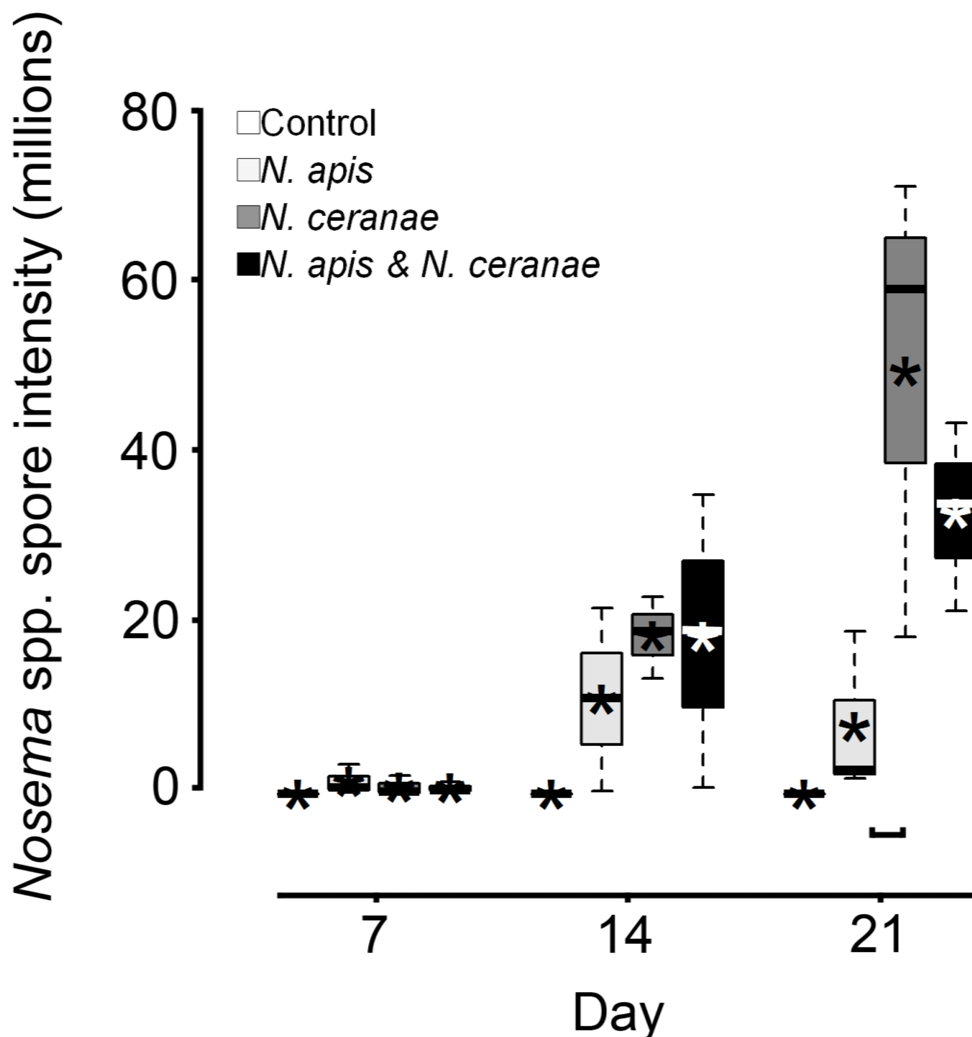

Figure 7.3 Effect *Nosema* infection development in live-sampled adult worker western honey bees. Infection intensity determined by spore counts at 7, 14, and 21 d post oral inoculation (Table 7.1). Boxplots show interquartile range (box), median (black or white line within interquartile range), data range (dashed vertical lines), and outliers (open dots); asterisks (black or white) represent means. Horizontal square parenthesis under boxplots indicates a significant difference; controls were excluded from analyses because no infections were observed.

In workers that died between 28 and 30 d post inoculation, *Nosema* spore intensities were significantly different among groups (Figure 7.4). Intensity in *N. ceranae* workers was greater than in *N. apis* workers (Tukey's HSD, adjusted $P = 0.03$), but not compared to workers from the mixed group (Tukey's HSD, adjusted $P = 0.60$). Additionally, no

difference in spore intensity was observed between workers from the *N. apis* and mixed treatments (Tukey's HSD, adjusted $P = 0.16$) (Figure 7.4). For the same workers, no difference in quantity of *N. apis* and *N. ceranae* DNA was observed between respective treatments (ANOVA, $F_{1,13} = 1.6$, $P = 0.23$) (Figure 7.5).

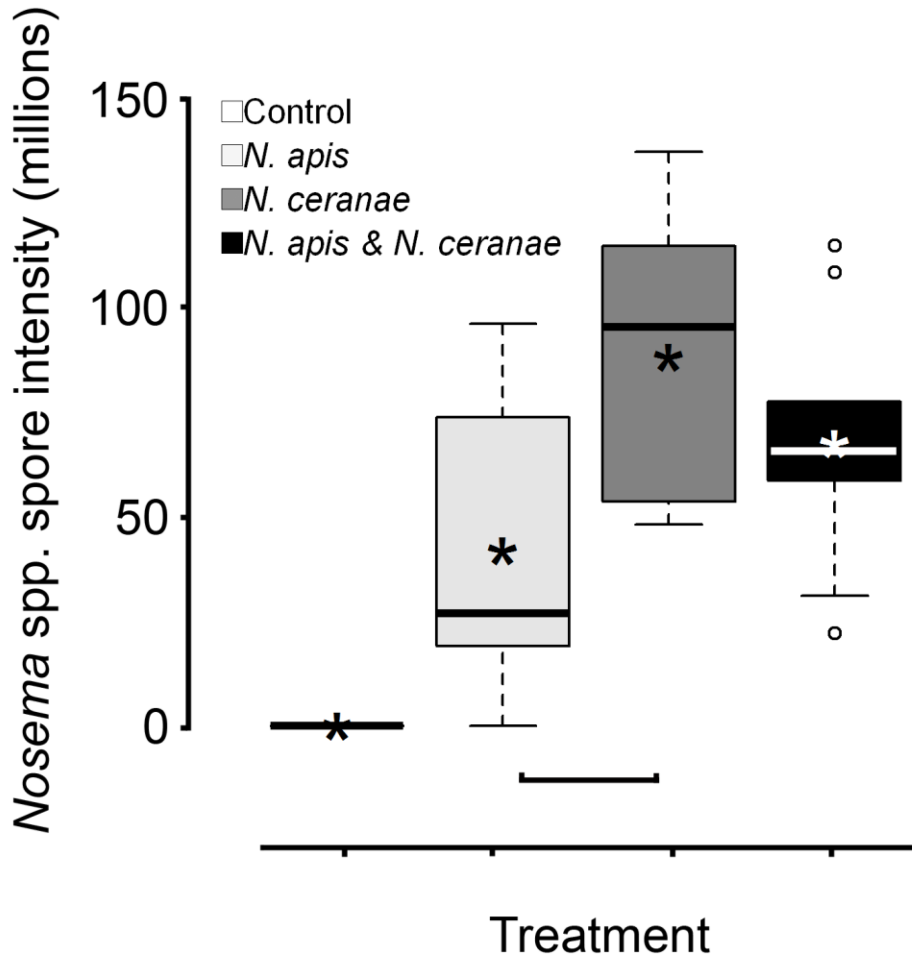

Figure 7.4 Level of *Nosema* infection in dead-sampled adult worker western honey bees four weeks post oral inoculation (Table 7.1). Infection intensity determined by spore counts using workers that died at between 28 and 30 d post inoculation. Boxplots show interquartile range (box), median (black or white line within interquartile range), data range (dashed vertical lines), and outliers (open dots); asterisks (black or white) represent means. Horizontal square parenthesis under boxplots indicates a significant difference; controls were excluded from analyses because no infections were observed.

7.4.3 Congener parasite interactions

No difference was observed between spore intensities from the mixed treatment and either *N. apis* or *N. ceranae* treatments (both Tukey's HSD, adjusted $P \geq 0.13$) (Figure 7.3). Additionally, no difference in the quantity of *N. apis* DNA was observed among *N. apis* and mixed treatments (all Tukey's HSD, adjusted $P \geq 0.50$), or for *N. ceranae* DNA quantity among *N. ceranae* and mixed treatments (all Tukey's HSD, adjusted $P \geq 0.62$) (Figure 7.5). Quantity of *Nosema* DNA between either species in workers from the mixed treatment collected at 28 d post inoculation (ANOVA, $F_{1,4} = 0.0$, $P = 0.91$), and in workers that died between 28 and 30 d (ANOVA, $F_{1,16} = 0.73$, $P = 0.41$), were also not significantly different (Figure 7.5).

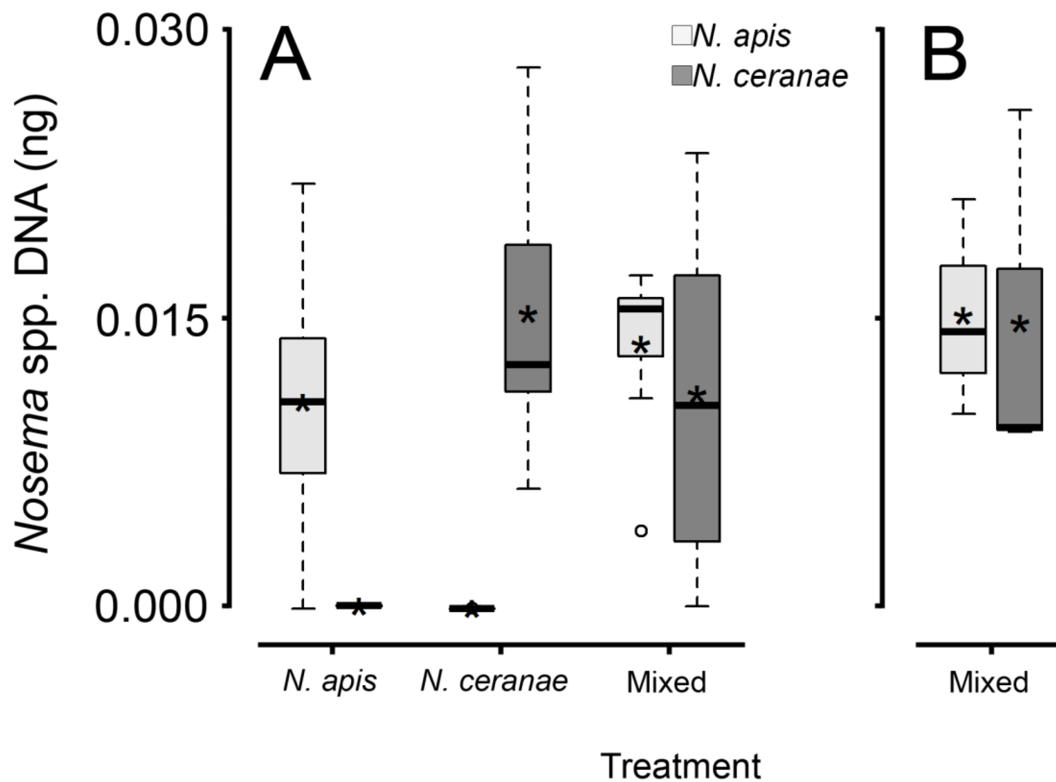

Figure 7.5 Levels of *Nosema apis* and *Nosema ceranae* DNA (square root transformed) in (A) dead and (B) live adult worker western honey bees four weeks post oral inoculation (Table 7.1). DNA quantity determined using quantitative PCR on: (A) dead workers from *N. apis*, *N. ceranae*, and mixed treatments collected at between 28 and 30 d post inoculation (same workers shown in Fig. 4) and (B) live workers collected 28 d post inoculation using one individual per cage. Boxplots show interquartile range (box), median (black or white line within interquartile range), data range (dashed vertical lines), and outliers (open dots); asterisks (black or white) represent means. No significant differences were observed in quantities of *N. apis* and *N. ceranae* DNA among treatments (when *N. ceranae* and *N. apis* from *N. apis* and *N. ceranae* treatments, respectively, were excluded from the analysis.

7.5 Discussion

Similar to other laboratory studies (e.g., Paxton et al., 2007; Forsgren and Fries, 2010; Martín-Hernández et al., 2011), we confirmed that infection by *Nosema* significantly reduced honey bee worker survivorship. Furthermore, *N. ceranae* reduced survivorship more than did *N. apis* or mixed *Nosema* infections; however, this result is not universal among previous investigations. Reasons for disparities among studies comparing mortality of infected workers could be genetic or environmental (including methodological). For example, as suspected for *Nosema bombi* microsporidians in bumble bees (Tay et al., 2005), genetic variants of *Nosema* species infecting honey bees may differ in virulence (Williams et al., 2008b; Chen et al., 2009a). Similarly, host genetics could also affect susceptibility (Dussaubat 2012a). Additionally, some commonly used agro-chemicals possibly interact with *N. ceranae* (Alaux et al., 2010a; Aufauvre et al., 2012; Pettis et al., 2012), and Deformed wing and Black queen cell viruses were negatively and positively correlated with *N. ceranae* and *N. apis*, respectively (Bailey et al., 1983; Costa et al., 2011). Unfortunately, broad-scale screening for these extrinsic factors in experimental workers, as well as their source colonies, is costly and not regularly performed during standard laboratory assays. Thus, it is not clear if these factors influenced this or previous results. Worker longevity from the mixed infections was similar to *N. apis* infections, but lower than for *N. ceranae* infections. It is possible that spore intensity in the *N. ceranae* treatment reached a critical

threshold that resulted in increased mortality that did not occur in workers infected by *N. apis* alone or both species together. Hence, mortality of workers in the latter two treatments was not statistically different.

We did not observe differences in energetic demand, as measured by sucrose consumption, among treatment groups. This was unexpected because parasites compete with their hosts for nutrients (Schmid-Hempel, 2005), thereby sometimes, but not always (Kyriazakis et al. 1998), increasing nutritional demand. In previous studies, *Nosema*-infected workers had significantly increased demand for energy, which was also measured by carbohydrate sucrose consumption (Mayack and Naug, 2009; Alaux 2010a), as well as increased sugar metabolism (Dussaubat et al., 2012b); workers were particularly needy when infected with *N. ceranae* compared to its congener (Martín-Hernandez et al., 2011). However, not all studies have observed these phenomena. For example, workers infected by *N. ceranae* in a laboratory in France did not exhibit increased demand for energy (Aufauvre et al., 2012). This is interesting because the extremely high *N. ceranae* spore intensities in workers studied by Aufauvre et al. (2012) were similar to spore intensities observed during our experiment (i.e., in some cases >100 million spores per bee), and therefore were expected to produce greater nutritional stress than lower spore densities observed in the other studies. It is also possible that nutrient reserves that workers stored during their development (Maurizio, 1959; Haydak, 1970) were sufficient in the face of parasite infection. This is unlikely due to the length of time experimental workers were observed, and because only carbohydrates and no other nutrients were provided. It would be interesting to compare, for example, nutrition and health status among treatments by assessing worker mass (Pettis et al., 2012) or vitellogenin gene (protects from oxidative stress) expression (Seehuus et al., 2006). It is also possible that aforementioned genetic and environmental factors could cause variation in nutritional demands of honey bees among the various laboratory studies.

Nosema spore intensities in workers that died between 28 and 30 d post-inoculation were consistent with spore intensity data collected from live workers at 21 d post inoculation, wherein *N. ceranae* fecundity was significantly greater than that of *N. apis*. Conversely,

quantity of *Nosema* DNA did not differ between congeners. It is likely that *Nosema* DNA that we detected represented immature stages within host cells rather than mature spores due to a dense wall surrounding each spore (Fries, 1993; Chen et al., 2009b). Spore dimorphism (thin walled spores germinate within hosts and those with thicker walls are released into the environment) are known from the family Nosematidae, including *N. apis* (Fries, 1993). It is possible that higher spore intensity of *N. ceranae* compared to *N. apis* is the result of a faster multiplication rate and a greater investment towards environmentally resistant spores that do not reinfect gut epithelial cells, but rather reside in the rectum until they are released into the environment via contaminated frass (i.e., faeces). Unfortunately, little is known about the biology, including life cycle and spore production, of *N. ceranae* in honey bees. Greater potential for faecal-oral horizontal transmission resulting from high levels of *N. ceranae* spores in the environment could explain why distribution of *N. ceranae* has increased rapidly in recent years (e.g., Klee et al., 2007), and why the parasite can be found in contaminated materials in the hive or on forage (Higes et al., 2008b; Copley et al., 2012). An alternative explanation for differences observed between spore and DNA results could be dysentery (i.e., diarrhea), which is often exhibited by *N. apis*-infected honey bees under natural conditions, ejecting spores from hosts (Fries, 1993); this sign is not observed in those parasitized by *N. ceranae* (Faucon, 2005), and high spore levels can be found in the hindgut (Bourgeois et al., 2012). It is possible that spores were ejected from workers infected by *N. apis* and not *N. ceranae* due to the length of the experiment, and hence not quantified when whole abdomens were crushed. This is unlikely because laboratory honey bees rarely defecate in cages, and few, if any, signs of frass were observed during our experiment.

To our knowledge this is the first laboratory study to follow simultaneously both *N. apis* and *N. ceranae* intensity beyond two weeks (cf., Paxton et al., 2007; Forsgren et al., 2010; Martín-Hernández et al., 2011). This is particularly important because mean honey bee worker longevity during the foraging season (when this study was performed) is between 15 and 60 d (Winston, 1987). Worker age could partially explain why we obtained spore intensities of ~90 million by the fourth week of the experiment. Although

much lower intensities typically occur fewer than 14 d after infection (e.g., Alaux et al., 2009; Pettis et al., 2012;), Aufauvre et al. (2012) had even higher spore intensities than we did after 22 d. Fully developed *N. apis* infections of ~20-30 million spores per individual occur within 14 d post inoculation, regardless of initial inoculum (Fries, 1988), and no difference in *N. ceranae* spore intensity was observed in workers 12 d post inoculation of ~33,333 or 333,333 spores (Pettis et al., 2012). Thus, it is unlikely that variation in spore intensities reflects initial spore inocula (e.g., Alaux et al., 2009; Pettis et al., 2012; Aufauvre et al., 2012). The *N. apis* spore intensity observed by Fries (1988) is similar to our observed mean spore intensity for that species of between 20-40 million at 14 and 21 d post inoculation, as well as in workers that died between 28 and 30 d.

It is possible that the discrepancy in *N. ceranae* spore intensities among laboratories could be the result of the type of honey bee tissue examined. Although laboratory methods are not always sufficiently described in the literature, we and Aufauvre et al. (2012) quantified parasite infection using whole abdomens, whereas others (e.g., Paxton et al., 2007) examined only the midgut. Furthermore, *N. ceranae* intensities were similar to ours when Paxton et al. (2007) examined whole honey bees and not just the midgut, although these individuals were from colonies rather than laboratory cages. This highlights the importance of standardising research methods for studying *Nosema* in honey bees to facilitate comparison of data among studies (e.g., Frost et al., 2012, Fries et al., 2013).

Based on spore intensity, it appears that carrying capacity within honey bees, or at least maximum population size, can be much greater for *N. ceranae* than for *N. apis*. Despite our extended observation of workers, neither our data nor that of previous studies that observed spore intensities regularly for shorter time periods obtained asymptotic *N. ceranae* intensities (Paxton et al., 2007; Alaux et al., 2009; Dussaubat et al., 2012a). It is possible that smaller spore size (Fries et al., 2006), broader tissue tropism (Chen et al., 2009b), and limited time for co-evolution (Poulin, 2007), at least compared *N. apis*, could help to explain this.

Although we observed higher spore intensities for *N. ceranae* compared to *N. apis*, results from mixed infections suggested competition between parasite species. If full infection occurs regardless of initial spore inocula, as discussed previously, we would expect parasite intensities from the mixed treatment to be the sum of both single *Nosema* treatments; this was clearly not observed as spore intensity was of intermediate intensity in mixed *N. apis* and *N. ceranae* treatments (although no statistical significance was observed). Unfortunately, similar size and shape of *N. apis* and *N. ceranae* spores did not make it possible to accurately distinguish species (Fries, 2006); therefore, we could not determine if symmetrical or asymmetrical competition occurred. Conversely, DNA quantities in single and mixed infections did not suggest competition as no difference in parasite intensity was observed, regardless of treatment. Forsgren and Fries (2010) similarly did not observe competition between *Nosema* species based on molecular methods; they did not investigate spore levels using light microscopy. This could suggest a functional response by one or both parasites, whereby host cells can be parasitized by *Nosema* but reproductive output (in terms of number of spores), is affected. It seems that the apparent displacement of *N. apis* by *N. ceranae* in many regions of the world as reported by Klee et al. (2007) could be explained by asymmetric competition resulting in greater spore production by *N. ceranae* that subsequently results in increased parasite horizontal transmission via the faecal-oral route. Although we cannot say how competition affects spore production of each species during co-infection, in Spain, for example, greater spore production by *N. ceranae* during single infections could provide an overall competitive advantage to that species for many parts of the year because unlike *N. apis*, it does not experience seasonal differences in prevalence (Martín-Hernández et al., 2012). Conversely, if full infection intensity is a function of initial spore quantity ingested, then mean spore intensity observed in the mixed *N. apis* and *N. ceranae* treatment suggests that no interaction between *Nosema* species occurred, whereas quantities of *Nosema* DNA suggest a synergism. Additional studies are required to better understand the relationship between infection dose and final infection intensities of the novel *N. ceranae* to better evaluate possible inter-specific interactions between *N. apis* and *N. ceranae* in honey bees.

Controversy remains over the role of *Nosema* gut parasites in the recent high honey bee colony mortalities observed in many parts of North America and Europe (e.g., Higes et al., 2008a; Bromenshenk et al., 2010; Genersch et al., 2010; Guzmán-Novoa et al., 2010; Williams et al., 2010b, 2010c). Here, in a long-term laboratory cage study using parasites and hosts residing outside of Europe, we demonstrated that parasitism by *Nosema* increases honey bee worker mortality, despite regional studies at the colony level suggesting that *N. ceranae* may not be the most economically important parasite of honey bees (e.g., Guzmán-Novoa, 2010; Williams et al., 2010c). In particular, honey bee survivorship was significantly reduced by the relatively novel *N. ceranae* compared to the historic *N. apis*. Additionally, we observed higher spore intensity in honey bees parasitized by *N. ceranae* compared to *N. apis* and a numerical response to spore production during co-infection; this is likely important to inter-host horizontal parasite transmission that relies on ingestion of spores. Although temperature (Fenoy et al., 2009; Fries, 2010; Gisder et al., 2010; Martín-Hernández et al., 2012), geographic isolation from the global apiculture industry (e.g., Williams et al., 2010d), and previously described genetic and other environmental factors all probably influence distribution, as well as virulence, of the two *Nosema* parasites in honey bees, variation in laboratory methods employed by researchers likely further contribute to our foggy understanding of these host-parasite systems. Recently Fries et al. (2013) and Williams et al. (2013) describe *in vitro* laboratory protocols for studying *Nosema* in honey bees. Following these protocols will facilitate comparison of results obtained among studies, and ultimately improve our knowledge of not only mechanisms responsible for differences in virulence of *Nosema* parasites, but also other factors that influence honey bee health in general.

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CHAPTER 8 CONCLUSIONS AND FUTURE DIRECTIONS

Part of the work presented in Chapter 8 appeared as my Preliminary Exam essay and report.

8.1 CONCLUSIONS

Detection of *Nosema ceranae* in honey bees during a period of dramatic population declines sparked a flurry of speculation regarding the contribution of this parasite to colony mortality. When I began my graduate studies very little was known about *N. ceranae* in honey bees apart from work that suggested that the parasite had recently displaced the historic honey bee microsporidian, *Nosema apis* (Klee et al., 2007). As a result, my thesis, as well as work by others in numerous laboratories around the world, sought to better understand the biology and management of *N. ceranae* in honey bees.

For the remainder of this section I will focus on highlighting key findings from my thesis work regarding the distribution, management, virulence, and inter-specific interactions of *N. ceranae* in honey bees. Additionally, I will connect my findings with those from other laboratories that simultaneously studied this parasite so as to provide a solid overview of the current state of knowledge of *N. ceranae* in honey bees, and to recommend future directions of research.

8.1.1 *N. ceranae* distribution

Within the last decade, *N. ceranae* has been detected on all continents where honey bees are maintained, while distribution of *N. apis* has receded (e.g., Klee et al., 2007; Chen et al., 2008; Invernizzi et al., 2009; Higes et al., 2009). In Maritime Canada, I found that *N. ceranae* was the most prevalent *Nosema* parasite in Nova Scotia and Prince Edward Island, but not in New Brunswick (Chapters 3, 4, and 6). Despite the admittedly limited scope of surveys in the latter two provinces, beekeeper operations sampled in Nova Scotia are likely representative of most colonies maintained in mainland Nova Scotia due

to the broad geographic area I sampled. This has potential important implications for species-specific management by beekeepers. Unfortunately, many surveys, including those of this thesis, lack appropriate longitudinal depth to truly understand long-term changes in parasite prevalence over time due to lack of properly preserved historical honey bee samples or laboratory resources. Investigations not included in my dissertation that I performed in Newfoundland, where only *N. apis* has been detected (Williams et al., 2010; Shutler et al., submitted), should provide adequate baseline information to properly study *N. ceranae* dispersal should it invade that region.

Although movements of honey bees, their products, and beekeeping equipment by humans have resulted in introductions of parasites to new geographic regions around the globe (Evans and Spivak, 2010, Lounsberry et al., 2010), most dispersal relies on natural routes of horizontal and vertical transmission to be successful. For honey bees, parasite transmission occurs among individuals within the same colony, or to new individuals in different colonies (i.e., inter-colony transmission). The latter can occur vertically by swarming, or horizontally via robbing, drifting, or forage-sharing (Fries and Camazine, 2001). Mechanisms of parasite transmission for *N. ceranae* are still relatively unclear; however, detection of *N. ceranae* in honey bees in Maritime Canada (Chapters 3, 4, and 6) suggests that both the parasite and host successfully fulfil encounter and compatibility filters described previously (Poulin, 2007). The laboratory study I performed (Chapter 7) also supports my field data, whereby *N. ceranae* can successfully reproduce in Maritime honey bees, even during co-infection with *N. apis*. High fitness (indexed by spore output) of *N. ceranae* compared to *N. apis* suggests a mechanism whereby exclusion of *N. apis* by *N. ceranae* could be the result of asymmetrical competition (i.e., unequal response by parasites) between species (Poulin, 2007) because as gut parasites (Fries, 1993; Higes, 2006), both occupy similar ecological niches.

In North America, it is possible that *N. ceranae* originated from isolated importations of infected honey bees or their products, possibly from central Europe (Chapter 2). At a coarse scale, *N. ceranae* likely dispersed throughout the United States during unrestricted inter-crop movement of infected migratory beekeeping colonies for commercial

pollination, but at a fine scale, natural transmission routes such as flower sharing (Higes, 2008b) and robbing (Giersch et al., 2009) likely transmitted the parasite to the local environment and to neighbouring colonies. *N. ceranae* could also have spread to Canada during importation events or from the United States by foraging workers. This could also explain why only the historic species, *N. apis*, can be found in isolated beekeeping areas of Canada such as the island of Newfoundland (Williams et al., 2010; Shutler et al., submitted).

8.1.2 *N. ceranae* management

Relatively little effort has been devoted to developing successful management techniques for *N. ceranae*. My research (Chapters 4 and 5), as well as that of Higes et al. (2008a), suggest that the same late summer dose of fumagillin recommended for *N. apis* is also suitable for *N. ceranae*. Additionally, 30 mg fumagillin per colony applied in four weekly treatments in spring is recommended by the manufacturer (Medivet Pharmaceuticals Ltd., 2010). This is supported by results I presented (Chapter 4), wherein fumagillin treatment applied in fall has no effect on *N. ceranae* spore intensity one year later. *N. ceranae* appears to be extremely resistant to desiccation and temperatures up to 60°C (Fenoy et al., 2009), just below the melting point of wax; therefore, heat treatment methods used for *N. apis* do not work for *N. ceranae* (Pernal et al., 2009). Comb disinfection studies revealed that electron beam irradiation successfully reduced *N. ceranae* spore viability, whereas 80 % acetic acid fumigation, that is effective against *N. apis*, did not (Pernal et al., 2009), thereby providing at least two options (i.e., chemotherapy and comb irradiation) for disease management. Although indoor over-wintering did not influence levels of *N. ceranae* parasitism, it nevertheless reduced colony mortality (Chapter 6), and therefore provides a good option for beekeepers that manage colonies perennially in temperate climates.

8.1.3 *N. ceranae* virulence

The few data currently available on *N. ceranae* pathogenicity in honey bees provide conflicting views. In the field, *N. ceranae* has been associated with increased colony

mortality in Spain (Higes et al., 2010), but not in Germany (Genersch et al., 2010). Field studies that I (Chapters 5 and 6) and Guzmán-Novoa et al. (2010) performed in Canada suggested that *N. ceranae* did not significantly influence colony health. In the laboratory, infection studies performed in Europe on individual honey bees also provide contradictory evidence of virulence and infectivity compared to uninfected or *N. apis*-infected individuals (Higes et al., 2007; Paxton et al., 2007; Forsgren and Fries, 2010). During similar experiments in Canada (Chapter 7), I observed that *Nosema* parasitism significantly increased honey bee mortality, in particular during *N. ceranae* infection. This was likely the result of tissue damage (Higes et al., 2007; Dussaubat et al., 2012a, 2012b) caused by high numbers of spores that were observed, but possible additional mechanisms contributing to increased mortality could be host immune suppression (Antúnez et al., 2009) or nutritional stress (Mayack and Naug, 2009, 2010; Alaux et al., 2010a). The disparity between results from my field and laboratory studies was intriguing. It is possible that colony-level disease resistance mechanisms (Evans and Spivak, 2010), present under natural conditions (Chapters 5 and 6) but not in the laboratory (Chapter 7), mitigated effects of parasitism by *N. ceranae.* Additionally, extremely high spore levels in laboratory-infected honey bees likely contributed to reduced survivorship (Chapter 7); such infection intensities may be rare under natural colony conditions, and were not observed during my field investigations (Chapters 5 and 6).

8.1.4 *N. ceranae* inter-specific interactions

A simultaneous increase in prevalence of *N. ceranae* and decrease of *N. apis* suggest that the introduced parasite is displacing the historic one (e.g., Klee et al., 2007; Chen et al., 2008; Invernizzi et al., 2009; Higes et al., 2009; Chapters 3, 4, 6). It is possible this resulted because of inter-specific competition for the same limited resources (Poulin, 2007), whereby *N. apis* has experienced a numerical response (i.e., decreased prevalence) in honey bees. Exclusion appears to be spatially heterogeneous, especially in Europe (Klee et al., 2007; Gisder et al., 2010; Higes et al., 2010), and *N. ceranae* is significantly more prevalent than *N. apis* in the North America (Chen et al., 2008; Chapters 3, 4, 6).

Unfortunately, many surveys lack appropriate longitudinal depth to truly understand changes in parasite prevalence over time from field observations due to lack of properly preserved historical western honey bee samples. Paradoxically, *N. ceranae* did not exhibit a competitive advantage over *N. apis* in a cage experiment by Forsgren and Fries, 2010, although my data suggest that competition between *N. ceranae* and *N. apis* resulted in overall decreased *Nosema* spore production (Chapter 7).

8.2 FUTURE DIRECTIONS

Despite intensive investigations into the biology of *N. ceranae*, its contribution to recent colony mortality is not clear due to conflicting results from multiple field and laboratory studies. Elucidating effects of this parasite on colonies is further complicated by the multiple additional pressures that no doubt also influence honey bee health; these factors may not be geographically homogeneous. Understanding factors influencing *N. ceranae* distribution and virulence, as well as strategies for parasite management, should be priorities.

8.2.1 Adoption of appropriate experimental approaches for *N. ceranae* research

Experimental design may contribute to our current foggy understanding of *N. ceranae*. For example, recent laboratory experiments by Higes et al. (2007), Paxton et al. (2007), Forsgren and Fries (2010), and I (Chapter 7) to understand *Nosema* virulence in honey bees highlight a diversity of study protocols (e.g., host and parasite genotypes, growth chamber temperature, food quality and quantity) employed to answer similar questions, but also a diversity of results. This makes teasing apart critical factors from those that are less important extremely difficult. Complementary laboratory (i.e., highly controlled settings) and field (i.e., real-world scenarios) investigations using standardized approaches are required. The COLOSS BEEBOOK chapters discussing *Nosema* (Fries et al., 2013) and laboratory (Williams et al., 2013) studies are a step towards this goal.

8.2.2 Development of an IPM strategy for *N. ceranae* control

Currently a limited strategy of IPM has been adopted by beekeepers for *N. ceranae* compared to the mite *Varroa destructor* (e.g., Calderone, 2005; Delaplane et al., 2005). This is largely due to a lack of knowledge of *N. ceranae* biology, as well as limited availability of preventative and control techniques.

Monitoring is the first step of any successful IPM programme (EPA, 2009). However, the largest adversaries to proper monitoring of *Nosema* in honey bee colonies are resources and time. Currently most beekeepers rely on researchers and extension personnel to assess parasite status (e.g., Chapters 1, 3, 4, and 6). Efforts should be made to develop simple diagnostic tools and sampling strategies to detect *Nosema* infection instead of conventional techniques such as light microscopy or PCR analyses that are impractical for most beekeepers.

Preventing and controlling pests using a variety of methods at appropriate times are the keystones to IPM, yet very little regional data on economic and treatment thresholds exist. Additionally, only one type of chemotherapy, Fumagilin-B®, is known to be effective for *N. ceranae* (Chapters 4 and 5) and *N. apis* control (Katznelson and Jamieson, 1952). Future attention should focus on determining ideal treatment regimes and economic injury levels that ensure adequate control but avoid over-dosing and promoting development of resistance.

Bee yard management plays a critical role in honey bee disease epidemiology (Fries and Camazine, 2001), including prevention of disease. Understanding sources of infection, as well as routes of transmission of *N. ceranae*, will possibly identify methods to prevent or reduce parasitism in colonies. It is likely that typical Good Apicultural Practices, such as regular replacement or sterilisation of old comb, removing dead-outs to reduce robbing, and appropriate colony orientation to limit drifting (Currie *et al.*, 1998), can all help to prevent *N. ceranae* parasitism. Ultimately, efforts should focus on identifying methods

for *N. ceranae* management that are practical to beekeepers and follow the four pillar framework of IPM.

8.2.3 Identification of the causes of variation in *N. ceranae* distribution, virulence, and inter-specific interactions

Encounter and compatibility filters are governed by host and parasite genotypes, in addition to surrounding environments (Mackinnon and Read, 1999; Ferguson and Read, 2002). This framework suggests a number of hypotheses that should be further investigated to explain heterogeneity of *N. ceranae* distribution and virulence in honey bees.

First, honey bees have significant genetic variation for mechanical, physiological, immunological, and behavioural responses to disease (Evans and Spivak, 2010). For example, multiple genetic variants can possibly explain regional differences in prevalence and virulence of the ectoparasite mite *Varroa destructor* (de Guzman et al., 1999; de Guzman and Rinderer, 1999). Similarly, variable genotypes of the microsporidian *Nosema bombi* may at least partly explain differences in bumble bee (*Bombus* spp.) susceptibility (Tay et al., 2005). Both I (Chapter 3) and Huang et al. (2008) detected genetic variants of *N. ceranae*, although it is not known if they correspond to differences in virulence or parasite fitness, or how they may interact with variants or subspecies of honey bees that are distributed throughout the world. Very little is known about variation in honey bee susceptibility to *Nosema* disease. Although differences in susceptibility to *N. apis* have been observed (Malone et al., 1995; Malone and Giacon, 1996), no genetic differences were found (Malone and Stefanovic, 1999). Additionally, Dussaubat *et al.* (2012a) recently suggested that host genotype, rather than parasite genotype, may play a more important role in determining virulence. It is possible that a lack of selection pressure due to unnatural management of colonies and reduced genetic diversity, particularly in North America where honey bees are not native, play some role in lowering natural resistance to disease (vanEngelsdorp and Meixner, 2010).

Second, climate may account for regional differences in *Nosema* prevalence, species composition, and pathology. Although honey bees maintain brood nests at ~34°C (Jones et al., 2004), peripheries of hives may vary considerably in temperature as ambient conditions fluctuate. Temperature significantly affected susceptibility of adult honey bees to tracheal mites (*Acarapis woodi*) (McMullan et al., 2010), and temperatures of 25 and 37°C restricted development of *N. apis*, but not *N. ceranae*, in workers (Martín-Hernández et al., 2009; Higes et al., 2010). Because contaminated wax is a significant source of *N. apis* infection (Bailey, 1953), conditions at which spores are maintained may also play an important role in *N. ceranae* transmission. Exposing spores to -18°C for seven days significantly reduced viability of *N. ceranae* compared to *N. apis* (Fries, 2010), and germination potential of *N. ceranae* spores was significantly lower than *N. apis* when exposed to 4°C for four days (Gisder et al., 2010). These data may partially explain why *N. ceranae* appears to have rapidly displaced *N. apis* in countries with warmer climates, such as the United States, Spain, and Italy, but not universally in more temperate locations such as Canada (Chapters 3, 4, and 6; Williams *et al.* 2010; Shutler et al., submitted), Sweden, and the United Kingdom (Klee et al., 2007; Gisder et al., 2010; Higes et al., 2010).

Third, interactions among environmental stressors such as parasites and pesticides that are regionally associated with honey bees (Ellis and Munn, 2005) could be influencing host susceptibility and parasite virulence. For example, *N. ceranae* synergistically interacted with a neonicotinoid pesticide (Alaux et al., 2010b), and is linked with an invertebrate iridescent virus in colonies exhibiting symptoms of Colony Collapse Disorder (Bromenshenk et al., 2010). Despite observing no competitive exclusion during my laboratory study (Chapter 7), genetic and environmental factors (e.g. temperature as discussed previously) experienced under natural conditions likely influence fecundity of both *N. apis* and *N. ceranae*. This possibly created opportunities for asymmetric competition, and subsequent spatial and temporal heterogeneous displacement of *N. apis*.

Fourth, differences in efficacy of Fumagilin-B® against *N. apis* and *N. ceranae* may promote one species over the other. Fumagilin-B® is commonly used in both the United States and Spain, where *N. ceranae* has a high prevalence; however, both species are common in Canada, where Fumagilin-B® is also routinely applied (CAPA, 2010) and effective against *N. ceranae* (Chapters 4 and 5). However, *N. ceranae* has a high prevalence in countries that outlaw the use of antibiotics, such as Switzerland, Greece, and Italy (Klee et al., 2007; Charrière and Neumann, 2010). It is therefore unlikely that management with Fumagilin-B® favours one *Nosema* species over the other; however, experiments are needed to confirm this and other potential management options.

Fifth, it is possible that limited investigations of *N. ceranae* do not adequately evaluate effects of parasitism on honey bees. As discussed earlier and briefly in Chapter 7, studies by various research groups that employ a diversity of designs and methods make comparisons difficult, and may inappropriately suggest variation in parasite development or virulence.

Because differences in parasite prevalence, virulence, and inter-specific interactions typically vary with place and time, depending on drivers involved (Polley and Thompson, 2009), it is critical to identify specific factors that may influence *N. ceranae* in honey bees.

8.3 FINAL REMARKS

Apiculture faces a monumental task in maintaining strong, healthy honey bee colonies; adoption of an IPM approach for all parasites remains the best strategy for mitigating colony losses. The first step is to accurately assess factors responsible for colony mortality (Chapter 2). Ultimately, how or if a parasite is managed by beekeepers depends on colony-level effects during typical apiculture scenarios directed towards pollination and honey production. Strategies for preventing and controlling *N. ceranae* at appropriate times using a variety of methods that target all life stages are required when infection contributes colony mortality or reduced productivity. Conversely, if *N. ceranae*

contributes little to colony vitality, attention should focus on other more economically important parasites.

Many questions still remain about the microsporidian parasites in honey bees before a proper IPM approach can or should be adopted. Crucially, a lack of standardized tests to tease apart factors influencing parasite development and virulence have hampered recent efforts due to the diversity of variables selected for in these studies and the conflicting results produced. Furthermore, studies to investigate the effects of *N. ceranae* on honey bees in both the laboratory and the field under a variety of genetic and environmental conditions, is needed. This is particularly important because of potential interactions of *N. ceranae* with other competiting parasites in honey bees around the globe.

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Williams, G.R., Tarpy, D.R., vanEngelsdorp, D., Chauzat, M.-P., Cox-Foster, D.L., Delaplane, K.S., Neumann, P., Pettis, J.S., Rogers, R.E.L., Shutler, D., 2010. Colony Collapse Disorder in context. BioEssays 32, 845-846.

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APPENDIX A PUBLICATIONS

The work presented in Chapter 2 also appears in:

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