

**Infection Cycle, Transmission Mechanisms, and Management of *Nosema ceranae* in
Apis mellifera Colonies**

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ABSTRACT

Nosema ceranae is a recently described, widespread microsporidian parasite of *Apis mellifera* that has raised concerns as to whether it is contributing to increased colony losses. To better understand this parasite, investigations were made into the seasonality of infections, alternative transmission mechanisms, and potential control approaches. All studies used real-time PCR with specific primers and probes for *N. ceranae*, as well as traditional spore analysis. Monthly colony monitoring in Virginia showed that *N. ceranae* was present yearlong with the highest levels observed in April-June and lower levels through the fall and winter. There was no difference in infection levels among bees sampled from different areas of the hive regardless of the time of year. Additionally, *N. ceranae* infects all castes of the colony. Drones of different ages, including pupae, in-hive, and flying drones, were found to be infected at low levels with infections most prevalent during peak annual levels in April-June. Approximately 5% of flying drones had moderate to high levels of infection indicating that flying drones, which would be the most likely age group to drift, could assist in the horizontal transmission of *N. ceranae* both within and between apiaries. Immature and mated queens were also found to be infected at low levels. Infection in the ovaries and spermathecae suggests the possibility for vertical transmission. Finally, control of *N. ceranae* is thought to improve the health of bees and to reduce colony losses. Fall fumagillin treatments and winter stimulative pollen feeding were compared. Neither treatment significantly lowered *N. ceranae* levels in colonies sampled 3-6 months later, nor did they significantly improve colony survival. Due to the high cost of

treatment and the time required, we do not recommend either treatment for *N. ceranae* infections during the fall. Colony winter losses due solely to *N. ceranae* seem unlikely because levels of *N. ceranae* were low. Impacts from *N. ceranae* infections were also minimal during the summer as productive colonies had some of the highest levels of infection. Although *N. ceranae* is prevalent throughout hives, it does not seem to be a major cause of colony losses.

DEDICATION

This is dedicated to all of those who believed in me when I did not believe in myself. Thank you.

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Several colleagues aided in the writing and research behind several of my chapters presented as part of this dissertation. A brief description of their contributions is included here.

Chapter 2: Seasonal Activity of *Nosema ceranae* in Honey Bee Colonies

Chapter 2 was submitted to the Journal of Invertebrate Pathology on 19 August 2011.

Matthew R. Williams, PhD (Department of Statistics, Virginia Tech) is currently a statistician at the Research and Development Division in the National Agricultural Statistics Service at the United States Department of Agriculture. Dr. Williams was a co-author on this paper, helped with the experimental design, and performed the statistical analysis.

Richard D. Fell, PhD (Department of Entomology, Cornell University) is currently an emeritus professor in the department of entomology at Virginia Tech. Dr. Fell was a co-author on this paper, principal investigator for the grants supporting the research, helped with the experimental design, sample collection, and provided editorial comments.

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TABLE OF CONTENTS

LIST OF FIGURES	x
LIST OF TABLES.....	xi
CHAPTER 1 Literature review.....	1
Honey bees and their importance.....	1
Honey bees.....	1
Honey bee development.....	2
Population decline.....	4
Possible causes for CCD.....	5
Microsporidia.....	5
<i>Nosema apis</i>	6
Life cycle	8
<i>Nosema ceranae</i>	9
<i>N. ceranae</i> life cycle.....	11
Detection of <i>N. ceranae</i> in other species	14
Transmission.....	15
Effects of <i>N. ceranae</i> infection.....	16
Treatment.....	17
Concerns	21
References.....	24
CHAPTER 2 Comparison of within hive sampling and seasonal activity of <i>Nosema ceranae</i> in honey bee colonies.....	37
Abstract.....	39
1. Introduction.....	40
2. Methods.....	43
2.1 Sample collection	43
2.2 Forager sampling.....	44
2.3 Genomic DNA extraction.....	44
2.4 Quantitative real-time PCR (qPCR).....	44
2.5 Spore counts.....	45
2.6 Statistical analysis	45
3. Results.....	46
3.1 Annual analysis for <i>N. ceranae</i>	46
3.2 Warmer months.....	47
3.3 Colder months.....	47
3.4 <i>Nosema</i> -negative samples.....	48
3.5 Infection levels and colony strength	48
3.6 Spore counts.....	49
4. Discussion.....	49
Acknowledgments.....	53
References.....	54
CHAPTER 3 <i>Nosema ceranae</i> in drone honey bees (<i>Apis mellifera</i>)	63
Abstract.....	65
1. Introduction.....	65

2. Materials and methods	67
2.1 Drone Sampling	67
2.2 Genomic DNA (gDNA) extraction and quantitative real-time PCR (qPCR).....	67
2.3 Spore counting	68
3. Results and discussion	68
Acknowledgments.....	70
References.....	71
CHAPTER 4 Low natural rates of <i>Nosema ceranae</i> in <i>Apis mellifera</i> queens	76
Abstract.....	78
1. INTRODUCTION	78
2. METHODS	81
2.1 Queen rearing.....	81
2.2 Genomic DNA (gDNA) extraction.....	82
2.3 Quantitative real-time PCR (qPCR).....	83
2.4 Brood nest bees	84
2.5 Flying drones	84
3. RESULTS	85
3.1 Brood nest bees from queen rearing hives.....	85
3.2 Immature queens and royal jelly samples.....	85
3.3 Newly emerged queens	85
3.4 Mated queens	86
4. DISCUSSION.....	86
ACKNOWLEDGEMENTS.....	89
REFERENCES	90
CHAPTER 5 Treatment for <i>Nosema ceranae</i> - fall fumagillin and winter pollen feeding.....	96
Abstract.....	98
1. INTRODUCTION	98
2. METHODS	102
2.1 Fumagillin treatment.....	102
2.2 Pollen substitute feeding.....	103
2.3 Genomic DNA (gDNA) extraction.....	103
2.4 Real-time PCR (qPCR).....	103
2.5 Spore analysis	104
2.6 Statistical analysis.....	104
3. RESULTS	104
3.1 Fall fumagillin treatment.....	104
3.2 Winter pollen feeding	105
3.3 Colony survival and <i>N. ceranae</i> levels	105
4. DISCUSSION.....	106
REFERENCES	110
CHAPTER 6 Summary.....	116
Chapter 2.....	117
Chapter 3.....	117
Chapter 4.....	118
Chapter 5.....	118
Future research.....	119

References.....	121
APPENDIX A <i>In situ</i> hybridization for the detection of <i>Nosema ceranae</i> in honey bee tissues	122
Introduction.....	122
Materials and methods	125
<i>Fixation and embedding</i>	125
<i>In vitro transcription of DIG-labeled probe</i>	126
<i>In situ hybridization</i>	126
<i>Microscopy</i>	127
Results.....	127
<i>In situ hybridization</i>	127
<i>Real-time PCR and spore counting</i>	127
References.....	130

LIST OF FIGURES

Chapter 2

Figure 1. Box plot of the seasonal cycle for *N. ceranae*..... 59

Figure 2. Box plot for workers sampled during different seasons..... 60

Chapter 3

Figure 1. Box plot for the infections in adult and immature drones. 74

Chapter 5

Figure 1. Presence or absence of spores for each treatment. 114

LIST OF TABLES

Chapter 1

Table 1. Molecular detection of <i>Nosema</i> spp. in countries across the world.....	10
---	----

Chapter 2

Table 1. The percentage of <i>Nosema</i> -negative samples in the different bees groups sampled.....	61
Table 2. The percentage of <i>Nosema</i> -negative samples by month.....	61
Table 3. Mean spore counts (\pm SE) ¹ from bees sampled in different areas of the hive from September 2009 to September 2010 in colonies positive for <i>Nosema</i> spp. spores.....	62

Chapter 3

Table 1. <i>Nosema ceranae</i> levels observed for each set of drones sampled. A total of 9 drone abdomens (pooled samples of 3) and 45 worker abdomens (pooled samples of 5) were examined from each colony sampled.....	75
---	----

Chapter 4

Table 1. <i>N. ceranae</i> mean DNA copy number (\pm SEM) in abdomens and heads of bees taken from the brood nest in queen rearing hives.....	94
Table 2. <i>N. ceranae</i> mean DNA copy number (\pm SEM) from individual in queen larvae and corresponding royal jelly samples.	94
Table 3. <i>N. ceranae</i> mean DNA copy number (\pm SEM) for newly emerged <i>Apis mellifera</i> queen tissues.....	94
Table 4. <i>N. ceranae</i> mean DNA copy number (\pm SEM) in mated queen organs. Organs from each queen were analyzed individually.....	95
Table 5. <i>N. ceranae</i> mean DNA copy number (\pm SEM) for tissues dissected from flying drones and the abdomens of workers taken from inside the hive.....	95

Chapter 5

Table 1. Mean <i>N. ceranae</i> DNA copy number (\pm SEM) and mean spore counts per bee (\pm SEM) for fall fumagillin treatment.....	115
Table 2. Mean <i>N. ceranae</i> DNA copy number (\pm SEM) and mean spore counts per bee (\pm SEM) for winter stimulative pollen feeding.....	115

CHAPTER 1

Literature review

Honey bees and their importance

Honey bees are essential for the pollination of many agriculturally important crops such as vegetables, fruits, and nuts and are a major component of the pollinator complex that provides more than 223 billion dollars in annual benefits to agriculture worldwide. These benefits account for about 9.5% of the total value of human food production (Gallai et al., 2009). Approximately 70% of crops used primarily for human consumption are dependent on pollinators (Klein et al., 2007) and an estimated 75% of flowering plants are pollinated by insects (National Academies of Science, 2006). In the United States, honey bees pollinate 95 fruit and vegetable species with an estimated value of pollinator services at 14.6 billion annually (Stokstad, 2007). Large monocultures of crops (i.e. apples, almonds, blueberries, and cranberries) require managed honey bees to be shipped from across the United States to facilitate in pollination (Klein et al., 2007). While there are native, unmanaged pollinators, their numbers are not sufficient to pollinate crops worldwide (Gallai et al., 2009). Both the numbers and diversity of native pollinators and honey bees have been decreasing due to a number of factors such as parasitic mites, pathogens, pesticides, the small hive beetle, and habitat loss (Klein et al., 2007; National Academies of Science, 2006).

Honey bees

The order Hymenoptera contains bees, wasps, and ants. There are approximately 20,000 species of bees in 7 families and over 150 genera (Caron, 1999). Bees evolved approximately 100

million years ago from wasps and paralleled the speciation and diversification of flowering plants (Caron, 1999). Honey bees are classified in the family Apidae and in the genus *Apis*. Within *Apis*, there are nine species of honey bees. Two species, *Apis cerana* and *Apis mellifera*, have been very important to humans because they store large amounts of honey and can be easily managed in man-made hives (Bailey, 1981). *A. cerana*, the Asian or eastern honey bee, is smaller than *A. mellifera* and is found throughout India, Sri Lanka, southeast Asia, Indonesia, the Philippines, China, Taiwan, and Japan (Caron, 1999). *A. mellifera*, the European or western honey bee, has been transported and introduced all over the world. Members of the genus *Apis* are eusocial and contain a single queen, sterile female workers, and male drones. Only queens have a fully developed reproductive system and are capable of laying eggs (Gould and Gould, 1988). Workers secrete wax, maintain the comb, tend to brood, and forage for pollen and nectar (Bailey, 1981). Drones serve one purpose which is to mate with virgin queens.

Honey bee development

Honey bee development has been reviewed by Bailey (1981), Winston (1987), and Winston (1992). A queen lays either a fertilized egg which will develop into a female or an unfertilized egg which will develop into a male. After an egg is laid, it takes three to hatch as a larva. Larvae have five developmental instars and are fed and reared by young workers called nurse bees. Worker larvae are fed worker jelly for part of their development while queen larvae are fed royal jelly throughout larval development. Larvae complete their development in 5 to 6.5 days with queens having the shortest development time followed by workers then drones. After the fifth instar, larvae develop into pre-pupae and the cell is capped by workers. Pupae take 7 days (for queens), 12 days (for workers), or 14.5 days (for drones) before emerging as adults. Total

development time is 16 days for queens, 21 days for workers, and 24 days for drones. All young immature stages of bees described above are referred to as brood.

Population numbers in a hive are seasonal. During the winter in temperate regions, bees cluster together over comb filled with honey and pollen with the queen in the center region of the cluster (Gould and Gould, 1988). Brood rearing is most prevalent in the late spring and summer when the weather is warm and food sources are more readily available. Drones are only reared in the warmer months when food is plentiful and are removed from the hive by workers in the fall in (Fukuda and Ohtani, 1977; Witherell, 1972).

Honey bees exhibit temporal polyethism in which the behavior of a bee changes with age [as reviewed by (Seeley, 1985; Winston, 1987)] and is controlled by juvenile hormone and vitellogenin (Guidugli et al., 2005; Nelson et al., 2007; Robinson et al., 1991; Whitfield et al., 2006). Bee behavior and associated jobs are concentrated in the center of the nest and as the bees age, their movement and jobs take them to the periphery of the nest and eventually outside the nest as foragers. Young workers clean the cell from which they emerged, feed and rear brood, cap brood, attend the queen, and groom other bees. Workers from age 11–20 days help ventilate the hive, start comb building, receive nectar, clean the hive, pack pollen, and guard the entrance of the hive. Finally, workers 20 days and older become foragers and leave the hive to collect water, propolis, pollen, and nectar. As bees mature, changes occur in their exocrine glands. As workers progress from nurse bees, to building comb, to guard bees, their hypopharyngeal glands, wax glands, and alarm pheromone glands become more or less active based on their age and duties (Seeley, 1985).

Population decline

Since the 1980s there has been a decrease in the number of bee colonies due to the introduction of the Varroa mite (vanEngelsdorp et al., 2007). Colony numbers have decreased from 4.5 million colonies in 1980 to 2.3 million in 2008 (NASS, 2009). Prior to the introduction of parasitic mites, winter losses were estimated to be between 5–10% while after the introduction of Varroa mites, losses ranged from 15–25% (vanEngelsdorp et al., 2008). In recent years there has been an accelerated decline in honey bee populations worldwide. The increased losses were first reported in 2006 from beekeepers in 22 states and across Europe (Oldroyd, 2007). Large scale colony losses are not a new phenomenon, and have occurred in the past. Heavy losses were reported in Ireland in 950, 992, and 1443, and Italy in 1690 (Fleming, 1871). In 1906, heavy colony losses were reported on the Isle of Wight in the United Kingdom (Silver, 1907). In the United States, heavy losses were reported in 1897, the 1960s and 1970s (Stokstad, 2007), 1903 in Utah (Critchlow, 1904), and 1995 in Pennsylvania (Finley et al., 1996). The alarming nature of the losses in the fall of 2006 to the spring of 2007 was more extreme than previously seen with many beekeepers reporting 80-100% colony loss (Oldroyd, 2007) with a national average of 45% in the United States (Cox-Foster et al., 2007). In Virginia, colony losses have averaged 30% over the past ten years.

Many of these losses have since been attributed to a new condition called colony collapse disorder (CCD). In CCD, there is a loss of adult bees with few if any dead bees in or around the hive. The queen is attended by only a few newly emerged workers, yet frames may be full of brood, honey and pollen (Cox-Foster et al., 2007). While CCD was first reported in 2006, symptoms matching CCD were noticed by some beekeepers in 2004 (Cox-Foster et al., 2007).

In Europe, honey bee losses have also been documented but symptoms are slightly different with a more gradual population decline. The condition is called honey bee colony depopulation syndrome (Higes et al., 2008a) and is characterized by a disappearance of adult bees, lack of attention to the brood, a decrease in colony strength and honey production, heavy winter mortality, and no other signs of pathogens (Higes et al., 2009b).

Possible causes for CCD

Many different factors have been suggested as potential causes of CCD. Researchers have hypothesized that pathogens, parasites, insecticides and other pesticides, genetically modified plants, stress from transport across the country, and nutrition may be involved. Previously unidentified pathogens may also play a role (Cox-Foster et al., 2007), a hypothesis supported by evidence that CCD can be transmitted by re-using equipment that has not been sterilized by acetic acid or irradiation (Pettis et al., 2007). Also, a compromised immune system due to multiple pathogens detected in CCD bees may be in part responsible. In a recent study, a whole-genome microarray was used to compare genes expressed in the guts of bees from CCD hives and from non-CCD healthy hives. Surprisingly the expression of detoxification and immune genes were similar in CCD and healthy bees; however, there was a difference in the expression of genes in CCD bees from various parts of the United States (Johnson et al., 2009). CCD, therefore, is most likely caused by multiple factors acting together.

Microsporidia

Microsporidia are eukaryotic obligate intracellular parasites with over 1200 species in 143 genera (Sagastume et al., 2011) and are particularly known for the infections they cause in honey

bees (*Nosema apis*), farmed fish (*Glugea atherinae*), silkworms (*N. bombycis*) (Germot et al., 1997), and as opportunistic parasites in AIDS patients (Orenstein, 1991). While not all bad, some have been used as biological control agents against insects [reviewed by (Coulson et al., 2000)] such as grasshoppers (*N. locustae*); the European corn borer (*N. pyrausta*); Anopheline mosquitoes (*N. algerae*); the cotton leaf borer (*N. scripta*); and the spruce worm borer (*N. fumigeranae*).

Unlike other eukaryotes, microsporidia lack mitochondria and peroxisomes (Hirt et al., 1999). Microsporidian nuclear division is primitive, their ribosomes and ribosomal RNA are more similar to prokaryotes (Curgy et al., 1980; Ishihara and Hayashi, 1968), and there is no 5.8S rRNA in the large ribosomal subunit (Vossbrinck and Woese, 1986). Microsporidia were classified as amitochondriate protists in the kingdom Archezoa and were thought to be ancient organisms that diverged before eukaryotes acquired mitochondria (Vossbrinck et al., 1987); however, recent research using the β -tubulin (Edlind et al., 1996; Keeling and Doolittle, 1996), Hsp70 (Germot et al., 1997; Hirt et al., 1997), and RNA polymerase I (Hirt et al., 1999) genes in phylogenetic analysis now classify microsporidia as Eukaryota in the rank Opisthokonta in the kingdom Fungi (Adl et al., 2005).

Nosema apis

The genus *Nosema* contains over 150 species that infect 12 orders of insects. *N. apis* is a microsporidian that develops only in the epithelial cells of the midgut in adult bees (Bailey, 1981; de Graaf and Jacobs, 1991). Bees that are infected do not exhibit any outward signs of disease, although the ventriculus may be white and swollen (Fries, 1993). *N. apis* inhibits the

development of the hypopharyngeal (Wang and Moeller, 1971), eggs fail to hatch (Hassanein, 1953), bees age earlier (Wang and Moeller, 1971), forage earlier (Fries, 1993), and workers have a decreased lifespan (Wang and Moeller, 1970) all of which can result in a decrease in colony population. Digestive disorders can occur from the degeneration of epithelial cells, resulting in a decrease in protein metabolism and decreased proteolytic activity in the midgut (Liu, 1984; Malone and Gatehouse, 1998). These impacts lead to a decrease in hemolymph which leads to fewer amino acids, decreased hypopharyngeal gland size and function, and decreased levels of protein in the fat bodies (Liu, 1990; Wang and Moeller, 1969, 1971).

N. apis infections can be triggered by stress (Doull, 1961), disturbing a colony in either the winter or summer (Oertel, 1967), transporting hives early in the season, and overcrowding colonies in one area (Bailey, 1981). Young, infected bees will start to perform duties typically performed by older bees (Wang and Moeller, 1970). It has also been demonstrated that infected bees fed pollen have an increased longevity, but an increase in protein also benefits *N. apis* development (Rinderer and Elliott, 1977). Queens and drones, in addition to workers, can be infected, although infections are not as common (Bailey, 1972a; Czekoska, 2000; Jay, 1966; Webster et al., 2004). Infected queens may stop laying eggs and die a few weeks after their initial infection (L'Arrivee, 1965). While *N. apis* rarely leads to colony death, infected colonies produce less honey because the life of the foragers has been reduced (Wang and Moeller, 1970) and collect less pollen (Anderson and Giacon, 1992).

Life cycle

N. apis infections are spread primarily through an oral-fecal route when young workers clean contaminated comb (Bailey, 1953a), ingest contaminated water (Bailey, 1981), through trophallaxis (Webster, 1993), and possibly in contaminated honey (Fries, 1993). When spores are ingested, they pass through the proventriculus into the midgut where conditions induce spore germination, causing the polar filament to extrude and inject its sporoplasm into an epithelial cell (Fries, 1993). The sporoplasm contains two nuclei which become enclosed in a plasma membrane after ejection from the spore (Weidner et al., 1984). Infections typically spread from the posterior to the anterior part of the gut (Bailey, 1955a; Fries, 1988). While in the cytoplasm of an epithelial cell, *N. apis* matures into meronts (Gray et al., 1969) which undergo nuclear division into merozoites (Fries, 1989). Merozoites develop into mature sporonts that then divide into two sporoblasts. *Nosema* spp. are disporous with two different types of spores formed. Mature spores can germinate in the same cell, spread between adjacent cells, or lyse the host cell and re-initiate the cycle (Bailey, 1981; Liu, 1990). The life cycle can be completed in 48–60 hours after the initial infection (Fries, 1993). Vegetative stages have been transmitted intracellularly *in vitro* in insect cell lines (Ishihara, 1969; Kawarabata and Ishihara, 1984; Kurtti et al., 1983), and *in vivo* with spores germinating inside their host cell and injecting their sporoplasm into neighboring cells (Fries, 1989; Fries et al., 1992; Iwano and Ishihara, 1991; Iwano et al., 1994). While one spore could trigger an infection, 20–90 spores are considered the lower threshold for triggering an infection (Bailey, 1972b; Fries, 1988). Spore counts can range from 30–50 million spores per bee (Bailey, 1981) and can there be more than 200 million in bees that have not defecated (Fries, 1993).

Nosema ceranae

Up until 15 years ago *N. apis* was the only microsporidian species known to infect honey bees, but in 1994, a new species, *N. ceranae*, was identified in *Apis cerana*, the Asian honey bee [reviewed by (Fries et al., 1996)]. This new microsporidia was classified in the genus *Nosema* based on the 16S small subunit ribosomal RNA. Mature spores are ovocylindrical 4.7 x 2.7 μm (fixed and stained 3.6 x 1.7 μm). The polar filament is isofilar with a diameter of 96-102 nm with 20-23 coils and a 55-60° angle. Merogonial stages and sporonts are diplokaryotic and disporoblastic. More than one merogonical cycle occurs with diplokaryotic meronts that divide by binary fission. Dividing sporonts are more commonly observed than meronts. Spores were only found in the intestinal tract in the epithelial cells of the ventriculus suggesting tissue specific infection. No intracellular germination of spores was observed as the infected cells lyse in the gut lumen. There are no outward clinical symptoms. The *N. ceranae* genome is estimated to be 7.4-15 MB long and is AT rich with no codon usage bias. Many repetitive elements and novel genes have been identified which may be virulence factors or candidate secretory proteins that interact with the host tissue (Cornman et al., 2009).

In 2006, *N. ceranae* was found to have jumped hosts to *A. mellifera* and was found in Spain and in Taiwan (Higes et al., 2006; Huang et al., 2007). Subsequent reports provided evidence based on molecular analysis that *N. ceranae* infects *A. mellifera* globally (Table 1), and indicate that *N. ceranae* is more widespread than originally thought and more prevalent than *N. apis*. *N. ceranae* was detected in 1998 in Europe, the early 1990s in the US, and in 1990 in Uruguay. Due to the global presence of *N. ceranae*, displacement of *N. apis* has been hypothesized (Chauzat et al.,

2007; Chen et al., 2008; Klee et al., 2007; Paxton et al., 2007); however, some believe it is still too early to tell if *N. apis* has been displaced (Gisder et al., 2010; Paxton, 2010).

Table 1. Molecular detection of *Nosema* spp. in countries across the world.

Country	Species	Reference
Europe	<i>N. ceranae</i>	(Higes et al., 2006)
Taiwan	<i>N. ceranae</i>	(Huang et al., 2007)
Uruguay	<i>N. ceranae</i>	(Invernizzi et al., 2009)
Spain	<i>N. ceranae/N. apis</i>	(Martin-Hernandez et al., 2007)
Switzerland	<i>N. ceranae/N. apis</i>	(Martin-Hernandez et al., 2007)
France	<i>N. ceranae</i>	(Martin-Hernandez et al., 2007)
Germany	<i>N. ceranae</i>	(Martin-Hernandez et al., 2007)
Croatia	<i>N. ceranae</i>	(Gajger et al., 2010)
Hungary	<i>N. ceranae</i>	(Tapasztai et al., 2009)
Turkey	<i>N. ceranae/N. apis</i>	(Giray et al., 2010; Muz et al., 2010)
Japan	<i>N. ceranae</i>	(Yoshiyama and Kimura, 2011)
Mexico	<i>N. ceranae/N. apis</i>	(Guzman-Novoa et al., 2011; Puc et al., 2011)
Algeria	<i>N. ceranae</i>	(Higes et al., 2009a)
France	<i>N. ceranae</i>	(Chauzat et al., 2007)
Finland	<i>N. ceranae</i>	Paxton et al., 2007)
Italy	<i>N. apis</i>	(Klee et al., 2007)
Hungary	<i>N. apis</i>	(Klee et al., 2007)
Denmark	<i>N. apis</i>	(Klee et al., 2007)
Sweden	<i>N. apis</i>	(Klee et al., 2007)
Northern Ireland	<i>N. apis</i>	(Klee et al., 2007)
Brazil	<i>N. ceranae</i>	(Klee et al., 2007)
USA	<i>N. ceranae</i>	(Klee et al., 2007)
Vietnam	<i>N. ceranae</i>	(Klee et al., 2007)
Finland	<i>N. ceranae</i>	(Klee et al., 2007)
Greece	<i>N. ceranae</i>	(Klee et al., 2007)
Serbia	<i>N. ceranae</i>	(Klee et al., 2007)
Spain	<i>N. ceranae</i>	(Klee et al., 2007)
Denmark	<i>N. ceranae/N. apis</i>	(Klee et al., 2007)
Germany	<i>N. ceranae/N. apis</i>	(Klee et al., 2007)
Sweden	<i>N. ceranae/N. apis</i>	(Klee et al., 2007)
Ireland	<i>N. apis</i>	(Klee et al., 2007)
New Zealand	<i>N. apis</i>	(Klee et al., 2007)
USA	<i>N. ceranae/N. apis</i>	(Chen et al., 2008; Traver and Fell, 2011a)
Costa Rica	<i>N. ceranae/N. apis</i>	(Calderón et al., 2008)
Australia	<i>N. ceranae/N. apis</i>	(Giersch et al., 2009)
Canada	<i>N. ceranae</i>	(Williams et al., 2008)
Thailand	<i>N. ceranae</i>	(Chaimanee et al., 2010; Suwannapong et al., 2011)

Without molecular detection, previous studies assumed that the microsporidia causing nosemosis was *N. apis* because of the low level infections in the summer and a small peak in the fall with a slow increase during the winter and then a rapid increase with brood rearing (Bailey, 1955b; Martin-Hernandez et al., 2007). PCR is very sensitive and allows the detection of *Nosema* spp. DNA in sampled bees. Since the description of *N. ceranae*, a number of different PCR-based assays have been developed to detect and discriminate between *N. ceranae* and *N. apis* [as reviewed by (Traver and Fell, 2011b)]. Conventional PCR followed by sequencing (Fries et al., 1996), PCR-RFLP (Klee et al., 2007; Tapaszti et al., 2009), species specific PCR (Chen et al., 2008; Hamiduzzaman et al., 2010; Higes et al., 2006), multiplex PCR (Martin-Hernandez et al., 2007), and real-time PCR (Bourgeois et al., 2010; Burgher-MacLellan et al., 2010; Chen et al., 2009a; Traver and Fell, 2011a) have all been used.

***N. ceranae* life cycle**

N. ceranae reproduction occurs in the midgut epithelial cells and causes lesions that result in permanent damage to the epithelial layer, which affects digestion. Spores have been observed in the epithelial cells of the ventriculus, but not in the epithelial cells of the malpighian tubules, small intestine, or rectum suggesting a tissue-specific infection (García-Palencia et al., 2010); however, PCR signals have been detected in other tissues. *N. ceranae* was detected in alimentary canals, malpighian tubules, hypopharyngeal glands, and salivary glands but not in the muscle tissues which could explain why crawlers are not observed (Chen et al., 2009b; Gisder et al., 2010). Detection of *N. ceranae* in other tissues is similar to *N. bombi* (Fries et al., 2001). Since the hypopharyngeal and salivary glands can be infected, royal jelly may be a potential transmission route (Chen et al., 2009b).

In cage studies of *N. ceranae*-infected honey bees, there were few infected midgut epithelial cells observed (4.4%) at 3 days post-infection (dpi) with increasing number of mature cells observed at 6dpi (66.4%) and at 7dpi (81.5%) (Higes et al., 2007). During the infection, the ventricular cells degenerate, leading to vacuoles in the cytoplasm, disruption of the cellular membranes, condensed and reduced nuclei, meronts in the basal infolds of the epithelial cells, extensive cell lysis, and a broken, fragmented, or absent peritrophic membrane (García-Palencia et al., 2010). Emptied spores were also observed, suggesting that autoinfective spores were able to transmit an infection to adjacent epithelial cells (Higes et al., 2007). The specific tissue lesions observed suggest that *N. ceranae* is highly adapted to the host metabolism in the epithelial cells, since the parasite requires high levels of oxygen for lipid and protein synthesis (Liu, 1984). Cell regeneration either ceases or the infection spreads faster than cell regeneration can occur, ultimately leading to the death of the infected bee. *N. ceranae* is thought to be more pathogenic than *N. apis* (Higes et al., 2007; Paxton et al., 2007) but not all studies agree (Forsgren and Fries, 2010).

During a *N. ceranae* infection, there is a higher percentage of immature stages present than mature stages which may explain why the pathology (i.e. increased pathogenicity and mortality) is so different from *N. apis* when similar spore counts are observed (Martin-Hernandez et al., 2009). Also, *N. ceranae* is able to infect more cells than *N. apis* in the same time frame; this is most likely due to autoinfective spores, which allow for quick reproduction and infection of tissues (Higes et al., 2007). If the infection spreads rapidly, host cell regeneration may not be possible and the result is cell death (García-Palencia et al., 2010). Since vegetative stages cannot

be counted on a hemocytometer, the actual number of cells infected cannot be determined by spore counts.

N. ceranae is eurythermal, tolerating a wide range of temperatures while *N. apis* is stenothermal and survives in a narrow range of temperatures (Higes et al., 2010). At 33°C, *N. apis* and *N. ceranae* developed at the same rate with similar total spore counts per day and both completed their life cycle within 2 days; however, there was a higher proportion of immature stages for *N. ceranae* (Martin-Hernandez et al., 2009). The biotic potential is higher for *N. ceranae* at 25°C and 37°C, meaning that *N. ceranae* can adapt and complete its endogenous life cycle at a broader range of temperatures than *N. apis*. This developmental difference represents an epidemiological difference between the species (Martin-Hernandez et al., 2009). During a coinfection, *N. ceranae* has been shown to have a 100-fold higher copy number than *N. apis*, demonstrating that it is not only more prevalent and more competitive, but also has better mechanisms to evade host immunity to allow for faster growth and reproductive capacity than *N. apis* (Chen et al., 2009a). Since *N. ceranae* can complete the endogenous life cycle at a higher biotic index and successfully outcompete *N. apis*, changes in climate may affect the distribution, seasonality, and severity of the disease.

N. ceranae spores are resistant to high temperatures (Fenoy et al., 2009a) which agrees with previous studies that *N. ceranae* adapts better to different climates than *N. apis* and explains why there is a higher prevalence in all seasons (Martin-Hernandez et al., 2007). It also helps explain why the wax in old combs from infected colonies could be a reservoir for spores. Freezing decreases spore viability which is good for management, but is an issue that needs to be resolved

to optimize preservation and storage of *N. ceranae* spores for scientific study (Fenoy et al., 2009b). Colonies that were overwintered either indoors or outdoors in nearctic temperatures did not affect *N. ceranae* intensity (Williams et al., 2010) but there was higher mortality on colonies wintered outdoors. There is also speculation that *N. ceranae* has different strains with different levels of virulence (Williams et al., 2010).

Detection of *N. ceranae* in other species

In Argentina, all castes of three native bumble bee species were found to be infected with *N. ceranae*, *Bombus atratus*, *B. morio*, *B. bellicosus*, no *N. bombi* or *N. apis* were detected (Plischuk et al., 2009). When examining samples from 1987, no *Nosema* spp. were detected suggesting a recent invasion, which could be devastating due to the widespread distribution of *Bombus* spp., and that *N. ceranane* may be highly pathogenic to *Bombus* spp.

In Thailand, *A. mellifera*, *A. cerana*, *A. florea*, and *A. dorsata* were all found to be infected with *N. ceranae* (Chaimanee et al., 2010; Suwannapong et al., 2010; Suwannapong et al., 2011). *N. ceranae*-infected *A. florea* had lower hypopharyngeal gland protein content compared to uninfected bees; these observations are consistent with *A. mellifera*-infected bees (Suwannapong et al., 2010; Suwannapong et al., 2011).

N. apis has not been detected in other species, suggesting *N. ceranae* is more adaptive and less host specific. Wide host range is uncommon for microsporidia. There appears to be no transmission barrier for *N. ceranae* between *A. mellifera* and *A. cerana* (Huang et al., 2008). *A. cerana* foragers also visit the same foraging areas and floral species as *A. florea* which could

allow for cross infection between species. Transfer of *N. ceranae* via infected feces at foraging areas or through robbing have also been suggested as possible mechanisms (Suwannapong et al., 2010).

Transmission

N. ceranae spores were detected in pollen found in pollen baskets and pollen traps (Higes et al., 2008c). This finding suggests that pollen stored in comb could function as a *N. ceranae* reservoir, a factor which has been previously observed for other pathogens (Gilliam et al., 1988; Mehr et al., 1976; Moffett et al., 1978). Spores in corbicular pollen most likely occurred during self contamination from saliva when the pollen was initially collected, and not from contaminated flowers (Higes et al., 2008c); although, some pathogens can be transmitted through contaminated flowers (Durrer and Schmid-Hempel, 1994; Singh et al., 2010).

N. ceranae spores have also been detected in regurgitated pellets from *Merops apiaster* and were still viable and infective 18 days after collection (Higes et al., 2008b). *M. apiaster* is an insectivorous, migratory bird that breeds in central and southern Europe, northern Africa, western Asia, and the United Kingdom. This species could be an additional route for transmission of *N. ceranae* to remote areas as the spores in the pellets could be a reservoir for *N. ceranae* (Valera et al., 2011). Apiaries are common stop over locations during migration as birds that feed nearby could aid in the dissemination of spores on a local scale; however, birds spend most of the time flying and searching for food, which would allow for spores to be dispersed over long distances. Viable *N. ceranae* spores were detected in pellets from the Iberian peninsula, central Europe, and central Asia (Valera et al., 2011).

Effects of *N. ceranae* infection

N. ceranae causes an energetic stress on infected bees, especially foragers, which have higher energy demands (Mayack and Naug, 2009). *N. ceranae*-infected bees were more likely to exhibit risky foraging in adverse conditions leading to a decreased lifespan (Naug and Gibbs, 2009). *N. ceranae*-infected bees have an increased appetite for sucrose, as exhibited by a stronger proboscis extension reflex, regardless of whether bees were satiated or starved (Mayack and Naug, 2009). With increased hunger, there should be increased trophallactic rates as bees are more likely to beg for food; however, bees are less likely to share due to their increased hunger, a factor which could affect the transmission of *N. ceranae* as the social interactions would be altered (Naug and Gibbs, 2009). When a host has a parasite induced energetic stress, two things occur (1) the parasite uses energy from the host and (2) the host expends energy for immune defense which makes it more susceptible to other pathogens (Mayack and Naug, 2009). The energetic stress may be greater in *N. ceranae*-infected bees versus *N. apis*-infected bees because of a new host-relationship.

N. ceranae-infected foragers take longer to return to their parent colony and many become disoriented or even fail to return from the field, either of which depletes the colony population of foragers (Kralj and Fuchs, 2010). In *N. apis*-infected bees, increased foraging was observed in adverse conditions (Woyciechowski and Kozowski, 1998). The infection level in *N. apis*-infected returning bees was 23% lower than the departing bees which would tend to decrease the disease levels in an individual colony (Kralj and Fuchs, 2010). The drifting of bees could facilitate the horizontal spread of pathogens to other colonies once they enter a non-parental hive.

N. ceranae suppresses the honey bee immune response which could increase honey bee susceptibility to other pathogens. In *N. apis* infections, the humoral response is activated with an up-regulation in abaecin, defensin, hymenoptaecin, and phenoloxidase, but either no change or a down-regulation occurs in *N. ceranae* infections (Antúnez et al., 2009). Vitellogenin and glucose dehydrogenase expression decreases in *N. ceranae*-infected bees with no change in *N. apis* infections, while lysozyme expression is not affected by either pathogen (Antúnez et al., 2009). *N. apis* activates the immune system with an increase in the expression of antimicrobial peptides and other immune related genes, while *N. ceranae* suppresses the immune response by decreasing the transcription of these genes, resulting in a partial suppression of both the humoral and cellular defenses. The decrease in vitellogenin is consistent with the observed decreased lifespan in *N. ceranae*-infected bees.

N. ceranae and the neonicotinoid imidacloprid, together were found to weaken honey bees by causing a high energetic stress leading to increased mortality (Alaux et al., 2010). The hemocyte number and phenoloxidase activity were not affected, contrary to previous findings (Antúnez et al., 2009), but the glucose oxidase activity decreased with the combination, suggesting a synergistic interaction (Alaux et al., 2010).

Treatment

Fumagillin is an antibiotic isolated from the fungus *Aspergillus fumigatus* (Hanson and Eble, 1949) and has been used to treat microsporidiosis in insects (Armstrong, 1976; Hartwig and Przelecka, 1971). Prior to 1951, there were no antibiotics for *Nosema* control in honey bees. Treatment with fumagillin proved to be very effective at decreasing the number of bees infected

with *N. apis* and increased the survival of bees despite a continuing, persistent low level infection (Bailey, 1953b; Katznelson and Jamieson, 1952). The infection was able to persist in the anterior part of the ventriculus within the esophageal invaginations that protect the epithelial cells (Bailey, 1953b). Fumagillin-treated bees lived longer than unfed, infected bees (Farrar, 1954) and the suppression of *N. apis* was directly proportional to the dose (Girardeau, 1972). Furthermore, the treatment was seen to target the parasite and not the host.

Fumagillin was thought to target the sporoplasm following germination of the spores inside the host epithelial cells in *N. apis* infections (Bailey, 1953b; Katznelson and Jamieson, 1952; McCowen et al., 1951). Infected host cells have a decreased rate of RNA synthesis and treatment with fumagillin allowed host cell RNA synthesis to return to normal and inhibited DNA replication in the parasite (Hartwig and Przelecka, 1971). Fumagillin treatment causes irregular shaped vegetative life stages and may possibly affect the parasite indirectly by targeting the host cell functions or growth (Didier, 1997). Following treatment for 24 hours, protein and lipid granules were seen in the host cell cytoplasm adjacent to the spores; 72 hours later, the number of protein granules increased in the host cell cytoplasm (Liu, 1973). Treatment restored lipid synthesis in host cells, so the lipids were not incorporated into parasite cells which is consistent with a non-uniform membrane observed in young spores (Liu, 1973). Ultrastructural analysis after fumagillin treatment revealed a decrease in cytoplasmic density, a decrease in the number of ribosomes and a clustering effect, and the nuclear membrane lost all definition and eventually disintegrated (Liu, 1990).

Fumagillin is easy to administer as it can be dissolved in sugar syrup and supplied to bees using a feeding container. Fumagillin administered in a sugar syrup is more effective than dusting or the use of candy patties (Furgala and Gochnauer, 1969) because it takes longer for bees to consume the syrup leading to greater exposure (Furgala and Gochnauer, 1969). However, a spring treatment with fumagillin in icing (powdered) sugar was effective when paired with a syrup administered the previous fall. Bees store the fumagillin syrup in the comb for consumption during the winter, while the spring dry sugar treatment helps keep *Nosema* levels low before an increase in bee population (Szabo and Heikel, 1987). In northern climates, fumagillin syrup can be difficult to feed during the winter, but added with pollen supplements in patty form during the spring was also effective and a possible alternative to fall treatments (Furgala and Gochnauer, 1969; Langridge, 1961).

Many compounds have been tested for treatment of *Nosema* disease but fumagillin is the most effective, non-toxic compound. Paromomycin also showed promise (Moffett et al., 1969); however, follow up studies found that paramomycin, as humatin, was not effective in either the laboratory or the field (Furgala and Boch, 1970). Itraconazole was effective because it targeted spores, causing deformities with some spores lacking a polar filament which would inhibit germination (Liu and Myrick, 1989). The mode of action for itraconazole was thought to be an inhibition of ergosterol synthesis resulting in membrane damage (Liu and Myrick, 1989). Sodium sulfathiazole and oxytetracycline on their own were ineffective at controlling *Nosema*, but in combination with fumagillin, enhanced the efficacy more than the use of twice the amount of fumagillin (Gochnauer and Furgala, 1981). Chimosin is a protein involved in the formation of the peritrophic membrane with the highest levels in 9–12 day old bees (Zherebkin, 1975).

This enzyme is thought to help form a resistant peritrophic membrane so that parasites cannot penetrate and infect the midgut epithelial cells. In older bees, the levels of chimosin were lower which could explain why older bees are more susceptible to high intensity *N. apis* infections (Zherebkin, 1975). Nosemactin, a fumagillin containing compound, showed promise but had little anti-*Nosema* activity (Furgala, 1962; Goetze and Zeuttschel, 1959) and in some cases was toxic to bees (Furgala and Boch, 1970).

Natural compounds have also been looked at as an alternative to chemical control. Thymol, resveratrol, vetiver essential oil, and lysozyme have been examined (Maistrello et al., 2008), but only thymol and resveratrol decreased the levels of *N. ceranae* and increased longevity in treated bees. Both compounds also had low toxicity and residual activity (Costa et al., 2010). Nozevit, an herbal preparation in patty form or sugar syrup, has also been examined and has been shown to decrease the number of spores present after treatment, but bees were not *Nosema*-free (Gajger et al., 2009a; Gajger et al., 2009b). Recently, surfactant molecules that are cyclic lipopeptides and have antifungal, antibacterial, antiviral, and antimycoplasmal activities have been tested and showed to have promise by decreasing *Nosema* intensity after treatment (Porrini et al., 2010).

Fumagillin only targets active infections within the honey bee midgut and not the resistant spores on equipment. Alternative options, both chemical and natural, have been investigated for decreasing the viability of *Nosema* spores. Replacing colonies lost during the winter is a standard practice but using the same combs that have been contaminated with spores from feces does not prevent infection of new package bees (Moeller, 1962). Transferring colonies to new comb after feeding has the potential to be a successful non-chemical control method, but can be

expensive and does not prevent re-infection (Bailey, 1955b). Fumigation of combs with formaldehyde, acetic acid, or formalin was effective and non-toxic to bees (Bailey, 1955b) . These fumigation methods were also effective at targeting the active infection inside the bees as well as the resistant spores on combs (Bailey, 1955a). Ethylene oxide was also examined for treatment because of its use in sterilizing items that cannot be sterilized by steam, dry heat, or liquid chemicals (Michael, 1964), but was later found to leave toxic residues (Hornitzky, 1986). Heat treatment was effective at killing spores when equipment was heated to 120°F for 24 hours, but a heat chamber is required (Cantwell and Lehnert, 1968; Cantwell and Shimanuki, 1969). Gamma irradiation has also been investigated as it does not induce radioactivity to items exposed and can kill spores and other parasites (Hornitzky, 1986). Gamma irradiation induced changes in all developmental stages and most notably resulted in an undeveloped polar filament (Liu et al., 1990). When exposed to freezing temperatures, *N. ceranae* spore viability decreases (Fenoy et al., 2009b) suggesting that equipment should be stored in areas that are not heated during the winter. A combination of hygienic treatment of equipment and antibiotic control are the best combinations for combating this disease.

Concerns

The recent discovery of *N. ceranae* in the U.S. has raised concerns among beekeepers as to whether this new pathogen could be contributing to CCD-related losses and other losses. The timing and widespread incidence of *N. ceranae* coincides with the first reports of CCD; however, subsequent analyses of older samples have shown that *N. ceranae* has been present in *A. mellifera* longer than previously thought. Since its discovery, *N. ceranae* has been linked with colony declines in Europe and associated with collapsing colonies in the U.S. (Cox-Foster et al.,

2007; Higes et al., 2008a; Higes et al., 2009b). Little is known about the impacts of this pathogen on colony health and whether it is a contributing cause to increased losses in the United States. *N. ceranae* has been found in CCD hives and in royal jelly from those hives, as well as non-CCD hives (Cox-Foster et al., 2007).

In response to concerns over *Nosema* spp., we initiated a statewide survey of Virginia colonies to determine whether honey bee colonies were infected with *N. ceranae* and/or *N. apis* and at what levels. We found a high percentage of colonies (70%) were infected primarily with *N. ceranae* and that (only 2.7% were infected with *N. apis* at low levels and only as a co-infection with *N. ceranae*). We also found that traditional diagnoses based on spore counts do not provide an accurate indication of colony infections with *N. ceranae* (Traver and Fell, 2011a).

N. apis infections peak in the early spring and decrease during the summer months (Bailey, 1955b). The decrease in infection level of *N. apis* in the summer is thought to be due to a number of factors: less stress, better nutrition, increased bee activity, and a reduced potential for comb contamination from defecation in the hive (Bailey, 1981). Currently little is known about the infection cycle or transmission of *N. ceranae* in honey bee colonies or its role in colony losses. In Spain, colonies were monitored for 18 months and no seasonality was found as infections were detected at high levels year round, but researchers only looked for spores (Martin-Hernandez et al., 2007). High levels of *N. ceranae* during the summer months (Higes et al., 2008a) suggest the possibility of transmission mechanisms other than ingestion of spores from feces. Infective spores have been found in pollen baskets (Higes et al., 2008c) and there have been studies to show that pathogens can be transmitted through pollinators visiting the same

flowers, although this is probably not a major factor in *N. ceranae* transmission (Durrer and Schmid-Hempel, 1994). Chen *et al.* (2009b) used PCR to detect *N. ceranae* in the hypopharyngeal and salivary glands of workers. Our preliminary findings suggest that brood food may also be contaminated with *N. ceranae*. Taken together, the infection of brood-food and/or the salivary glands could provide alternate routes for the spread of *N. ceranae* and explain why high levels of infection are seen in hives observed during the warmer months. Additionally, queens and drones could be infected and help facilitate *N. ceranae* transmission. Understanding the annual disease cycle is key to the development of effective control procedures and the prevention of negative impacts on colony health.

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CHAPTER 2

Comparison of within hive sampling and seasonal activity of *Nosema ceranae* in honey bee colonies

The following chapter was formatted for publication in the Journal of Invertebrate Pathology. This work has been accepted for publication on 13 October 2011 and is currently *in press*. As an author, I retain the right to include the journal article, in full or in part, in a thesis or dissertation.

Comparison of Within Hive Sampling and Seasonal Activity of *Nosema ceranae* in Honey Bee Colonies

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Abstract

Nosema ceranae is a microsporidian parasite of the European honey bee, *Apis mellifera*, that is found worldwide and in multiple *Apis* spp.; however, little is known about the effects of *N. ceranae* on *A. mellifera*. Previous studies using spore counts suggest that there is no longer a seasonal cycle for *N. ceranae* and that it is found year round with little variation in infection intensity among months. Our goal was to determine whether infection levels differ in bees collected from different areas of the hive and if there may be seasonal differences in *N. ceranae* infections. A multiplex species-specific real-time PCR assay was used for the detection and quantification of *N. ceranae*. Colonies were sampled monthly from September 2009-September 2010 by collecting workers from honey supers, the fringe of the brood nest, and the brood nest. We found that all bees sampled were infected with *N. ceranae* and that there was no significant difference in infection levels among the different groups of bees sampled ($P = 0.74$). However, significant differences in colony infection levels were found at different times of the year ($P < 0.01$) with the highest levels in April-June and lower levels in the fall and winter. While our study was only performed for one year, it sheds light on the fact that there may be a seasonality to *N. ceranae* infections. Being able to predict future *N. ceranae* infections can be used to better advise beekeepers on *N. ceranae* management.

Keywords: *Nosema ceranae*, sampling, real-time PCR, *Apis mellifera*, seasonality

1. Introduction

Microsporidia are obligate intracellular parasites and are classified as Fungi (Adl et al., 2005). Members of the genus *Nosema* have two primary stages in the lifecycle, a vegetative stage that yields merozoites and a sporogonic phase in which mature spores are produced (Higes et al., 2010). Spores are the infective stage of *Nosema* spp. and are capable of surviving outside of the host. *N. ceranae* is a pathogen of the European honey bee *Apis mellifera* (Higes et al., 2006; Huang et al., 2007), as well as other Asian honey bee species (Botias et al., 2009; Chaimanee et al., 2010; Fries et al., 1996). While *N. ceranae* seems to be a relatively recent pathogen of *A. mellifera*, studies show that *N. ceranae* has been present in Uruguay since 1990 (Invernizzi et al., 2009), in Europe since at least 1998 (Paxton et al., 2007), and in the United States since 1985 (Accession No. FJ416497.1). Currently, *N. ceranae* has been described ubiquitously where beekeeping is practiced (Adl et al., 2005; Chen et al., 2008; Giersch et al., 2009; Higes et al., 2009; Invernizzi et al., 2009; Klee et al., 2007; Martin-Hernandez et al., 2007; Paxton et al., 2007; Williams et al., 2008) and has been hypothesized to be displacing the other *Nosema* species of honey bees, *N. apis*, on a global level (Klee et al., 2007; Martin-Hernandez et al., 2009; Paxton et al., 2007; Tapasztai et al., 2009). Others believe that it is still too early to determine whether *N. apis* has been displaced (Gisder et al., 2010; Higes et al., 2010).

Prior to the description of *N. ceranae*, *N. apis* was thought to be the only microsporidian that infected *A. mellifera*. Using standard phase contrast microscopy, it is difficult to distinguish between spores of the two species. Only under transmission electron microscopy can the different morphology be seen. *N. apis* spores are larger and have more polar filament coils within the spore (Chen et al., 2009b; Fries, 1989; Liu, 1984). The use of molecular diagnostics

has greatly facilitated parasite detection and quantification. *N. apis* was first detected molecularly in 2004 (Webster et al., 2004). Since then, standard PCR assays using species specific primers followed by separation on agarose gels (Chen et al., 2008; Klee et al., 2007; Martin-Hernandez et al., 2007) and real-time PCR (Bourgeois et al., 2010; Burgher-MacLellan et al., 2010; Chen et al., 2009a; Cox-Foster et al., 2007; Traver and Fell, 2011) have improved detection and species identification. Before the use of molecular assays for species determination, it is possible that *N. ceranae* infections prior to 1996 may have been improperly diagnosed as *N. apis* (Fries et al., 2006).

Due to its recent discovery, knowledge on the transmission and dissemination of *N. ceranae* within colonies has been lacking. Transmission of *N. apis* is primarily through an oral-fecal route (Fries, 1993), and similar routes of transmission are thought to exist for *N. ceranae*; however, factors contributing to the transmission of *N. ceranae* within hives are unknown (Fries, 2010). Bees can be exposed to *Nosema* by cleaning contaminated comb and through trophallaxis (Higes et al., 2010). Infected bees that are crushed during hive manipulation by beekeepers can also be a source of infective spores (Malone et al., 2001). Pollen can act as a reservoir for pathogens and *N. ceranae* spores have been found in corbicular pollen (Higes et al., 2008). Honey and royal jelly may also be a source of spores (Cox-Foster et al., 2007) but studies are needed to determine if such spores are viable. Furthermore, movement of bee products, equipment (Klee et al., 2007), queens, and their attendants may facilitate the spread of *N. ceranae* (Giersch et al., 2009). In addition worker activity may affect the distribution and transmission of *N. ceranae*. Honey bees exhibit age polyethism which involves a temporal division of labor based on the colony needs and physiological development. Maturation of

honey bees from nurses to foragers is regulated by vitellogenin, juvenile hormone, and primer pheromones (Amdam et al., 2003; Amdam and Omholt, 2003; Guidugli et al., 2005; Nelson et al., 2007; Robinson et al., 1991; Whitfield et al., 2006), but workers are capable of performing different tasks if required by colony needs (Haydak, 1932; Nelson, 1927). Based on the age and task of the worker, exposure to *N. ceranae* and the level of infection may be variable.

N. apis infections have been shown to have a seasonal cycle, with levels peaking in the spring, decreasing through the summer, followed by a secondary smaller peak in the fall before levels decrease in the winter and then build up in the spring (Bailey, 1959; Doull and Cellier, 1961; Dyess, 1978; Morison et al., 1956; Mussen et al., 1975; Oertel, 1964; Pickard and El-Shemy, 1989). Studies in Spain have shown a lack of seasonality for *N. ceranae* infections (Martin-Hernandez et al., 2007); however, previous findings based on a statewide survey of Virginia honey bee colonies led us to speculate that there was a seasonality for *N. ceranae* infections. Both spore and qPCR data suggested that *N. ceranae* has an infection cycle that peaks in the late spring or early summer and then decreases for the remainder of the year (Traver and Fell, 2011) which is similar to the infection cycle observed in Germany (Gisder et al., 2010). Our study examined in-hive bees as opposed to trapped, returning foragers to provide a more accurate estimation of a colony level infection. Foragers only represent 25% of a colony population (Seeley, 1995) and are not present during winter months. Additionally, by collecting and examining bees from different areas of the hive, we hoped to improve the reliability of diagnostic sampling techniques for beekeepers. Our other goal was to examine whether there could be a seasonal cycle for *N. ceranae* and if so, when infection levels peak. We determined that although *N. ceranae* is detectable year round, there are significantly higher levels in April

through June. While only performed for one year, this is the first report of a comprehensive study using systematic monthly sampling that is not based solely on spore counts, but rather a quantitative method to determine *N. ceranae* infection levels in honey bee colonies.

2. Methods

2.1 Sample collection

Sampling was started in September 2009 and completed in September 2010. Sample colonies were located in southwest Virginia in apiaries owned by Virginia Tech in Blacksburg. A total of 10 colonies were sampled. Samples of approximately 60 honey bees per sample area were collected during the third week of each month and stored in 70% ethanol. Samples were taken from the honey supers (the topmost hive bodies containing frames with honey), the fringe of the brood nest (defined as a full frame out from the outermost part of the brood nest), and the brood nest in the warmer months (September and October 2009 and April-September 2010). During the winter when bees cluster to survive the cold temperatures, samples were taken from the inner and outer parts of the winter cluster. Winter sampling (November 2009-March 2010) was done as carefully and quickly as possible to minimize colony disturbance. Brood pattern and colony strength (based on the number of frames with brood) were recorded for each colony from spring through fall when samples were collected. Brood frames were examined visually and the amount of brood on each side was estimated to the nearest quarter of a frame, such that a frame that had approximately half of the cells filled on each side would be rate as a half a frame of brood.

2.2 Forager sampling

Nine hives (the seven that made it through the 13-month study and an additional two from the same apiaries) were sampled for foragers and in-hive bees (from the fringe of the brood nest) in April 2011 to see if *Nosema* levels differed between the two groups. Foragers were trapped and collected at the hive entrance at midday. Bees from the fringe of the brood nest were then sampled to provide an in-hive bee sample for comparison. Workers were stored in 70% ethanol. A total of 30 foragers and 30 in-hive bees from each hive (for each sample six sets of five abdomens) were analyzed using qPCR and two sets of 10 bee abdomens for each area sampled (a total of 40 per hive) were analyzed for spore numbers.

2.3 Genomic DNA extraction

Genomic DNA was extracted from three sets of five bee abdomens from each area of the hive sampled (15 bees per area for a total of 45 bees per hive in the warmer months and 30 in the colder months) as previously described (Traver and Fell, 2011). Briefly, abdomens were crushed in Bender Buffer, followed by a proteinase-K-phenol:chloroform extraction. DNA was precipitated using isopropanol and resuspended overnight in DEPC-treated water at room temperature. DNA quantification was performed on a Nanodrop2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.4 Quantitative real-time PCR (qPCR)

A multiplex real-time PCR assay was used as described previously (Traver and Fell, 2011) to quantitate levels of both *N. ceranae* and *N. apis*. Standard curve quantitation using ten-fold serial dilutions of plasmid DNA from each species was performed in each PCR reaction to create

standard curves relating C_T values to copy number for both *N. ceranae* and *N. apis*. All samples were run in triplicate and an average C_T value was obtained and converted to an average copy number.

2.5 Spore counts

Two sets of ten bee abdomens per area sampled (for a total of 60 bees in the warmer months and 40 bees in the colder months) were crushed in 1 ml of distilled water using a 1 ml pestle. Sample lysates were diluted 1:10 and loaded onto a Bright-line hemacytometer (Hausser Scientific, Horsham, PA) to determine the number of spores per bee. Each sample was counted four times and the average number of spores per bee calculated as previously described (Cantwell, 1970).

2.6 Statistical analysis

All statistical analyses were performed using SAS 9.2. Data were transformed using log (mean copy number +1). To compare infection levels among the months, hives, and bees sampled, a generalized linear model following a gamma distribution was used, followed by a likelihood ratio test using a χ^2 probability distribution (analogous to an F-test in ANOVA). Bees were placed into groups depending on whether they were sampled in warmer or colder months. This grouping allowed the comparison of bees from the honey supers, the brood nest fringe, and brood nest to one another. Bees sampled from the inner and outer portions of the winter cluster were similarly compared. To analyze *Nosema*-negative samples, infection level data were first converted into presence/absence data (infected or not infected). Then a logistic regression was used to model the proportion of the population that was infected based on hive, month, and bees

sampled as the factors. For both analyses, *post-hoc* tests were performed using the least squares means and significance was selected as $\alpha = 0.05$.

3. Results

3.1 Annual analysis for *N. ceranae*

All ten colonies monitored throughout the 13-month period were infected with *N. ceranae*; however, three hives died between March and April of 2010. One hive died of unknown causes while another hive died due to exposure to rain and freezing winter temperatures after the cover blew off during a winter storm. In both of those hives, dead bees were present in the bottom of the hive. The third hive was abandoned and had no bees or food when examined in April. Thus the sample size from September 2009-March 2010 and April 2010-September 2010 was 10 and 7 colonies, respectively. *N. apis* was only detected in January at very low levels (mean copy number $4.65 \pm \text{SEM } 0.27$) and as a co-infection with *N. ceranae*. The multiplex qPCR assay used for all of our analyses contained primers and probes for both *Nosema* species, thus *N. apis* would have been detected if present.

When all months and workers sampled throughout the different areas of the hive were included in the gamma regression model, there were significant differences in infection levels between months ($\chi^2 = 169.05$, $df = 11$, $P < 0.01$) and hives ($\chi^2 = 57.69$, $df = 9$, $P < 0.01$), but not among the different groups of bees sampled ($\chi^2 = 1.24$, $df = 3$, $P = 0.74$). We observed a significant difference in *N. ceranae* levels throughout the months sampled, indicating seasonality for *N. ceranae* (Figure 1). Infections were significantly lower in September-March, then peaked April-June, and decreased July-September. Additionally, a non-parametric 3-way ANOVA was

performed using month, hive, and bees sampled as factors. The results were similar to the gamma regression analysis (data not shown). Analysis using a gamma regression was used because the data were highly skewed and once transformed, followed a gamma distribution giving this analysis more power and allowing for prediction of future *N. ceranae* cycles. The analysis was then split between the two different sampling time periods: the warmer months (September-October 2009 and April-September 2010) and the colder months (November 2009-March 2010) based on the bees being sampled in the hive.

3.2 Warmer months

Workers sampled during the warmer months were collected in September and October 2009 and April-September 2010. During these months, workers were spread throughout the hive so that samples from honey supers, the fringe of the brood nest, and the brood nest could be easily obtained (Figure 2A). There was also a significant difference in *N. ceranae* infection levels between months ($\chi^2 = 172.66$, $df = 7$, $P < 0.01$), but not among bees sampled from any of the three areas ($\chi^2 = 0.06$, $df = 2$, $P = 0.97$). There was a significant difference in infection levels in different colonies sampled ($\chi^2 = 72.35$, $df = 9$, $P < 0.01$), as expected, since infection levels vary between colonies.

3.3 Colder months

Workers sampled during the colder months included those from November 2009-March 2010 (Figure 2B) and were based on when bees formed a winter cluster. There was a significant difference in infection levels between months sampled ($\chi^2 = 11.73$, $df = 4$, $P < 0.02$) and hives ($\chi^2 = 20.20$, $df = 9$, $P = 0.02$), but there was no significant difference in infection levels between

bees sampled from the inner or outer part of the winter cluster ($\chi^2 = 0.72$, $df = 1$, $P = 0.4$). Interestingly, *N. apis* was only detected in January in two hives (both in the inner and outer part of the winter cluster) at very low levels. This finding agrees with our statewide survey of Virginia for *Nosema* spp. where *N. apis* was found in 2.7% of colonies (Traver and Fell, 2011).

3.4 *Nosema*-negative samples

In the analysis, gamma distributions only use values greater than zero, so *Nosema*-negative samples (as indicated by a zero copy number) were analyzed separately. *Nosema*-negative samples were found in bees from all areas within the hive in all colonies sampled (Table 1) and in all months except in March 2010 (Table 2) when there were no *Nosema*-negative samples. The proportion of *Nosema*-negative samples was not significantly different among bees sampled (Table 1- $\chi^2 = 1.3$, $df = 3$, $P = 0.73$); however, the proportion significantly varied over months for both seasons (Table 2; warmer months- $\chi^2 = 74.09$, $df = 7$, $P < 0.01$; colder months- $\chi^2 = 43.85$, $df = 4$, $P < 0.01$). The trend in occurrence of *Nosema*-negative samples also corresponded with when *N. ceranae* levels peaked with fewer *Nosema*-negative samples when the highest levels of *N. ceranae* were observed.

3.5 Infection levels and colony strength

From May-September 2010, colony strength was recorded when samples were collected. Strength ratings were based on the number of frames with brood. A non-parametric ANOVA (Kruskal-Wallis Test) was performed to determine whether there was a relationship between colony strength as indicated by the number of frames with brood and *N. ceranae* levels. No significant relationship was found between colony strength and *N. ceranae* levels ($\chi^2 = 32.36$, df

= 30, $P = 0.36$) indicating that weak colonies can be heavily infected with *N. ceranae* as can strong colonies, which is in agreement with a previous finding (Traver and Fell, 2011).

3.6 Spore counts

Mean spore counts (\pm SE) for colonies positive for *Nosema* spp. spores are reported in Table 3. We did not find a strong correlation (Spearman's rank correlation coefficient $\rho = 0.1319$) between spores and qPCR results, which agrees with our previous finding (Traver and Fell, 2011). This poor correlation was due to the numerous samples with no detectable spores. Additionally, there was a negligible correlation between spores and qPCR results when individual months, hives, and bees were examined (data not shown).

4. Discussion

N. ceranae has been detected year round and based on this finding it has been suggested that there is no seasonality to infections and therefore no annual infection cycle (Martin-Hernandez et al., 2007); however, this previous study was done only using spore counts. Our study used both spore counts and qPCR data and we saw a clear seasonal trend in infection levels using qPCR data (Figure 1). While our study was only for a 13-month period, it supports a previous finding that levels peak late spring and early summer (Traver and Fell, 2011) and agrees with data reported from Germany looking at *N. ceranae* levels over a five year period (Gisder et al., 2010). We did not find a significant correlation between spore counts and *N. ceranae* copy number. qPCR is more sensitive than using spore counts and will detect infections 40% more frequently. Previous studies have reported that *N. apis* infections are low or non-detectable in the summer but increase during the fall and winter, exhibiting a peak in the spring before the winter bees die

(Bailey, 1955; Doull and Cellier, 1961; Furgala and Boch, 1970; Morison et al., 1956). Fries (2010) has suggested that seasonality of *Nosema* spp. may be dependent on climatic conditions, although others have suggested that seasonal differences may have existed previously but no longer occur (Martin-Hernandez et al., 2007). Since we found low levels of *N. ceranae* in the winter, winter colony losses due solely to *N. ceranae* seem unlikely. The fact that infection levels remained low during the winter suggests that stress from colony disturbance during sample collection had no effects on the intensity of *N. ceranae* infections. Also, the higher levels of *N. ceranae* infection during the spring and summer months may provide a competitive advantage over *N. apis*, which is not normally found during the summer, and thus help explain the apparent displacement of *N. apis* by *N. ceranae*.

Moeller et al. (1956) found that in an *N. apis* infected colony, infected bees were more frequently found in the warmer part of the winter cluster and suggested that sampling bees from the top bars in the center of the winter cluster is sufficient for an accurate diagnosis of *N. apis*. We found that there was no significant difference between bees sampled in the inner or outer parts of the winter cluster. Several studies have indicated that during warmer weather, colony level *N. apis* infections cannot be accurately diagnosed by sampling bees from the brood nest (L'Arrivee, 1963; Taber and Lee, 1973). Instead, spore counts from returning foragers have been found to be higher than those of bees collected at the hive entrance or from the brood nest and thus provide a better indicator of *N. apis* levels (El-Shemy and Pickard, 1989). However, spore counts start to decrease with the onset of foraging because foragers defecate more regularly during flights (El-Shemy and Pickard, 1989). Our data suggest that *N. ceranae* spores are a poor

indicator of infection levels, most likely for low level infections, in colonies regardless of where the worker samples were collected, as we only find spores 30.6% of the time.

Recently the reliability of spore counts for the diagnosis of *N. ceranae* infections has been questioned. Significant differences have been observed in spore counts between in-hive bees and foragers, as well as variation in spore numbers based on the time of day or week samples were collected (Meana et al., 2010). Samples from the central parts of a hive were found to be unsuitable as indicators of disease level and that older bees must be sampled for an accurate diagnosis; however, only one hive was used to make this determination (Meana et al., 2010). Contrary to previous findings, when we compared foragers to in-hive bees, there was no significant difference between the bees for *N. ceranae* infection levels based on qPCR results ($\chi^2 = 3.71$, $df = 1$, $P = 0.054$) and if using a non-parametric ANOVA, there was no significant difference ($P = 0.4166$). The average copy number ($\pm SE$) for the in-hive bees was $3.06 \times 10^4 \pm 3.05 \times 10^4$ while the average for the foragers was $1.01 \times 10^5 \pm 7.10 \times 10^4$. When examining for the presence or absence of spores, we detected significantly more spores in in-hive bees 88.9% (16/18) of the time compared with 61.1% (11/18) of the time for foragers ($\chi^2 = 4.59$, $df = 1$, $P = 0.03$). Spore analysis had an average ($\pm SE$) of $2.78 \times 10^4 \pm 1.05 \times 10^4$ and $3.72 \times 10^5 \pm 3.71 \times 10^5$ spores per bee for in-hive and foragers, respectively.

Martin-Hernandez et al. (Martin-Hernandez et al., 2009) found that unlike bees infected with *N. apis* which have an equal proportion of immature and mature stages present, a *N. ceranae* infection has a higher proportion of immature stages and spore counts do not accurately reflect the level of infection. However, our qPCR allows for the detection of immature stages during

the proliferative phase when vegetative cells are being produced. The increased sensitivity of the analysis shows that young bees can be and are infected. We also believe that the use of hive bees provides a better indication of the overall level of infection within a colony and a better representation of the colony population. When conditions are right, there are 25% foragers in a hive at any given point (Seeley, 1995) so sampling only from foragers biases the population of bees being examined. Using foragers for a *Nosema* diagnosis increases the likelihood of finding spores but it biases the sample to the most heavily infected bees.

One of our goals was to determine whether sampling from different areas of the hive would result in different levels of *N. ceranae*. We found that there was no significant difference in infection levels between bees sampled from different locations in the hive. Movement of bees occurs within a hive during sampling so we cannot definitively conclude that only young bees were sampled from the brood area or that older workers were sampled from the honey supers; however, we can conclude that random samples from the hive should be suitable for *N. ceranae* diagnosis, although it may depend on the time of year the bees are sampled. This approach is more convenient for beekeepers as they only need to sample in-hive bees rather than trapping foragers or trying to sample only from the oldest bees in the hive which is more subjective based on beekeeper experience. Also, there is a need for standardized sampling techniques. Current recommendations are that only foragers should be trapped, but our findings show that this is unnecessary as an in-hive sample can be used for diagnostic purposes. Despite having only one sampling year of data, we did observe a seasonal trend for *N. ceranae* infections. We were able to detect *N. ceranae* year round, but levels did peak and were significantly higher from April-June when compared to other months. However, further studies are needed to establish an

annual infection cycle for *N. ceranae* and gain a better understanding of the changes in infection intensity within colonies during the year.

Unfortunately, the highest levels of *N. ceranae* were seen during the spring, just before and during peak nectar flows in Virginia. This seasonality could be problematic for beekeepers, in the United States where fumagillin is still approved for use, since treatment with fumagillin should not be made immediately before or during a honey flow. However, we observed very little impact of *Nosema* infections on either colony growth or productivity, suggesting that even though we found higher levels of infection, treatment is not necessary. The low levels of infection during the fall and winter also indicate that the use of fall treatments is unnecessary. Current recommendations for treatment are based on the *N. apis* cycle which has higher infection levels during the winter (Bailey, 1955). Treating in early March may be beneficial for weak colonies with high levels of *N. ceranae*, but are otherwise unnecessary.

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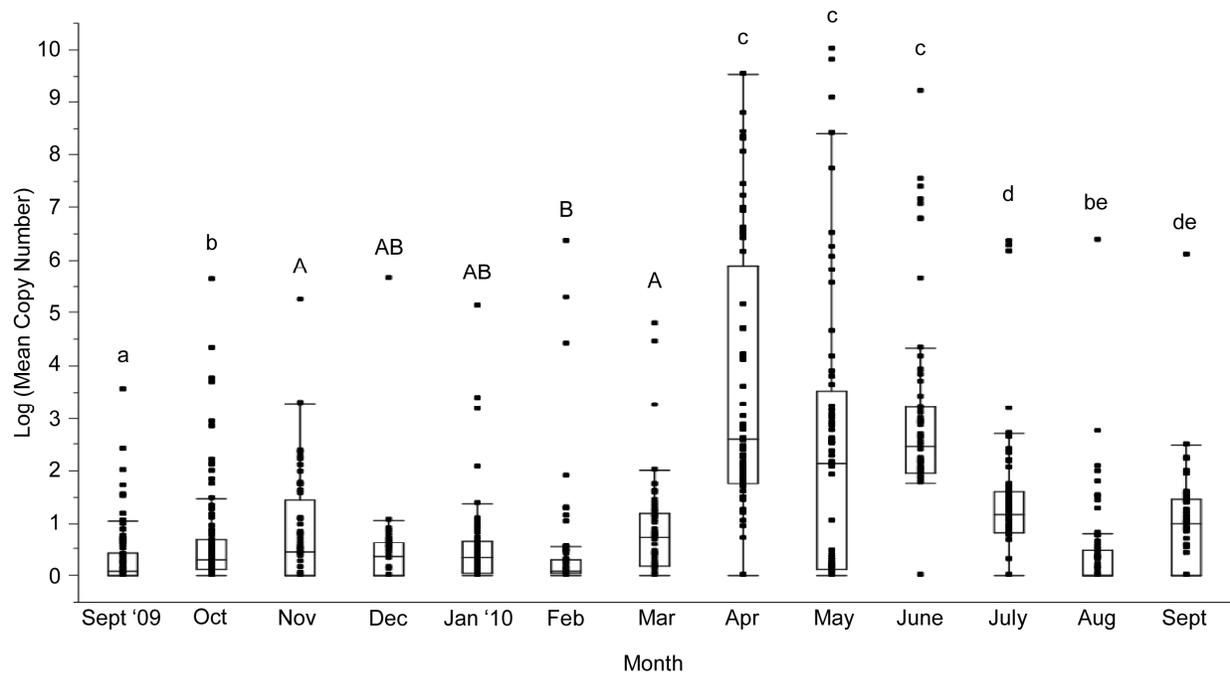


Figure 1. Box plot of the seasonal cycle for *N. ceranae*. On the y-axis is the *N. ceranae* infection level (indicated by the log of the mean DNA copy number) and month is on the x-axis. Sampling started in September 2009 and ended in September 2010. All data from each hive and all bees sampled are represented for each month. The horizontal lines represent the 25th, median, and 75th quartiles. Whiskers encompass data points falling within the (upper quartile + 1.5 x interquartile range) and (lower quartile – 1.5 x interquartile range). Lower case letters indicate significant differences between samples from warmer months and upper case letters indicate significant differences between samples sampled from the colder months.

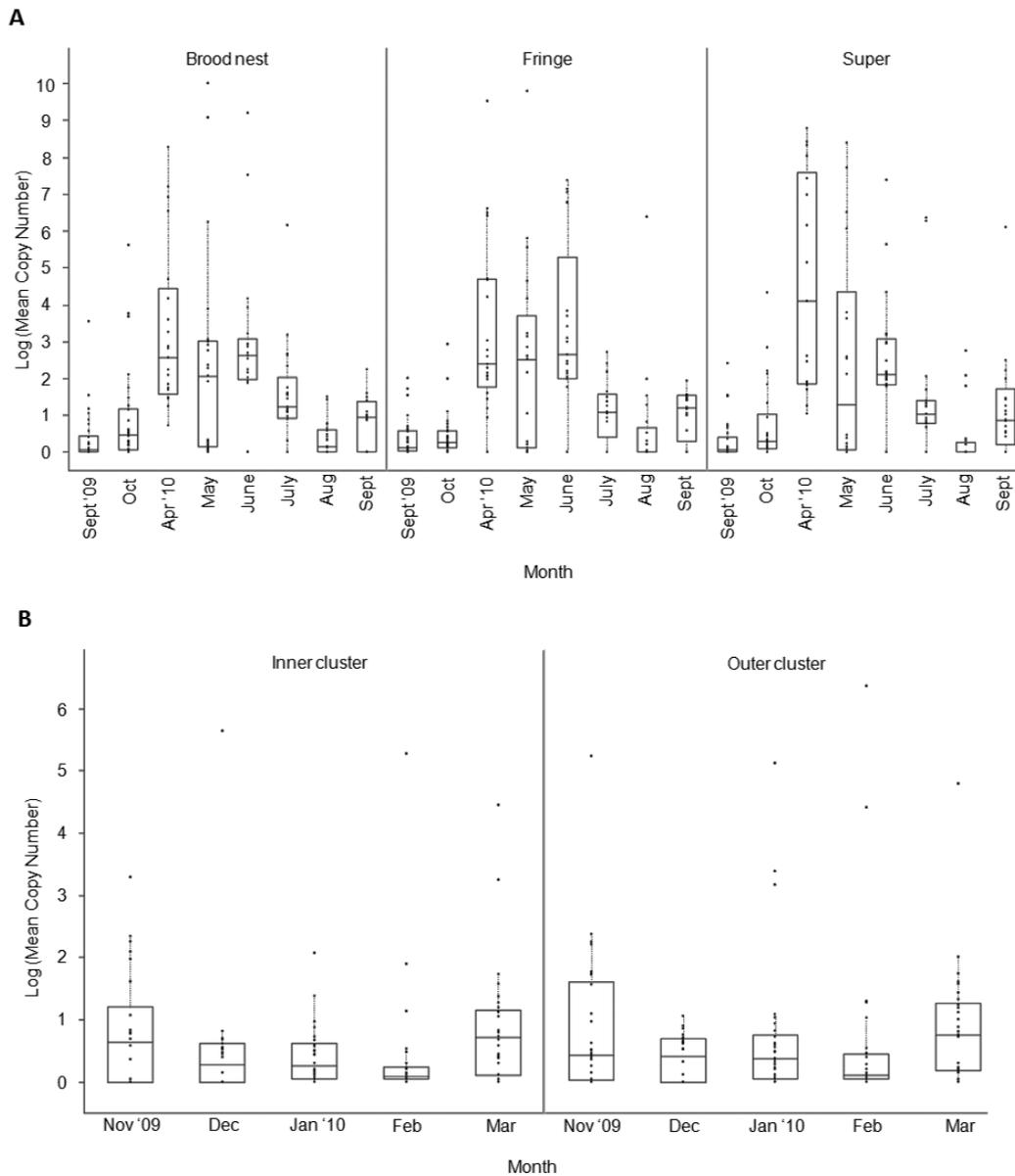


Figure 2. Box plot for workers sampled during different seasons. On the y-axis is the *N. ceranae* level represented as the log of mean DNA copy number. On the x-axis are workers sampled from different areas of the hive. Within each group of workers sampled, the x-axis is broken down into the months sampled. The horizontal lines represent the 25th, median, and 75th quartiles. Whiskers encompass data points falling within the (upper quartile + 1.5 x interquartile range) and (lower quartile – 1.5 x interquartile range). (A) Workers sampled from the brood nest, the fringe of the brood nest, and the honey supers during September-October 2009 and April-September 2010. (B) Workers sampled during the colder months (November 2009-March 2010) from the inner and outer sections of the winter cluster.

Table 1. The percentage of *Nosema*-negative samples in the different bees groups sampled.

Bees sampled	% <i>Nosema</i>-negative samples
Brood Nest	18.8
Super	19.3
Fringe	20.7
Inner Cluster	16.7
Outer Cluster	12.7

Table 2. The percentage of *Nosema*-negative samples by month.

Month sampled	% <i>Nosema</i>-negative samples
September 2009	19.0 a ¹
October	10.8 bc
November	21.7 AB ²
December	35.0 B
January 2010	5.0 C
February	11.7 AC
March	0.0 D
April	1.4 d
May	5.1 bd
June	12.3 ab
July	13.6 ab
August	43.2 e
September	22.7 ac

¹The lower and ²upper case letters indicate significant differences between samples from warmer months and colder months, respectively.

Table 3. Mean spore counts (\pm SE)¹ from bees sampled in different areas of the hive from September 2009 to September 2010 in colonies positive for *Nosema* spp. spores.

Month	Brood Nest ²	N ³	Fringe	N	Super	N
September '09	$2.76 \times 10^5 \pm 2.17 \times 10^5$	10/20	$6.44 \times 10^5 \pm 4.05 \times 10^5$	10/20	$1.74 \times 10^6 \pm 1.29 \times 10^6$	11/19
October '09	$5.68 \times 10^4 \pm 1.47 \times 10^4$	11/23	$4.94 \times 10^5 \pm 4.29 \times 10^5$	9/23	$4.75 \times 10^4 \pm 6.92 \times 10^3$	10/23
April '10	$2.15 \times 10^6 \pm 6.29 \times 10^5$	6/15	$3.64 \times 10^6 \pm 2.99 \times 10^6$	2/14	$8.72 \times 10^6 \pm 1.10 \times 10^6$	10/12
May '10	$2.71 \times 10^5 \pm 1.41 \times 10^6$	2/14	$4.43 \times 10^6 \pm 2.51 \times 10^6$	3/14	7.50×10^6	1/12
June '10	2.50×10^4	1/14	0	0/14	$6.00 \times 10^5 \pm 5.50 \times 10^5$	2/14
July '10	$8.10 \times 10^5 \pm 7.73 \times 10^5$	5/14	$1.20 \times 10^6 \pm 1.18 \times 10^6$	3/14	$5.00 \times 10^4 \pm 1.44 \times 10^4$	3/14
August '10	$3.57 \times 10^4 \pm 5.05 \times 10^3$	7/14	$7.50 \times 10^4 \pm 2.70 \times 10^4$	4/14	$7.00 \times 10^4 \pm 5.00 \times 10^3$	5/14
September '10	$7.50 \times 10^4 \pm 0$	2/14	$3.25 \times 10^5 \pm 2.92 \times 10^5$	4/14	$1.68 \times 10^5 \pm 1.50 \times 10^5$	6/14

Month	Inner cluster	N	Outer cluster	N
November '09	$1.08 \times 10^5 \pm 2.98 \times 10^4$	9/20	$1.14 \times 10^6 \pm 6.58 \times 10^5$	11/20
December '09	$1.03 \times 10^6 \pm 4.47 \times 10^5$	5/20	$1.45 \times 10^5 \pm 8.42 \times 10^4$	5/20
January '10	$1.10 \times 10^6 \pm 1.13 \times 10^6$	6/19	$7.06 \times 10^5 \pm 4.06 \times 10^5$	13/20
February '10	$9.80 \times 10^5 \pm 9.30 \times 10^5$	5/23	$5.86 \times 10^5 \pm 5.14 \times 10^5$	11/23
March '10	$3.06 \times 10^5 \pm 1.76 \times 10^5$	12/30	$6.28 \times 10^5 \pm 3.86 \times 10^5$	15/30

¹Mean spore counts were calculated from two sets of ten worker abdomens for each area sampled and from each colony sampled

²Spore counts are divided by the season sampled (warm versus winter months). The table only includes data from positive spore counts.

³Sample size that had spores out of the total number of samples for each groups of bees examined.

CHAPTER 3

Nosema ceranae in drone honey bees (*Apis mellifera*)

The following chapter was formatted for publication in the Journal of Invertebrate Pathology. This work was originally published by Traver and Fell in Volume 107, Issue 3, July 2011, pp. 234-236 (DOI:10.1016/j.jip.2011.05.016) in the Journal of Invertebrate Pathology. As an author, I retain the right to include the journal article, in full or in part, in a thesis or dissertation.

Nosema ceranae in drone honey bees (*Apis mellifera*)

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Abstract

Nosema ceranae is a microsporidian intracellular parasite of honey bees, *Apis mellifera*. Previously *Nosema apis* was thought to be the only cause of nosemosis, but it has recently been proposed that *N. ceranae* is displacing *N. apis*. The rapid spread of *N. ceranae* could be due to additional transmission mechanisms, as well as higher infectivity. We analyzed drones for *N. ceranae* infections using duplex qPCR with species specific primers and probes. We found that both immature and mature drones are infected with *N. ceranae* at low levels. This is the first report detecting *N. ceranae* in immature bees. Our data suggest that because drones are known to drift from their parent hives to other hives, they could provide a means for disease spread within and between apiaries.

Keywords: *Nosema ceranae*, *Nosema apis*, drones, drifting, *Apis mellifera*, real-time PCR

1. Introduction

Nosema apis is a microsporidian pathogen of honey bees, *Apis mellifera*, and was thought to be the only microsporidia that infected honeybees until 1996 when *Nosema ceranae* was discovered in *Apis cerana* (Fries et al., 1996) and was later found to naturally infect *A. mellifera* (Higes et al., 2006; Huang et al., 2007). *N. ceranae* is now widespread and can be found on all continents where beekeeping is practiced (Adl et al., 2005; Klee et al., 2007; Martin-Hernandez et al., 2007; Paxton et al., 2007; Chen et al., 2008; Williams et al., 2008; Giersch et al., 2009; Higes et al., 2009; Invernizzi et al., 2009). Due to the rapid spread of *N. ceranae* it has been hypothesized that, with a few exceptions, it is displacing *N. apis* (Klee et al., 2007; Paxton et al., 2007; Martin-Hernandez et al., 2009; Tapaszti et al., 2009) in many countries. Exceptions include Germany,

the UK, and Switzerland although displacement there may be occurring currently (Gisder et al., 2010; Higes et al., 2010).

Transmission of *N. apis* occurs primarily through an oral-fecal route within hives (Fries, 1993) when bees clean contaminated comb, consume contaminated water, or exchange food by trophallaxis. Transmission of *N. ceranae* is thought to be similar but additional routes of spread may be involved, especially between hives by drifting bees. Drifting is a behavior in which bees, both workers and drones, return to the wrong hive after leaving their parent colony (Free, 1958). It is a common occurrence in apiaries, especially when hives are placed in rows with few landmarks or orientation cues for the bees. This behavior can cause problems by increasing the spread of disease within apiaries. Drones begin drifting between 6 and 7 days of age when initiating mating flights. The percentage of drifting increases until drones are 15 days of age after which it decreases (Currie and Jay, 1991). Drones drift 2–3 times more frequently than workers with anywhere from 3–89% drifting with an average occurrence of 50% (Free, 1958; Witherell, 1965; Currie and Jay, 1991; Neumann et al., 2000). In addition, 21% of drones will drift more than once (Currie and Jay, 1991). Drifting by drones could increase *Nosema* spp. transmission between hives.

Drones are as susceptible to *N. apis* infection as workers but are less frequently infected because they are not involved in cleaning combs (Bailey, 1972). However, drones may become infected when fed by workers that have cleaned contaminated comb (Free, 1957; Bailey, 1972) and thus they could play a role in the transmission within and between hives. Currently, the prevalence of drone infection by *N. ceranae* is unknown. Since Free (1958) suggested that drones could be a

reservoir for *N. apis* spores, it has been speculated that they could also vector diseases within an apiary (Fantham and Porter, 1912; Bailey, 1955; Hanko and Lemakova, 1971). Our goal was to determine whether drones are naturally infected with *N. ceranae* and at what levels.

2. Materials and methods

2.1 Drone Sampling

Colonies were located in three apiaries owned by Virginia Tech in Blacksburg, VA. All of the colonies were found to be naturally infected with *N. ceranae* and were ranked as moderate to strong colonies based on the number of frames with brood and taking into account the time of year. Apiaries had a standard arrangement with one to two colonies per hive stand. Hive stands were separated by three meters. Drone adults and immatures were sampled, when available, from nine colonies during September 2009–September 2010. Drone pupae were collected from capped cells at the purple eye stage (with body pigmentation) and were approximately 17–23 days old. Adult drones were collected in-hive and at the entrance after flight. In-hive drones were randomly collected from frames throughout the hive. Flying drones were collected on their return to the hive after a piece of queen excluder was used to block the hive entrance. The collection of flying drones was made between 2 and 4 pm when mating flights most often occur (Howell and Usinger, 1933; Oertel, 1956) and to decrease the chance of collecting younger drones making orientation flights.

2.2 Genomic DNA (gDNA) extraction and quantitative real-time PCR (qPCR)

Genomic DNA extraction and qPCR were performed as previously described (Traver and Fell, 2011). Briefly, gDNA was extracted from the abdomens of nine drones per hive (three sets of

three drone abdomens) using a Bender buffer lysis, phenol:chloroform extraction, and isopropanol precipitation. Drone pupae were washed in a 5% bleach solution followed by a rinse in distilled water before removing the abdomen to ensure external DNA contamination was minimized. Additionally, gDNA from 45 worker abdomens (pools of 5 female abdomens) from each colony was extracted to estimate the natural infection level for the colony. A total of 50 ng gDNA was used in a duplex qPCR using TaqMan assays (Applied Biosystems, Foster City, CA). This allowed for the simultaneous detection of *N. apis* and *N. ceranae* using species specific primers and probes designed from sequences of the 16S rRNA genes. All reactions were performed on a StepOne Real Time PCR System (Applied Biosystems). Standard curve quantitation was used to estimate average copy number for each species. All samples were run in triplicate.

2.3 Spore counting

Two sets of five drone abdomens were used for spore counting as previously described (Traver and Fell, 2011) for each sample of in-hive and flying drones to estimate the number of spores per drone. Drone pupae were not spore counted due to the limited sample size of pupae at the desired age.

3. Results and discussion

Drones were found to be naturally infected at low levels with *N. ceranae* (Fig. 1, Table 1). No *N. apis* infections were detected in drones of any age. Average spore counts were 9436 ($n = 102$, $SEM = 2794$) and 13,839 ($n = 28$, $SEM = 3362$) spores per bee for in-hive and flying drones, respectively. Only 19.6% of in-hive drones had sufficient spore numbers to count, with all such

samples collected in September and October 2009. In flying drone samples, 50% had spores and all were collected in July and August 2010, with no observed spores from samples collected in June 2010. Additionally, workers were also found to be naturally infected with *N. ceranae* from all of the colonies sampled.

Drone pupae were infected at low levels and most frequently in May and June (Table 1) and this is the first report that has detected *N. ceranae* in immature bees. If pupae are infected before emergence, they may be infected through brood food or contamination in their cells. We speculate that the low level infections found in pupae could be due to developmental changes associated with pupation and reorganization of the alimentary canal. In-hive drones had the highest infections in June (Table 1). For both drone pupae and in-hive drones, the highest levels of infection coincided with high levels of infection in the sampled hives. Mature, flying drones were also infected, but generally at lower levels (Table 1) and may be due to flying drones not returning to the hive or because heavily infected drones do not survive as long. However, of all the drones examined, the highest levels of *N. ceranae* were observed in flying drones; 4.2% of the samples had copy numbers over 4000 (Figure 1, outliers). Drifting by these drones could aid in the dispersal of *N. ceranae* to other hives in an apiary.

The trend of increasing *N. ceranae* infections in drones during the spring and early summer coincides with the peak in the annual infection cycle of workers (Traver and Fell, in preparation) and agrees with the trend seen in the workers sampled from each colony. The peak in infections also corresponds with observations that bees emerging later in the season drift less than those emerging in the spring and early summer, May–August (Free, 1958; Jay, 1965). While our goal

of determining whether drones are naturally infected with *N. ceranae* was accomplished, additional research is needed to determine whether drones contribute to the spread of *N. ceranae* within apiaries.

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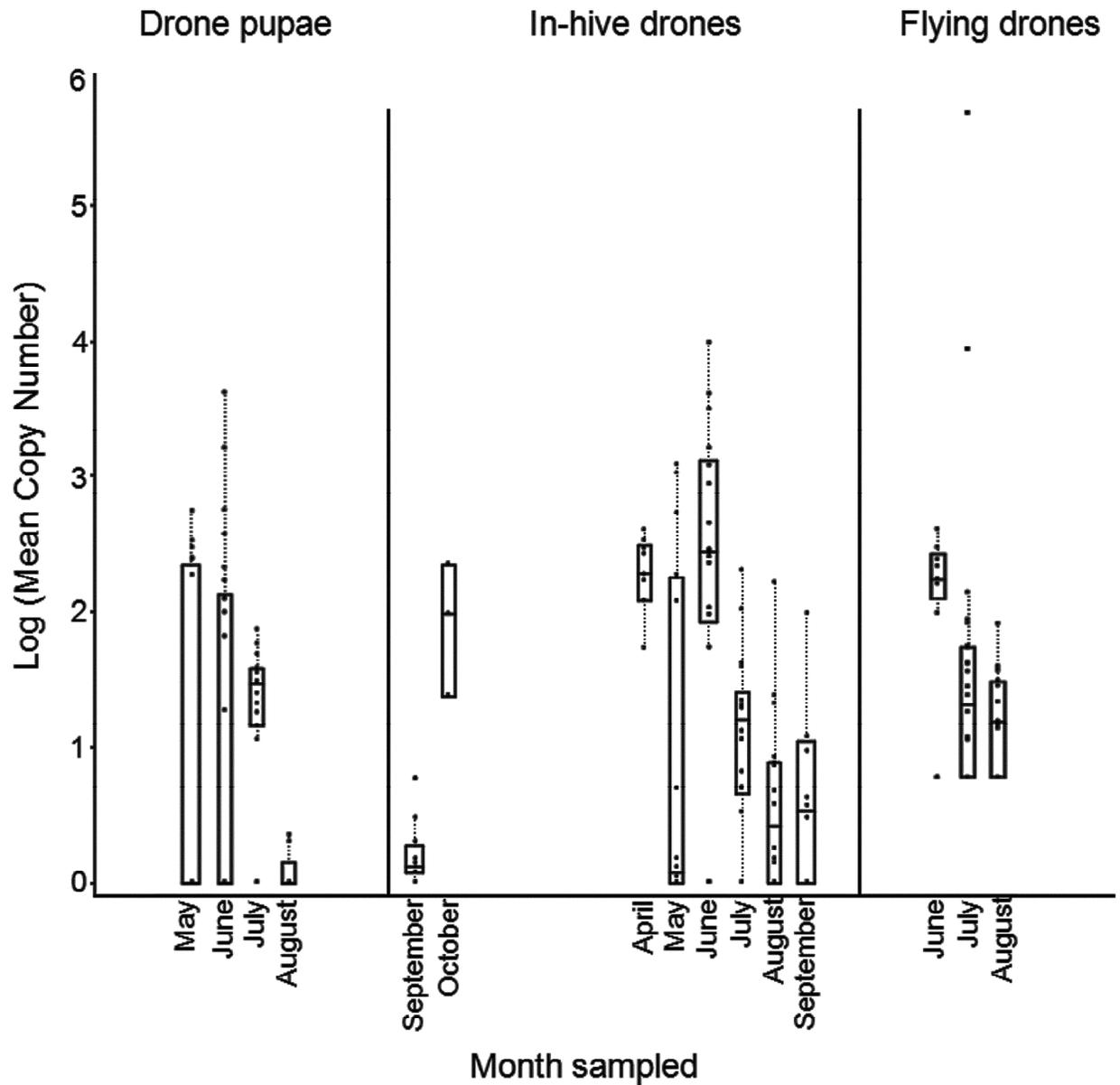


Figure 1. Box plot for the infections in adult and immature drones. On the y-axis is the *N. ceranae* infection level (indicated by the log of the mean copy number) and month sampled is on the x-axis. Sampling started in September 2009 and finished in September 2010. Data from all drones sampled in each month is shown. The lines for the box represent the 25th, median, and 75th quartiles. Whiskers encompass data points falling within the (upper quartile + 1.5 x interquartile range) and (lower quartile – 1.5 x interquartile range).

Table 1. *Nosema ceranae* levels observed for each set of drones sampled. A total of 9 drone abdomens (pooled samples of 3) and 45 worker abdomens (pooled samples of 5) were examined from each colony sampled.

Drone sample	N ^a	Month sampled	Avg. copy No.	SE ^b	Avg. hive copy No. ^c	SE
Drone pupae	6	May '10	87.12	151.78	2.84 x 10 ⁸	5.01 x 10 ⁸
	7	June '10	286.84	847.19	2.71 x 10 ⁷	6.59 x 10 ⁷
	4	July '10	29.33	19.73	8.80 x 10 ⁴	1.13 x 10 ⁵
	3	August '10	0.25	0.50	3.82 x 10 ⁴	1.01 x 10 ⁵
In-hive drones	3	September '09	0.8	1.4	57.37	136.54
	1	October '09	114.3	101.7	6.24 x 10 ³	1.62 x 10 ⁴
	3	April '10	214.4	114.1	7.6 x 10 ⁷	1.42 x 10 ⁸
	5	May '10	182.4	370.8	2.84 x 10 ⁸	5.01 x 10 ⁸
	6	June '10	1.25 x 10 ³	2.38 x 10 ³	2.71 x 10 ⁷	6.59 x 10 ⁷
	6	July '10	28.8	49.6	8.80 x 10 ⁴	1.13 x 10 ⁵
	6	August '10	13.3	37.8	3.82 x 10 ⁴	1.01 x 10 ⁵
	4	September '10	11.2	27.2	2.00 x 10 ⁴	5.29 x 10 ⁴
Flying drones	3	June '10	55.8	21.3	83.86	13.13
	6	July '10	1.81 x 10 ⁴	8.80 x 10 ⁴	8.80 x 10 ⁴	1.13 x 10 ⁵
	3	August '10	4	5.0	3.82 x 10 ⁴	1.01 x 10 ⁵

^aN represents the number of different hives sampled

^bSE is the standard error of the mean

^cAverage copy number from workers sampled in each hive for the given month

CHAPTER 4

Low natural rates of *Nosema ceranae* in *Apis mellifera* queens

The following chapter was formatted to facilitate publication in the journal *Apidologie*. This work was submitted by Traver and Fell on 15 September 2011.

Low natural rates of *Nosema ceranae* in *Apis mellifera* queens

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Nosema ceranae in honey bee queens

Abstract

The global incidence of *Nosema ceranae* is of concern as it seems to have displaced a closely related microsporidian parasite of *Apis mellifera*, *N. apis*. Queens are the primary female reproductive individuals in a honey bee colony and while they are generally free from *N. ceranae* infection, they are nevertheless susceptible. We sought to determine whether queen honey bees are naturally infected by *N. ceranae*, as these infections could be a factor in the rapid spread of this parasite. Samples were analyzed using real-time PCR and included immature larval queens, newly emerged queens, and older mated queens. In addition, analyses of the head, thorax, abdomen, ovaries, and spermathecae of adult queens were performed. Overall, we found that all tissues were infected with *N. ceranae* at low levels. The infection of the ovaries and spermathecae suggests the possibility of vertical transmission of *N. ceranae*.

Keywords: *Nosema ceranae*/real-time PCR/queens/*Apis mellifera*

1. INTRODUCTION

Nosema ceranae is a microsporidian pathogen of honey bees first described in *Apis cerana* (Fries et al., 1996) and later in *A. mellifera* (Fries et al., 2006; Huang et al., 2007). Detection of *N. ceranae* in honey bees dates back to 1998 in Europe (Paxton et al., 2007), the early 1990s in the United States (Chen et al., 2008), and to 1990 in Uruguay (Invernizzi et al., 2009). Due to the global prevalence of *N. ceranae*, displacement of *N. apis*, a closely related microsporidian species, has been hypothesized (Chauzat et al., 2007; Chen et al., 2008; Klee et al., 2007; Paxton et al., 2007). The rapid spread of *N. ceranae* may have been due to additional routes of transmission such as through foodborne transmission or through vertical transmission.

N. apis-infected nurse bees have reduced hypopharyngeal glands, which results in a decrease in brood food production (Wang and Moeller, 1969, 1971). It has therefore been hypothesized that the higher infection of *N. ceranae* in in-hive bees, the more at risk the queen is to infection (Higes et al., 2009). *N. ceranae*-infected in-hive bees could thus be a route by which the queen becomes infected; because nurse bees do not generally clean combs, they have a decreased risk for contracting an infection (Doull and Eckert, 1962). As with *N. apis*-infected workers, mature spores of *N. ceranae* were only found in the epithelial cells of the queen's ventriculus, not in the crop, proventriculus, Malpighian tubules, small intestine, rectum, ovary, or fat body (Higes et al., 2009; Webster et al., 2008).

N. apis has been shown to be primarily transmitted horizontally via foodborne transmission during trophallaxis of contaminated food or ingestion of spores during cleaning (Doull and Eckert, 1962; Higes et al., 2009). *N. ceranae*, unlike *N. apis*, has been detected in the hypopharyngeal and salivary glands (Chen et al., 2009; Gisder et al., 2010) and also in royal jelly (Cox-Foster et al., 2007), the primary food provided to developing queen larvae. While no spores have been found in royal jelly, it is possible that vegetative stages are present and could be a mechanism for the horizontal transmission of *N. ceranae* through brood food. Infection of immature developing queens is one consequence; another could be the transmission of *N. ceranae* to all immature developing workers and drones.

Queens may be involved in the transmission of *N. ceranae* through a horizontal mechanism and/or through vertical transovarial transmission. Previous research has shown that *N. apis* naturally infected queen honey bees (Webster et al., 2004), yet there was no evidence for vertical

transmission. While there is precedent for other *Nosema* spp. to be transmitted vertically from infected females to their eggs (Han and Watanabe, 1988; Nordin, 1975; Pavenstadt-Grupp and Ruthmann, 1989; Streett et al., 1993; Walters and Kfir, 1993), there is no evidence, based on PCR analysis of ovaries, of transovarial transmission in honey bees (Webster et al., 2008).

Queens are susceptible to *N. apis* and become infected in the same way as workers, through ingestion of spores (Fyg, 1964; Hassanein, 1951). The queen is the primary female reproductive in the colony and has a very specialized role. She is fed by workers and does not clean comb, so she is less susceptible to infection by *Nosema* (Hassenein, 1951). Queens have no natural defense to prevent *N. apis* infection, and heavily infected queens may have smaller ovaries and empty ovarioles (Hassenein, 1951). Oocytes degenerate and possibly undergo resorption, (Liu, 1992) leading to infertility, and more frequent supersedure (Farrar, 1942, 1947; Hassanein, 1951); however, there are differing reports as to whether *N. apis* infection causes supersedure. Queens experimentally infected with *N. apis* have been shown to lay normally after two weeks with no deterioration of the ovaries and no supersedure after the queens were accepted. There were also no outward pathological signs of infection (Czekoska, 2000; Doull and Eckert, 1962). *N. apis* has not been found in eggs, larvae, or pupae from colonies with infected queens (Hassenein, 1951; Webster et al., 2008).

Little is known about *N. ceranae* infections in queens. Previous studies, with the exception of Delaney et al. (2011), examined experimentally infected queens. In that study, neither *N. apis* nor *N. ceranae* were detected in the ventriculi of any queens from 12 different queen breeders; however, conventional PCR was used in this study, which is not as sensitive as real-time PCR for

this particular assay. Previously, we have shown that low level infections may not be detected when resolving PCR products on an agarose gel because the staining is dependent on the amount of ethidium bromide or other similar stain that intercalates into the DNA present (Traver and Fell, 2011a). Therefore in samples with very low levels of *Nosema* DNA, a band may not be visualized. *N. ceranae* spores have also not been detected in queen tissues (Delaney et al., 2011; Higes et al., 2009; Webster et al., 2004; Webster et al., 2008), but vegetative stages could still be present. High levels of queen failure (vanEngelsdorp et al., 2008) suggest that undetectable levels of pathogens may be affecting queen health and contributing to the increased colony losses observed during the past few years. The objective for this research was to investigate whether queens are naturally infected with *N. ceranae* and, if so, to identify the stage during development. We were also interested in determining whether *N. ceranae* could infect other tissues which might be involved in vertical transmission, such as the ovaries and/or spermatheca.

2. METHODS

2.1 Queen rearing

Five separate queen rearing hives (A-E) were set up for the study in Blacksburg, VA during the summers of 2009-2010. Queen rearing hives A and B were run in 2009 while hives C-E were used in 2010. Approximately 1-day old larvae were grafted into artificial queen cups and placed in the hives according to the Doolittle grafting method (Laidlaw, 1992). Different hives were used as a source of larvae for each of the queen rearing hives; the queen larvae, royal jelly, and adult queens produced in these hives were used as the sources of queens for the study.

For larval queens, cells from each rearing hive were removed before cell capping (n = 47), were snap frozen in liquid nitrogen, and stored at -80°C. Royal jelly (n = 46) from each cell was also removed and stored at 4°C. Newly emerged queens were obtained from queen cells that had been capped and were close to emergence. Ten cells from four rearing hives were removed from the grafting bar and placed in individual cages (with a piece of sterile gauze saturated in 1M sucrose solution to provide a food source for the newly emerged queen). Cages were placed in an incubator (94°C, 50% humidity) where the queens (n = 39) were allowed to emerge. Queens were killed 24 hours post-emergence and stored in 70% ethanol at room temperature. Mated queens (n = 17) were obtained from hives in the Virginia Tech apiaries in Blacksburg, VA. All mated queens had been laying for a minimum of several months and some for over a year.

2.2 Genomic DNA (gDNA) extraction

gDNA was extracted from royal jelly, 4-5 day queen larvae, newly-emerged queens, and mated queens. Adult queen abdomens were dissected before gDNA extraction so that the ovaries and spermathecae could be analyzed separately. Only the abdomen was dissected because we were interested in examining the reproductive organs to see if vertical transmission could be a possibility in vertical transmission of *N. ceranae*. gDNA from the head, thorax and remaining abdominal tissue containing the digestive tract (both mated and newly-emerged queens), and the ovaries of mated queens were prepared as previously described (Traver and Fell, 2011a), using a Bender Buffer lysis followed by a phenol:chloroform extraction. Isopropanol was used to precipitate the DNA, which was then re-suspended overnight at room temperature with DEPC-treated water. The smaller tissues samples (heads and ovaries of newly emerged queens, and

spermathecae and eggs dissected from the ovarioles of mated queens) were extracted using a commercially available kit (Qiagen DNeasy Blood and Tissue kit, Valencia, CA, USA).

A total of 39 newly emerged virgin queens and 17 mated queens were dissected and analyzed. Queens were stored in 70% ethanol until dissection. Prior to dissection, queens collected in 2010 were rinsed in distilled water to remove ethanol, washed in a 5% bleach solution, and then rinsed in distilled water to remove any external *Nosema* spp. DNA contamination from the external parts of the body.

For each queen cell, gDNA was extracted separately from the royal jelly and the corresponding larva as previously described (Traver and Fell, 2011a). For the 2010 larval samples, the larvae were washed in distilled water, then a 5% bleach solution, and then rinsed in distilled water before extraction to remove any external *N. ceranae* contamination and ensure that any *N. ceranae* DNA extracted was from an internal infection. Between 8 and 10 larvae/royal jelly samples were obtained from each queen-rearing hive for a total of 47 larvae and 46 royal jelly samples. All extracted DNA was quantified on a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.3 Quantitative real-time PCR (qPCR)

qPCR was performed on a StepOne Real Time PCR system (Applied Biosystems, Foster City, CA, USA). TaqMan assays (Applied Biosystems) using species-specific primers and probes were used to detect and quantify *N. ceranae* and/or *N. apis* in the extracted gDNA from the

queens. Standard curve quantitation was used to determine infection levels. All reactions were performed as previously described (Traver and Fell, 2011a).

2.4 Brood nest bees

From each queen rearing hive, approximately 60 bees from the brood nest were sampled and stored in 70% ethanol. Abdomens and heads were analyzed for *Nosema* spp. as described above. Six pools of five abdomens from each rearing hive were analyzed with the exception of queen rearing hive B, in which only three pools were analyzed due to insufficient sample size. From rearing hives in 2010, six pools of five heads from the corresponding abdomens extracted were also analyzed except for queen rearing hives A and B where the heads were not saved for analysis.

2.5 Flying drones

From three colonies (1-3), flying drones were sampled in the afternoon between 3:30 and 4:30 PM. A queen excluder was used to block the entrance of the hive and catch flying drones returning to the hive. Approximately 50 in-hive workers were also sampled and both the workers and drones were stored in 70% ethanol. Three sets of five worker abdomens from each hive sampled were analyzed for *Nosema* spp. as described above. Eight drones from each of the three colonies were dissected to remove the midgut, seminal vesicle, and mucus gland. Prior to dissection, drones were rinsed in distilled water followed by a 5% bleach wash and a final rinse in distilled water. Each gland was analyzed separately by qPCR for *Nosema* spp. analysis.

3. RESULTS

3.1 Brood nest bees from queen rearing hives

We first sought to determine whether in-hive workers taken from the brood nest of queen rearing hives were naturally infected with *N. ceranae*. We found that worker abdomens had varying levels of *N. ceranae* infections. Additionally, in 2010, we examined heads of nurse bees because these contain the brood food glands (hypopharyngeal and mandibular glands). We also found that the heads were infected with *N. ceranae* at low levels (Table 1).

3.2 Immature queens and royal jelly samples

We analyzed larval queens and newly emerged queens to determine whether *N. ceranae* can be transmitted to developing queens, i.e. through brood food. *N. ceranae* was found in royal jelly; however, we cannot exclude that this was due to contamination since royal jelly samples were not decontaminated with bleach. A subsample of royal jelly was used for spore counting, but spores were not observed in any sample. We did find that the larval queens were infected at low levels and that as a trend, the corresponding royal jelly sample from the queen cell was found to have a higher level of *N. ceranae* than the larva (Table 2).

3.3 Newly emerged queens

We found that abdomens, thoraces, heads, ovaries, and spermathecae were infected at low levels with *N. ceranae* (Table 3). Overall trends reflect that abdomens tend to have higher levels of infections compared to other tissues; though in some cases, ovaries were found to have a higher level of infection than the other tissues examined. The spermathecae (n = 7) exhibited a low level infection (*N. ceranae* DNA mean copy number $8.1 \pm \text{SEM } 6.7$), but data were only from

one queen rearing hive in 2009. Since the queens were not mated, this result may have been due to external body contamination as samples analyzed in 2009 were not subjected to a bleach wash. Additionally, the spermathecae were only analyzed from one queen rearing hive and in one year since spermathecae were not dissected in other queens.

3.4 Mated queens

The analysis of mated, laying queens would indicate the prevalence of *N. ceranae* in producing queens and whether infections spread to other tissues such as the ovaries. *N. ceranae* infection levels in older, mated queens were generally lower than those observed in newly emerged queens (Table 4).

Because spermathecae were found to be infected with *N. ceranae* in mated queens, we sampled flying drones from three hives known to have a *N. ceranae* infection. We did this to determine whether the spermatheca was infected due to a queen infection or whether it could have been infected venereally. In-hive workers from the three hives sampled had low levels of *N. ceranae* (Table 5). *N. ceranae* was detected in midguts, seminal vesicles, and mucus glands from flying drones at low levels (Table 5). *N. apis* was detected in three drones, two from hive 1 and one from hive 2. All detections were in midgut samples while no other part of the drone was found infected with *N. apis*.

4. DISCUSSION

Queens are at lower risk for *N. ceranae* infection than other bees because of their role in the hive as the primary female reproductives. They are not responsible for feeding immature bees, cleaning comb, or foraging, so queens are only likely to become infected when attended by

infected bees during feeding or grooming. *Nosema*-infected bees, however, are physiologically older and less likely to attend the queen (Wang and Moeller, 1970), thereby reducing her risk of exposure. Since the heads of nurse bees and royal jelly samples were found to be infected with *N. ceranae*, brood food could provide a mechanism for the horizontal transmission of *N. ceranae* to immature developing bees, including workers, drones, and queens. *N. ceranae* has been found in honey samples (Giersch et al., 2009) and honey may represent an additional reservoir for this parasite, especially because queens may feed themselves from stored honey when they are not laying eggs and before they mate (Free, 1959). While we did analyze all samples for the presence of *N. apis*, no queen samples were positive for *N. apis*.

N. ceranae introduction into other countries has been suggested to be a result of the importation of infected queens and their attendants. For example, the United States and Australia are the primary exporters of queens to Japan, where there are no commercial queen breeders. SSU rRNA gene sequence analysis showed that *N. ceranae* isolates from Japan were identical to the U.S. and Australia (Yoshiyama and Kimura, 2011). Other examples include Finland with the importation of queens from southern Europe (Paxton et al., 2007) and Costa Rica (Calderón et al., 2008). Infected queens could also have been responsible for the spread of *N. ceranae* through Australia; queen attendants and queens shipped from New South Wales were thought to have been the source of infection (Giersch et al., 2009). Honey and pollen could also be a reservoir for *N. ceranae* (Giersch et al., 2009; Higes et al., 2008) although infectivity studies are still needed.

Even though low levels of *N. ceranae* were found in the ovaries, this finding suggests that vertical transmission could contribute and facilitate the spread of *N. ceranae*. When parasites are transmitted vertically, low infection levels would be expected since a decrease in virulence is favored (Fries and Camazine, 2001) which could also explain why high levels in younger queens were not observed. Young queens with high levels of infection may not survive as the queen ages, she may be better able to fight off a *N. ceranae* infection as her vitellogenin and antioxidant capacity increases (Alaux et al., 2011).

Additionally, the spermathecae, seminal vesicles, and mucus glands were found to have low levels of *N. ceranae*, suggesting that mating could also be a transmission route as drones have been shown to be naturally infected (Traver and Fell, 2011b). Evidence exists for venereal transmission of viruses in honeybees as drones, semen, and the spermathecae of queens have been found infected (Chen et al., 2006a; Chen et al., 2005; Chen et al., 2006b; Yue et al., 2006). Whether such transmission leads to infection in a queen and/or vertical transmission through eggs is unknown. Our study sought to determine whether any of the reproductive organs were infected with *N. ceranae* and it would be interesting to examine semen and the stored sperm in a spermatheca to see if the fluid is infected.

While *N. ceranae* infections may not cause major health issues in colony populations, more subtle effects may be occurring with respect to queen health. Queens infected with *N. ceranae* were not weaker as would be expected; instead, infected queens had increased physiological functions. Increases in the vitellogenin titer, the antioxidant capacity, and the queen mandibular pheromone were observed, while the fat body content was unaffected (Alaux et al., 2011).

Higher levels of queen mandibular pheromone in *N. ceranae*-infected queens may reduce the attractiveness of a queen (Richard et al., 2007).

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Table 1. *N. ceranae* mean DNA copy number (\pm SEM) in abdomens and heads of bees taken from the brood nest in queen rearing hives.

Queen Rearing Hive	N ^b	Abdomens	N	Heads
A ^a	6	77727.2 \pm 35769.1	-	-
B	3	188.0 \pm 36.2	-	-
C	6	19815.9 \pm 19812.0	6	19.7 \pm 9.1
D	6	1.6 \pm 0.7	6	7.6 \pm 4.8
E	6	13.7 \pm 10.9	6	8.4 \pm 5.4
Totals	27	19549.3 \pm 11125.8	18	11.9 \pm 6.4

^a Queen rearing hives A and B were from 2009 and C-E were from 2010

^b N represents the number of pooled worker abdomens or heads examined

Table 2. *N. ceranae* mean DNA copy number (\pm SEM) from individual in queen larvae and corresponding royal jelly samples.

Queen Rearing Hive	N ^a	Royal Jelly	N	Larvae
A	7	80.3 \pm 21.8	8	95.0 \pm 15.9
B	10	134.4 \pm 17.3	10	130.2 \pm 64.3
C	10	444.5 \pm 176.1	9	12.1 \pm 11.3
D	8	37.5 \pm 16.3	9	1.0 \pm 0.6
E	10	56.3 \pm 11.2	10	1.0 \pm 0.5
Totals	45	150.2 \pm 48.5	46	47.9 \pm 18.5

^a N represents individual royal jelly or larval queen samples

Table 3. *N. ceranae* mean DNA copy number (\pm SEM) for newly emerged *Apis mellifera* queen tissues.

Queen Rearing Hive	N ^a	Abdomen	Thorax	Head	Ovaries
B	7	55.1 \pm 49.6	0.0 \pm 0.0	0.0 \pm 0.0	3.5 \pm 3.5
C	11	89.0 \pm 148.4	244.5 \pm 177.3	121.5 \pm 80.6	63.8 \pm 32.8
D	11	581.3 \pm 497.6	30.4 \pm 30.4	104.4 \pm 55.4	1332.1 \pm 1308.1
E	10	170.9 \pm 84.2	72.6 \pm 51.7	171.1 \pm 73.2	56.8 \pm 30.9
Totals	39	249.1 \pm 195.0	86.9 \pm 64.8	99.3 \pm 52.3	364.1 \pm 343.8

^a N represents the number of individual tissues examined from each queen from each queen rearing hive

Table 4. *N. ceranae* mean DNA copy number (\pm SEM) in mated queen organs. Organs from each queen were analyzed individually.

Queen Tissue	N ^a	Mean
Abdomen	17	26.6 \pm 15.8
Head	17	22.8 \pm 10.3
Thorax	17	48.4 \pm 32.7
Ovaries	18	42.3 \pm 27.6
Eggs	5	60.8 \pm 48.2
Spermatheca	15	19.0 \pm 7.9

^a N represents the number of individual tissues examined from each mated queen

Table 5. *N. ceranae* mean DNA copy number (\pm SEM) for tissues dissected from flying drones and the abdomens of workers taken from inside the hive.

Hive	N ^a	Drones			Workers	
		Midgut	Seminal vesicle	Mucus gland	N	Midgut
1	8	7.6 \pm 2.7	7.3 \pm 2.6	5.2 \pm 1.8	3	8.2 \pm 4.7
2	8	0.6 \pm 0.2	3.5 \pm 1.2	5.0 \pm 1.8	3	6.1 \pm 3.5
3	8	7.8 \pm 2.9	29.5 \pm 10.4	20.6 \pm 7.3	3	3.7 \pm 1.7
Totals	24	5.3 \pm 1.9	14.4 \pm 4.8	11.0 \pm 3.6	9	5.8 \pm 3.3

^a Tissues from eight drones were analyzed, with the exception of seven for the midgut

CHAPTER 5

Treatment for *Nosema ceranae*- fall fumagillin and winter pollen feeding

The following chapter was formatted to facilitate publication in the journal Apidologie.

Treatment for *Nosema ceranae*- fall fumagillin and winter pollen feeding

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Nosema ceranae control

Abstract

Fumagillin is the only antibiotic approved to treat *Nosema*. Fumagillin has been used to control *Nosema apis* infection and was the first choice for controlling *N. ceranae*. A real-time PCR assay was used to determine whether colonies are infected before any treatments, and if so, to quantify the levels of *N. ceranae* present and establish a baseline level. We found that a fall fumagillin treatment did not significantly lower *N. ceranae* levels 6-months post-treatment. We also tested a non-chemical approach by feeding colonies pollen patties in January and found that supplemental feeding also did not significantly decrease *N. ceranae* levels 3-months post-treatment. Furthermore, both treatments did not significantly improve colony survival. Treating colonies with fumagillin and/or feeding with pollen is time consuming and expensive. We do not recommend either approach as a means to control *N. ceranae*; however, pollen feeding during a time when *N. ceranae* levels are naturally low may improve colony health and assist with spring colony build-up.

1. INTRODUCTION

Fumagillin was isolated in 1949 from *Aspergillus fumigatus* (Hanson and Eble, 1949) and had little antibacterial or antifungal activity, but did have amebicidal activity (Eble and Hanson, 1951; Killough et al., 1952; McCowen et al., 1951). Since that discovery, fumagillin analogs have been used as angiogenesis inhibitors (Ingber et al., 1990), treatment for amebiasis (Griffith et al., 1997; Killough et al., 1952; McCowen et al., 1951), and microsporidiosis in humans (Molina et al., 2002), especially in immunocompromised patients (Didier, 1997). Fumagillin has also been used to treat microsporidiosis in insects (Armstrong, 1976; Hartwig and Przelecka, 1971). Prior to 1951, there were no antibiotics for *Nosema* control in honey bees. Treatment

with fumagillin proved to be very effective at decreasing the number of bees infected with *Nosema apis* and increased the survival of bees despite a continuing, persistent low level infection (Bailey, 1953; Katznelson and Jamieson, 1952).

Many compounds have been tested for treatment of *Nosema* disease but fumagillin is the most effective, non-toxic compound. Natural compounds have been looked at as an alternative to chemical control. Thymol, resveratrol, vetiver essential oil, and lysozyme have been examined (Maistrello et al., 2008), but only thymol and resveratrol decreased the levels of *N. ceranae* and increased longevity in treated bees with both low toxicity and residual activity (Costa et al., 2010). *Lauris nobilis* extract inhibited *N. ceranae* development at low concentration (Porrini et al., 2011a). Nozevit, an herbal preparation in patty form or sugar syrup, has also been examined and has shown to decrease the number of spores present after treatment, but bees were not *Nosema*-free (Gajger et al., 2009a; Gajger et al., 2009b). Recently, surfactant molecules that are cyclic lipopeptides and have antifungal, antibacterial, antiviral, and antimycoplasma activities have been tested and showed promise as there was a decrease in *Nosema* intensity after treatment (Porrini et al., 2010).

Fumagillin targets active infections within the honey bee midgut and not the resistant spores on equipment. Alternative options, both chemical and natural, have been investigated for control and to decrease the viability of *Nosema* spores. Transferring colonies to new comb after feeding has the potential to be a successful non-chemical control method, but can be expensive and does not prevent re-infection (Bailey, 1955). Fumigation of combs with formaldehyde, acetic acid, and formalin are effective and are not toxic to bees (Bailey, 1955). Heat treatment is also

effective at killing spores when heated to 120°F for 24 hours, but a heat chamber is required (Cantwell and Lehnert, 1968; Cantwell and Shimanuki, 1969). Gamma irradiation is also effective (Hornitzky, 1986) as it causes an undeveloped polar filament (Liu et al., 1990). When exposed to freezing temperatures, *N. ceranae* spore viability decreases (Fenoy et al., 2009) suggesting that equipment should be stored in areas that are not heated during the winter. Recently RNA interference has been shown to be effective at inhibiting *Nosema* levels and could be a possible method for control (Paldi et al., 2010). A combination of hygienic treatment for equipment and antibiotic control seem to be the best combinations for combating this disease.

One issue with fumagillin is proper storage. Exposure to light, the temperature at which it is stored, and the type of medium, sugar patty or syrup, used to administer the treatment (Higes et al., 2011) are all factors that affect the efficacy. When stored at 4°C for 24 hours, fumagillin was no longer active and at higher temperatures there was a more rapid degradation (Hartwig and Przelecka, 1971). When exposed to UV, there was also a decrease in concentration of fumagillin (Higes et al., 2011). HPLC analysis showed that fumagillin rapidly degrades after one day at 37°C with intense light, but degrades slower under fluorescent and daylight conditions (Assil and Sporns, 1991). The recommended treatment is 1.5 g fumagillin in a 50% sugar water solution fed weekly in 250 ml portions, four times, and administered in amber containers (Higes et al., 2011). HPLC methods have detected fumagillin in honey down to 100 parts per billion (Assil and Sporns, 1991), but later studies did not detect fumagillin residues in honey (Higes et al., 2011).

Since fumagillin has been successfully used to control *N. apis* infections, it was naturally considered as a control agent for *N. ceranae*. In Spain, fumagillin was shown to control *N. ceranae* when colonies were treated in January, but was not able to prevent re-infection after 4 to 6 months (Higes et al., 2008; Higes et al., 2009; Higes et al., 2011). In Canada, however, fall fumagillin treatment did lower *N. ceranae* levels significantly the following spring (Williams et al., 2008), but follow-up studies were highly variable depending on the beekeeper operation and location (Williams et al., 2011). Also, fumagillin was not effective at controlling *N. bombi* in *Bombus occidentalis*, most likely due to the infection location as it reproduces in the Malpighian tubules (Whittington and Winston, 2003). The inability of fumagillin to control a closely related *Nosema* spp. suggests that fumagillin may not be appropriate for treatment of all *Nosema* spp. such as *N. ceranae*.

Supplemental feeding of bees on different diets has also been tried to reduce *N. ceranae* levels. When comparing three diets (high fructose corn syrup (HFCS) + bee bread; HFCS + amino acids + vitamins; HFCS) there was no significant difference in the mortality of *N. ceranae* infected bees (Porrini et al., 2011b). In bees fed pollen, *N. ceranae* was found to develop more rapidly (Porrini et al., 2011b). Since *N. ceranae* depends on the host's health status, bees fed pollen or given supplements could lead to increased parasite development and spore production. However, an additional study did not find increases in spore production when bees were fed pollen. Feeding pollen prior to inoculation (7 days prior) also did not prevent infection, but these studies were performed in cages and not in the field (Porrini et al., 2011b). Differences in the success of treatment may be due to climatic and geographic differences and/or different haplotypes of *N. ceranae*, which may result in different pathogenicities.

While previous studies have looked at the efficacy of fumagillin treatment for *N. ceranae* (Higes et al., 2008; Higes et al., 2009; Higes et al., 2011; Williams et al., 2011; Williams et al., 2008), all relied on spore counts as indicators of infection. We have previously found that spore counts are unreliable and may not reflect the level of disease in a colony and real-time PCR can be a more sensitive and accurate way to detect infections using this assay (Traver and Fell, 2011). In this study, we sought to determine if a fall fumagillin treatment was successful in decreasing *N. ceranae* levels and improving survival of colonies. We also investigated whether winter supplemental pollen feeding could serve as an alternative to antibiotic control and to improve colony health and colony build-up in the spring to offset the effect of *N. ceranae*.

2. METHODS

2.1 Fumagillin treatment

A total of 16 colonies were treated with fumagillin (Fumagillin-B, Meivet, High River, Alberta, Canada) in the fall of 2010 (October or November 2010). Fumagillin was administered in one gallon feeder pails. A 52% sucrose syrup was used to dissolve 5 grams of fumagillin per gallon. Colonies were treated twice, with two weeks between treatments. Ten of the treated hives were located in Woolwine, VA and the other 6 were located in Richmond, VA. Additionally, at each site, equal numbers of hives were left untreated as controls to compare whether treatments had a significant impact on *N. ceranae* levels and colony survival. Colonies were randomly assigned a treatment or control. Before treatment, random samples of approximately 60 workers were collected from each colony and stored in 70% ethanol. Six months after treatment, colonies were re-sampled to compare pre- and post-treatment *N. ceranae* levels. Post-treatment sampling times were chosen so that sampling would occur at the peak of a natural *N. ceranae* infection.

2.2 Pollen substitute feeding

In January 2011, 15 colonies were fed MegaBee pre-mixed pollen patties (Dadant, Hamilton, IL) as a stimulative feeding approach and alternative to using an antibiotic. Six treated colonies were located in Blacksburg, VA and the other 9 colonies were located in Clover Hill, VA. An equal number of colonies were left untreated as a control group at each location. Random bee samples were collected from each colony at the time of feeding and stored in 70% ethanol. Colonies were re-sampled a second time, 3 months post-treatment to determine if pollen feeding had an impact on *N. ceranae* levels and colony survival. Again, post-treatment sampling times were chosen so that sampling would occur at the peak of a natural *N. ceranae* infection.

2.3 Genomic DNA (gDNA) extraction

Six sets of five worker abdomens (total of 30 worker abdomens) were analyzed from each hive as described previously (Traver and Fell, 2011). Bees were crushed in Bender Buffer and incubated with proteinase K overnight at 55°C. A phenol:chloroform extraction was used followed by an isopropanol precipitation for gDNA purification. gDNA was resuspended in DEPC-treated water overnight at room temperature. Quantification of all gDNA was performed on a Nanodrop2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.4 Real-time PCR (qPCR)

TaqMan assays (Applied Biosystems, Foster City, CA, USA) were used for *N. ceranae* infection levels and species determination as previously described (Traver and Fell, 2011). Two different fluorescent labeled probes were used for the detection of *N. ceranae* and *N. apis*. Standard curve quantitation was used to estimate infection levels.

2.5 Spore analysis

Two sets of ten abdomens (total of 20 abdomens from each colony) were analyzed to estimate a spore count per bee as previously described (Cantwell, 1970).

2.6 Statistical analysis

A non-parametric analysis of covariance (ANCOVA) using the rank transformation was used for analysis as previously described (Conover and Iman, 1982). This analysis was used to determine if there was a significant difference in the *N. ceranae* levels pre-and post-treatment. The pre-treatment *N. ceranae* levels were used as a covariant. JMP 8.0 (SAS Institute, 2010) was used for all analyses.

To determine if there was a difference in survival between fumagillin-treated and non-treated or pollen-fed and non-fed, a χ^2 analysis was performed.

3. RESULTS

The goal was to see if *N. ceranae* levels could be decreased before the height of the infection which starts in April, increases through June, and then starts to decrease (Traver et al, *submitted*, Ch. 2). Spore data were not analyzed statistically because many of the observations had zero spore counts (Figure 1).

3.1 Fall fumagillin treatment

qPCR data demonstrated that *N. ceranae* levels were not significantly lower in the spring following fall treatment ($F = 0.4470$, $df = 1$, $P = 0.5146$; Table 1) and agree with spore data,

except for the untreated colonies, where no spores were observed 6-months post-treatment (Table 1).

3.2 Winter pollen feeding

qPCR data demonstrate that *N. ceranae* levels were not significantly reduced after pollen feeding ($F = 0.5555$, $df = 1$, $P = 0.4647$; Table 2); however, data show that there was an increase in *N. ceranae* levels after pollen feeding, although this increase was not significant. Pre-treatment spore data were only based on 12 colonies (6 fed, 6 unfed) because hives from Clover Hill, VA were too weak to sample larger numbers of bees and the beekeeper only collected small samples, so these hives were only analyzed by qPCR. In control unfed hives, it appeared as though the spore counts increased after three months while spore counts for pollen fed colonies decrease three months post-treatment (Table 2).

3.3 Colony survival and *N. ceranae* levels

Colonies treated with fumagillin in the fall or fed pollen patties in the winter did not have a higher percent survival compared to control untreated/unfed hives ($\chi^2 = 0.570$, $df = 1$, $P = 0.4504$). Colonies fed pollen patties had slightly higher survival rates (73.3%) compared with the controls that were not fed (66.7%), but the survival difference was not significant. The survival was higher in both the control and treated groups in colonies fed pollen in the winter versus those treated with fumagillin (37.5%) or left untreated (58.8%).

4. DISCUSSION

Since *N. ceranae* was discovered recently (Higes et al., 2006; Huang et al., 2007), little was known about this parasite. Treatment approaches have been based on *N. apis* control, but *N. ceranae* and *N. apis* present different diseases. Fumagillin has been used to successfully control *N. apis*; however, we found that treatment of colonies with fumagillin did not reduce *N. ceranae* levels significantly when colonies were re-examined and did not result in significantly higher winter survival with the untreated hives having a slightly higher survival (Table 3). Our study was initiated because previous reports relied on using spore counts and we have shown that spore counts can be unreliable in diagnosing *N. ceranae* infections (Traver and Fell, 2011). With increasing concerns about residues being found in honey and resistance to antibiotics, we also looked into whether winter stimulative pollen feeding could serve as an alternative to fall fumagillin treatment and to increase colony health and spring build-up. We determined that supplemental pollen feeding did not significantly reduce *N. ceranae* levels as compared with control unfed colonies.

Bees are nutritionally stressed in the winter as they sustain themselves on pollen and honey reserves and need to initiate brood rearing. A poor diet in addition to stress may cause them to be more susceptible to diseases. Feeding pollen patties before brood rearing commences may help with spring colony build-up at a time when *N. ceranae* levels are low (Traver et al, Ch. 2, accepted); however, supplemental feeding of pollen patties in January did not lead to significantly lower levels of *N. ceranae* when they were re-examined, and there was only a slightly higher colony survival that was not statistically different. Previous studies have shown that bees fed pollen in the fall did not increase the number of winter bees or increase the protein

content of workers' heads nor did it give them an advantage in the spring (Mattila and Otis, 2006). When pollen was fed to *N. apis*-infected bees, there was an increase in longevity but also an increase in the *N. apis* spore development (Rinderer and Elliott, 1977). Similar results were observed in *N. ceranae*-infected bees fed pollen. *N. ceranae* was found to develop quickly as spore production is probably influenced by food quality of the host so a highly nutritious diet promotes a higher proliferation of the parasite (Porrini et al., 2011b). It was hypothesized that feeding protein increases the size of the midgut, which then provides more surface area for infection, resulting in a higher spore production thus acting as a supplement for both the host and parasite. Our results are consistent with previous findings as we observed higher levels of *N. ceranae*, although not significant, following pollen feeding. While there was no reduction of *N. ceranae* levels when feeding colonies pollen as a supplement, the secondary goal was to improve spring build-up at a time when *N. ceranae* levels are low. Even though the survival of pollen fed colonies was not significantly higher than the control unfed colonies, pollen feeding could help honey bees with spring colony build-up as colonies are stressed once brood rearing starts. Pollen feeding may also help with overall colony health.

Damaging thresholds for *N. ceranae* have yet to be determined. The next step to manage this disease would be to determine what levels cause damage and how best to control the disease. Our studies have found that treatment with fumagilin in the fall and pollen supplement feeding in the winter did not result in a decrease in *N. ceranae* levels. Furthermore, the survival of the colonies was not increased significantly when treatment or feeding was administered suggesting that regardless of *N. ceranae* levels, treatment may not be effective. Testing different

concentrations of fumagillin as well as testing treatments at different times of the year could prove more effective at reducing *N. ceranae* levels.

Previous studies have found no relationship between fall spore counts and winter mortality or a difference in colony strength or mortality in infected colonies treated with fumagillin (Williams et al., 2011). Even as a prophylactic treatment, fumagillin did not increase the survival of colonies nor did supplementing colonies with vitamins and protein in the fall, but this study only determined that *N. ceranae* was present and did not look at the levels, nor did they determine whether *N. ceranae* was present after treatment and if so, at what levels (Pajuelo et al., 2008).

We have observed strong colonies (determined by frames of brood, adult bee population, and surplus honey production) that have very high levels of *N. ceranae*, and these colonies have thrived and showed no symptoms of collapse as previously reported by Spanish researchers (Higes et al., 2008). The low levels of *Nosema* infections observed during the course of this study suggest that *N. ceranae* infections are not causing serious problems with honey bees during either the winter or summer months. We do not find that *N. ceranae* is a major factor, on its own, with colony losses in Virginia; however, whether this parasite has destructive interactions with other parasites, pathogens, or pests remains to be determined. Treatment is expensive and time consuming as it requires multiple trips to the colonies. Depending on the size of the operation, treatment may not be economically feasible. Also, without knowing if a colony is infected and if the levels are high enough to warrant treatment is important in determining a management strategy. This leads us to recommend not treating hives unless high levels have

been observed and other issues are at play (i.e. high mite loads, decreased build up in the spring, viral infection, weak colony strength, fecal streaking indicative of *N. apis*, etc).

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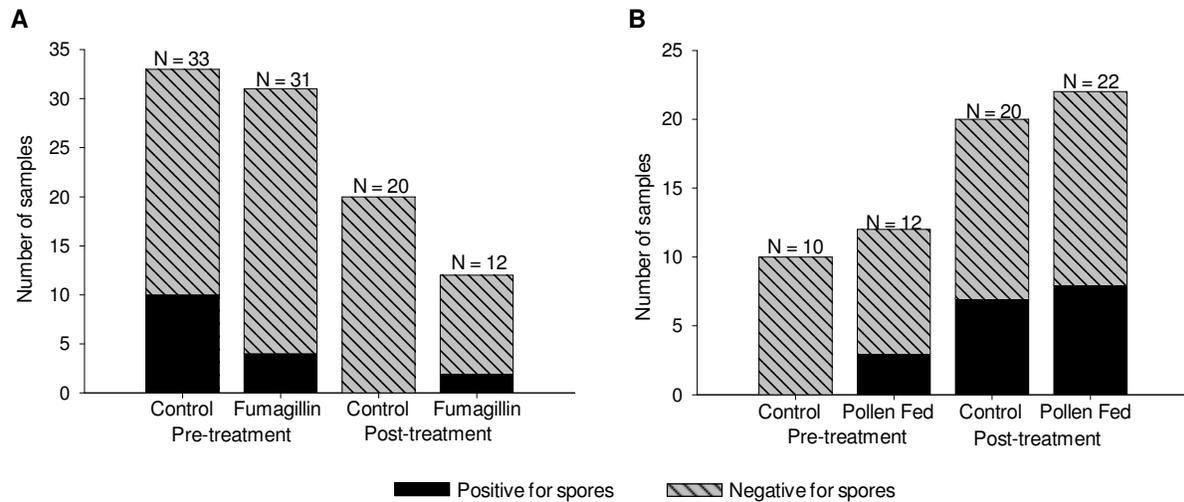


Figure 1. Presence or absence of spores for each treatment. Here N represents the total number of samples spore counted for each treatment. (A) Fall fumagillin feeding. The decrease in sample size was due to colonies that died during the winter. (B) Winter stimulative pollen feeding. The small sample size for pre-treatment sampling was due to no spore counting performed at one sampling location because not enough bees were available for spore counting.

Table 1. Mean *N. ceranae* DNA copy number (\pm SEM) and mean spore counts per bee (\pm SEM) for fall fumagillin treatment.

	Mean copy number				Mean spore count/bee			
	N	Control	N	Fumagillin	N	Control	N	Fumagillin
Pre-treatment	51	$1.5 \times 10^5 \pm 5.8 \times 10^4$	48	$1.2 \times 10^5 \pm 4.5 \times 10^4$	33	$3.9 \times 10^5 \pm 1.7 \times 10^5$	31	$3.2 \times 10^5 \pm 2.3 \times 10^5$
Post-treatment	60	$8.0 \times 10^4 \pm 3.6 \times 10^4$	36	$1.7 \times 10^5 \pm 8.0 \times 10^4$	20	0 ± 0	12	$4.0 \times 10^5 \pm 3.9 \times 10^5$

Table 2. Mean *N. ceranae* DNA copy number (\pm SEM) and mean spore counts per bee (\pm SEM) for winter stimulative pollen feeding.

	Mean copy number				Mean spore count/bee			
	N	Control	N	Pollen Fed	N	Control	N	Pollen Fed
Pre-treatment	72	$4.7 \times 10^4 \pm 2.7 \times 10^4$	84	$5.5 \times 10^4 \pm 2.48 \times 10^4$	10	0 ± 0	12	$2.0 \times 10^6 \pm 1.4 \times 10^6$
Post-treatment	60	$4.2 \times 10^4 \pm 1.1 \times 10^4$	66	$9.9 \times 10^4 \pm 3.85 \times 10^4$	20	$6.7 \times 10^5 \pm 2.7 \times 10^5$	22	$7.8 \times 10^5 \pm 3.1 \times 10^5$

CHAPTER 6

Summary

Nosema ceranae is a microsporidian parasite of *Apis mellifera* that was and is thought to be partially responsible for increased colony losses and a factor in Colony Collapse Disorder. The goal for this research was to determine whether *N. ceranae* is playing a significant role in colony losses in Virginia. *N. ceranae* prevalence and possible routes for transmission were examined to ultimately lead to a better understanding of the parasite and to develop control practices for beekeepers. *N. ceranae* was found year round, with significantly higher levels in April–June and lower levels through the fall and winter. *N. ceranae* was found to infect drones and queens at low levels, and both could contribute to the transmission of *N. ceranae* either horizontally or vertically. Treatment for *N. ceranae* is problematic as the highest infection levels coincide with the peak nectar flow. Neither fall treatment with fumagillin or winter stimulative pollen feeding significantly reduced *N. ceranae* levels 3–6 months post-treatment; this does not mean that levels were not initially reduced, only that reduced *N. ceranae* levels could not be sustained as levels naturally peaked in the spring. Additionally, neither approach improved colony survival. While *N. ceranae* is prevalent year-round and found in all castes, it is not as devastating as previously thought in Virginia. Productive, strong colonies with a surplus honey had some of the highest levels of *N. ceranae* and are currently thriving. Furthermore, these colonies have not been treated for *N. ceranae* suggesting that treatment may not be necessary and that other factors interacting with *N. ceranae* may be responsible for colony losses. Colony losses due solely to *N. ceranae* seem unlikely in Virginia.

Chapter 2

Previous reports using spore counts demonstrated that there was no longer a seasonality for *N. ceranae* infections and that the most accurate way to diagnose an infection is by sampling foragers. Using real-time PCR, we found that there was a seasonality for infections with significantly higher levels in April-June and lower levels in the fall and winter. When sampling bees from various areas within the hive (i.e. brood nest, fringe of the brood nest, the honey supers, and the inner or outer winter cluster), all bees were infected and there was no significant difference in infection levels among the different groups of bees sampled. There was no significant difference between foragers and in-hive bees when using real-time PCR, while in-hive bees had significantly more spores than foragers. This one year study agrees with findings in Germany and a previous study in our laboratory (Gisder et al., 2010; Traver and Fell, 2011).

Chapter 3

The rapid spread of *N. ceranae* suggests that there are additional mechanisms for transmission. Drifting is a potential mechanism for transmission of the disease within and between apiaries. Drones of different ages (pupae, in-hive drones, and flying drones) were analyzed and found to be infected with *N. ceranae*. This is the first report of analyzing immature bees for *N. ceranae*. The highest levels of infection were observed in the flying drones. These drones are the most likely to drift and represent those most likely to transmit *N. ceranae* to other colonies.

Chapter 4

Queen larvae and the corresponding royal jelly, newly emerged virgin queens, and older mated queens could provide alternate transmission mechanisms and were analyzed to detect *N. ceranae* infections and levels. From mature queens, the head, thorax abdomen, ovaries and spermatheca (in mated queens only) were dissected and used for real-time PCR analysis to determine the levels of *N. ceranae*. Queens of all ages were found to be infected. All tissues were infected at low levels. The seminal vesicle, mucus gland, and midgut of sexually mature drones were also analyzed. Low level infections found in the reproductive organs, the spermatheca and ovaries, suggest that vertical transmission could be involved in *N. ceranae* infections.

Chapter 5

Treatment for controlling *N. ceranae* infections is important for decreasing colony losses. Previous studies have shown that fumagillin is effective at controlling *N. ceranae*, but these studies only focused on spore counting. Fumagillin was administered in the fall and did not significantly reduce *N. ceranae* levels 6-months post-treatment. As an alternative to chemical control, winter stimulative feeding with a pollen patty was also tested. Pollen feeding did not significantly reduce *N. ceranae* levels 3-months post-treatment and neither treatment improved colony survival. Due to the time and costs associated with treatment, these results indicate that treatment for *N. ceranae* is not necessary as it will not reduce levels or improve winter survival.

Future research

While drones and queens were found to be infected, studies validating whether they play a role in the transmission of *N. ceranae*, both horizontally and vertically, are still needed. Examination of sperm from drones and stored semen from the spermatheca could help determine whether transovarial or venereal transmission is involved. Since *N. ceranae* has been detected in both the hypopharyngeal glands of workers (Appendix A) and in royal jelly samples (Chapter 4), the feeding of larvae could be an additional mechanism for transmission of *N. ceranae* to all developing bees, including drones and queens; however, follow up studies with negative controls are required.

Neither treatment with fumagillin in the fall or winter stimulative pollen feeding reduced *N. ceranae* levels or improved colony survival when examined 3–6 months post-treatment. Treatment is expensive and requires multiple trips to an apiary, and depending on how many colonies there are, can be time consuming. In our study, treatment may not have been effective because infection levels were not high enough to see a difference. Damaging levels of *N. ceranae* have yet to be determined so that treatment decisions can be made. Currently, all recommendations for treatment are based on *N. apis* infection levels. While there are recommendations on the label for the use of fumagillin in the United States, standardized dosing and administration of fumagillin should be performed in order to compare efficacy across studies. Even though the highest levels of *N. ceranae* were observed during the peak nectar flow, treatment with fumagillin in March may be beneficial for weak colonies. With weak colonies, survival is the primary concern and no surplus honey would be expected so any residual fumagillin in the honey would not be an issue. Winter stimulative pollen feeding at a

time when *N. ceranae* levels are naturally low may be beneficial for overall colony health and for early spring colony build-up. Follow up studies monitoring colony health would be required to determine if pollen feeding provided an advantage for spring colony build-up.

Although this was outside of the scope of this project, sequencing and comparisons of *N. ceranae* isolates from different geographic locations are important and need to be examined. If there are different haplotypes associated with certain geographic regions, it could help explain the different pathologies observed. Also, it is important to understand the interactions between different strains of *N. ceranae* and how they interact with their host, especially in different honey bee species and subspecies.

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APPENDIX A

In situ hybridization for the detection of *Nosema ceranae* in honey bee tissues

Introduction

Two important glands involved with feeding, the mandibular and hypopharyngeal glands, are located in the honey bee head. The mandibular glands are located at the base of the jaw and are sac-like structures attached to each of the mandibles by a duct (Ribbands, 1964; Winston, 1987). In workers, this is one of the glands responsible for producing and secreting brood food, either royal and/or worker jelly in bees of brood rearing age. The hypopharyngeal glands are the largest glands in the head located in the anterior part of the head on the dorsal surface of the brain (Ribbands, 1964). Each gland is made up of lobes and lobules with two collecting ducts that open into the pharynx (Hassanein, 1952). Each acinus contains 8-12 granular cells that are all connected to a duct cell (Deseyn and Billen, 2005). The size of acini changes with age, as does the amount of secretion which is positively correlated with acini size (Deseyn and Billen, 2005). These glands also produce and secrete brood food.

Proper amounts of protein are required for the development of the hypopharyngeal glands so bees must consume enough protein within their first two weeks of life (Deseyn and Billen, 2005; Free, 1961). Bees fed only carbohydrates after emergence did not develop their hypopharyngeal glands and became foragers earlier, probably because they skipped their nursing jobs due to lack of gland development (Free, 1961).

The activity of these glands are dictated by worker bee age and the presence of larvae, but not by eggs and pupae, which helped develop the hypothesis that feeding behavior induces gland

activation (Huang and Otis, 1989). Gland activity increases during the first three days after emergence, with the most activity when rearing brood, and a decrease with the onset of foraging or no brood present (Huang and Otis, 1989). Hypopharyngeal glands are larger in younger bees that are actively producing brood food while in older workers the glands resorb, but can be activated if required (Free, 1961).

Brood food is made up of proteins, lipids, sugars, cholesterol, amino acids, vitamins, and water (Deseyn and Billen, 2005). Royal jelly is made up of proteins (12.34%), carbohydrates (12.49%), lipids (5.465), and minerals (0.82%) with the remaining part being moisture (66.05%) (Liu, 1990). Nurse bees feed developing larvae from food produced in the hypopharyngeal and mandibular glands. A clear substance is secreted from the hypopharyngeal glands which may be combined with honey, digestive enzymes, water, and a milky white substance from the mandibular gland (Winston, 1987). Worker larvae are fed 20-40% of the white substance and 60-80% from the clear substance for the first two days as larvae (Winston, 1987). After that, the amount of mandibular food decreases and most food is from the hypopharyngeal glands. The amount and quality of brood food fed to female larvae will determine whether they develop into queens or workers.

In honey bees infected with *Nosema apis*, there are both behavioral and physiological changes. There is a positive correlation between the number of spores in the midgut and the degradation of hypopharyngeal glands (Wang and Moeller, 1969). *N. apis*-infected bees have malformed glands with smaller lobes (Hassanein, 1952), decreased volumes of the lobules, disintegrated nuclei, condensed chromatin, a decrease in size of the cytoplasm, and smaller secretory globules

(Wang and Moeller, 1969). The shape changes from grape-like clusters to irregular formations and the length of the secretory cycle is decreased (Wang and Moeller, 1969). In *N. apis*-infected bees, foraging begins earlier (Wang and Moeller, 1970) and the hypopharyngeal glands become atrophied (Wang and Moeller, 1971). In the atrophied glands, the secretion mixture was still present but at lower levels versus healthy bees and could represent a change in the quality of the secretion mixture (Liu, 1990). By 10 days post-infection, glands were severely affected with almost complete gland disruption and no royal jelly secretion (Wang and Moeller, 1971). Since the secretory cycle becomes disrupted, it may explain why diseased workers do not feed the queen which would be a behavioral not a functional change.

Nosema ceranae has been detected in tissues other than the midgut, such as the hypopharyngeal glands, the salivary glands, the mandibular glands, and the malpighian tubules (Chen et al., 2009; Gisder et al., 2010a). Spores have not been observed in these tissues yet through PCR, *N. ceranae* has been detected. If *Nosema* spp. can be transmitted from cell to cell by a vegetative stage *in vitro* (Ishihara, 1969; Kawarabata and Ishihara, 1984; Kurti et al., 1983), it is possible that this type of infection could occur *in vivo*. This has not been observed for *N. apis* where the primary infective source are spores and merozoites which do not infect intracellularly (Fries, 1989). Intracellular germination of spores has been observed which could explain the rapid intercellular spread where the first spores produced germinate within the cell and spread to adjacent cells (Fries et al., 1992). Infected workers could secrete contaminated royal jelly and possibly transmit *Nosema*.

In situ hybridizations have been used to identify chronic bee paralysis virus (Olivier et al., 2008), American foulbrood (Yue et al., 2008), and *Nosema ceranae* (Gisder et al., 2010b) infections in

honey bee tissues. Since *N. ceranae* has been detected in the heads via PCR, I wanted to use an in situ hybridization, as an alternative to transmission electron microscopy, to see if *N. ceranae* vegetative stages could be detected in the hypopharyngeal glands.

Materials and methods

All bees used for this objective were from colonies sampled from Objective 1. Honey bee hypopharyngeal glands were dissected from bees sampled from three different hives and from different areas within the hive. All hives used were previously known to have high levels of *Nosema* infection at the time of sampling (based on previous results, Chapter 2).

Fixation and embedding

Whole tissues were dissected and fixed in 4% paraformaldehyde in 1.5 ml centrifuge tubes for 24 hours at 4°C. The samples were rinsed three times in DEPC-treated water and then stored in 70% ethanol. Samples were shipped to the Beltsville USDA-ARS Bee Laboratory and processed by Judy Chen. Tissue dehydration was carried out by successive incubation in ethanol (70%, 95% and 100%) and xylol (2 x 5 min each) and embedded in paraffin. Paraffin sections were cut (10 um) and mounted on poly-L-lysinated slides and stored at 4 °C overnight. The sections were then rehydrated through a descending ethanol (100% and 95%) and xylol and incubated with proteinase K (10 ug/ml) for 30 minutes post-fixed and acetylated with 0.33% (v/v) acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) prior to hybridization.

In vitro transcription of DIG-labeled probe

Purified *N. ceranae* specific amplicons (Chen et al., 2009. Journal of Invertebrate Pathology 101:204–209) were individually incorporated into a pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Recombinant plasmids were linearized with *Bam*HI (New England Biolabs, Ipswich, MA) at 37 °C for two hours. The linearized DNAs were extracted once with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated by ethanol and dissolved in nuclease water. The DIG-labeled RNA probe complementary to *N. ceranae* genomic DNA was synthesized using DIG-RNA Labeling Kit (T7) (Roche Applied Science, Indianapolis, IN) following manufacturer's protocol.

In situ hybridization

Tissue sections were pre-hybridized in pre-hybridization solution (50% formamide, 5X SSC, 40 ug/ml salmon sperm) at 58 °C for two hours. Tissues were then hybridized with the probe solution at a concentration of 200-400 ng/ml probe in pre-hybridization solution at 58 °C overnight. After hybridization, the sections were washed twice in a low stringency wash solution (2X SSC, 0.1 % SDS) at room temperature for five minutes and then washed twice in high stringency wash solution (0.1 X SSC, 0.1 % SDS) at 52 °C for 15 minutes. The hybridization signals were detected with Alkaline phosphatase (AP)-labeled sheep anti-DIG antibody conjugate (Roche Applied Science). The conjugate solution was added to the dry sections and incubated at 4 °C for two hours in a humid chamber. The slides were rinsed three times with washing buffers. The color development was performed by adding the buffer solution containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP) on the tissue sections and incubated for three to six hours at room temperature in the dark. The color

reaction was stopped by a 5-minute wash in Tris/EDTA (0.1 mM, pH 8.0). The non-specific staining was removed in 95% ethanol overnight. The sections were rehydrated for through successive incubation in ethanol (70%, 95%, and 100%) and xylol (2x15 min each), and mounted in Eukitt resin.

Microscopy

In situ hybridization slides were observed under a light microscope (Nikon, Eclipse TE 300) and photographed with a Nikon Digital Camera (DXM 1200). The hybridization signals were shown by purple to blue color sites where the DIG-dUTP labeled probe had bound to *N. ceranae* DNA.

Results

In situ hybridization

The *in situ* hybridization using a DIG-dUTP labeled probe against *N. ceranae* was successful. The images shown in Figure 1 demonstrate that *N. ceranae* is present in the hypopharyngeal glands. While all of the hypopharyngeal glands were positive for *N. ceranae*, we had hoped that Sample A would serve as a negative control, but as is shown in Figure 1, Sample A also had *N. ceranae* in the hypopharyngeal glands. With the high prevalence of *N. ceranae*, it is difficult to find a *N. ceranae* negative bee.

Real-time PCR and spore counting

qPCR results demonstrated that the corresponding abdomens from the dissected heads had very low level infections with an average DNA copy number of 0.84 ± 0.36 (standard error of the

mean). This is interesting because two samples were negative by qPCR for *N. ceranae* yet there was a signal using an in situ hybridization. Abdomen lysates from the first round of phenol:chloroform extraction were used for spore counting. Excess phenol was solubilized by adding 95% ethanol prior to spore counting. No samples had any spores present.

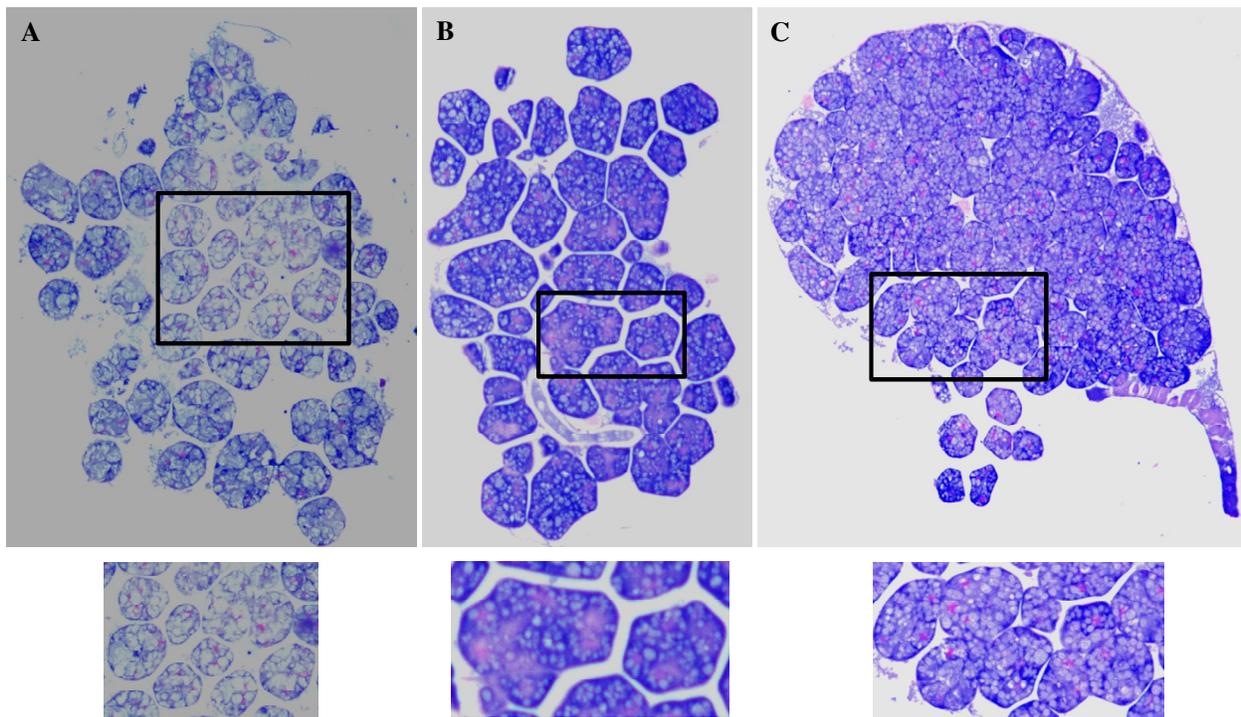


Figure 1. Hypopharyngeal glands from three honey bees. The images on top show the entire slice of tissue while the smaller images are enlarged areas within the black box. The purple color indicates where the Nosema-DIG labeled probe was able to hybridize indicating *N. ceranae* was present in the tissues processed. (A) A From a colony (KF 67) sampled in December 2009 from the inner part of the winter cluster (B) From a colony (PF 1) sampled in April 2010 from the honey supers (C) From a colony (MF 79) sampled in June 2010 from the brood nest.

Detection of *N. ceranae* in the hypopharyngeal glands of honey bees is interesting. Furthermore, because there are no spores present, a vegetative life stage present in the glands is the most likely form of *N. ceranae*. This would help explain why royal jelly is often found to be positive with *N. ceranae*. Further research needs to be done to use bees, or tissues that are not infected with *N.*

ceranae as negative controls, especially since two out of the six samples analyzed were negative by qPCR yet positive by in situ hybridization.

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