DISTRIBUTION OF MICROSPORIDIA, *NOSEMA* SPP., AND CO-INFECTION WITH ACARINE PARASITES IN PACIFIC NORTHWEST HONEY BEE (*APIS MELLIFERA* L.) COLONIES.

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of MATTHEW DIXON SMART find it satisfactory and recommend that it be accepted.

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Abstract

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Two species of *Nosema*, *Nosema apis* Zander and *Nosema ceranae* Fries are known to infect the western honey bee, *Apis mellifera*. Spores of both species are ingested and infect and reproduce in the epithelial cells lining the midgut, decreasing bee longevity and colony strength, particularly over the winter. One species, *N. ceranae*, was first identified in 1996 infecting the eastern honey bee, *Apis cerana* F. Since 2006, *N. ceranae* has been detected in populations of *A. mellifera* around much of the world including North and South America, Europe, Asia, Africa, and Australia. This initial appearance of *N. ceranae in A. mellifera* coincided with the sudden disappearance of honey bees in the U.S. and Europe characterized by the absence of the majority of the worker bees and the presence of the queen and healthy brood. Due to its high virulence and persistence throughout the year, *N. ceranae* was initially implicated as a potential cause of Colony Collapse Disorder (CCD). Since that time the list of factors potentially contributing to CCD has continued to grow to include exposure to chemicals in and outside the hive, poor nutrition, *Varroa destructor* mites, viruses, and low genetic variability, with no one factor solely predicting the occurrence of CCD.

The focuses of this research were to (1) determine the distribution of *Nosema* in Washington and the PNW by sampling stationary and migratory beekeeping operations and quantifying infection levels and *Nosema* species identity in the apiaries, (2) determine the infection levels and proportion of *N. ceranae*-infected individuals in samples as cohorts of *A. mellifera* aged, and (3) study whether colony level inoculation with *N. ceranae* and co-infection with *V. destructor* mites affects honey bee colony health through the summer and the following spring.

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Dedication

This thesis is dedicated to my mother and father who have always thoroughly supported my educational pursuits and taught me to follow my dreams.

Molecular detection and distribution of *Nosema* spp. in Pacific Northwest stationary and migratory honey bee (*Apis mellifera L.*) colonies

Summary

Two groups of colonies were sampled monthly from April 2008 – April 2009 to determine infection intensity, proportion of colonies infected, and species identity of *Nosema* present. One stationary apiary of 20 colonies was located on the campus of Washington State University in Pullman, WA. The other group of colonies was embedded in a migratory operation, undergoing the usual practices of the migratory beekeeper using the colonies for pollination services and honey production. Additionally, samples sent by beekeepers from around the Pacific Northwest (PNW) were tested for the presence and intensity of *Nosema* infection. Samples from stationary and migratory apiaries exhibiting spores of *Nosema* spp. were analyzed using a PCR-based method for *Nosema* species determination. Results indicated that *N. ceranae* was the dominant species, present in all months and in 97% of the samples tested. *Nosema apis* was detected three times, twice of which occurred in mixed infections with *N. ceranae*. The general trend in seasonal distribution showed that *N. ceranae* increased over the winter into the spring (particularly in the WA stationary colonies) and subsequently decreased through the summer and fall. WA stationary colonies and WA migratory colonies differed in the time of peak infection level and proportion of colonies infected.

Introduction

Two species of microsporidia are known to infect honey bees, *Nosema apis* Zander (1909) and *Nosema ceranae* Fries et al. (1996). Both species are obligate intracellular parasites, infecting the epithelial cells of the honey bee midgut (Larsson 1986). Once ingested, the spores germinate in the lumen of the midgut of adult honey bees and rapidly produce more spores intracellularly (Bailey 1955; Fries 1988; Fries 1989; Fries et al.1992). *Nosema* spores are thought to be acquired by young house bees when they take up and remove fecal deposits, dead and diseased bees, and other foreign or contaminated materials from the hive (Fries 1997). Spores may also be ingested via contaminated food and at common water sources (Fries 1997).

Nosema apis is able to complete its developmental cycle within 36 hours of inoculation (Fries et al. 1992), and spores of both species build up to extremely high levels in the lumen and within the midgut epithelial cells. Foragers inoculated with 10^4 spores had detectable *N. apis* spores three days later, and developed a ten-fold increase in spore load by day seven (Higes et al. 2007a). High levels of infection ultimately decrease the life-spans of infected bees (Fries 1988; Fries et al. 1992; Higes et al. 2007a; Higes et al. 2007b; Paxton et al. 2007, Woyciechowski and Moron, 2009), and are also known to cause higher supercedure rates and greater overwintering losses than in uninfected colonies (Farrar 1942). Elevated infection levels may also severely interfere with bees ability to absorb nutrients (Malone and Gatehouse 1998).

In 1996, a new *Nosema* species, *N. ceranae*, was identified parasitizing the eastern honey bee, *Apis cerana*, in China (Fries et al. 1996). Since 2007, *N. ceranae* has been found in populations of the western honey bee, *A. mellifera*, throughout much of the world including the U.S., Canada, South and Central America, Europe, North Africa, and Australia (Higes et al. 2006; Huang et al. 2007; Klee et al. 2007; Paxton et al. 2007; Calderon et al. 2008; Chen et al.

2008; Williams et al. 2008; Giersch et al. 2009; Higes et al. 2009). The current distribution of *N. ceranae* worldwide appears to be the result of a host switch or range expansion, although when this event occurred remains unclear (Chen et al. 2008; Klee et al. 2007; Paxton et al. 2007). However, recent evidence suggests that *N. ceranae* existed in U.S. managed honey bee populations as far back as the 1990s (Chen et al. 2008).

Cage studies have shown *N. ceranae* to be more virulent than *N. apis* (Higes et al. 2007a; Paxton et al. 2007) and Martin-Hernandez et al. (2007) found it to have a more sustained presence in the hive throughout the year, thus alarming beekeepers and scientists alike to the potential negative consequences of a new omnipresent microsporidian parasite of honey bees. The relatively recent detection of *N. ceranae* in populations of *A. mellifera* implicated it as a potential contributing factor to Colony Collapse Disorder (CCD) (Higes et al. 2007a; Martin-Hernandez et al. 2007; Cox-Foster et al. 2007).

In the Pacific Northwest, stationary over-wintering colonies of *A. mellifera* are confined within the hive and remain clustered for extended periods of time. Traditionally, the effects of *N. apis* abate as temperatures increase and bees are able to leave the confines of the hive to defecate outside (Fries 1997). Indeed, previous surveys to detect the distribution of *N. apis* suggest that it follows a seasonal trend, reaching its highest intensity (or mean infection level) and proportion of colonies infected in midwinter to spring, although it was detected in most of the months sampled (Farrar 1944; Jaycox 1960; Duoll and Cellier 1961; L'Arrivee 1963; Taber and Lee 1973; Pickard and El-Shemy 1989). On the other hand, migratory honey bee colonies providing pollination services in California and the PNW are not exposed to a prolonged time of freezing temperatures which require bees to cluster inside the hive, and seasonal variations of *Nosema* could therefore differ between the two types of beekeeping.

Migratory colonies are subjected to a very different management style than that for stationary honey bee colonies. Colonies are loaded onto trucks in November and transported south from the Pacific Northwest to overwinter in holding yards in California where they are fed sugar syrup and pollen supplementation, and treated for elevated mite infestations and *Nosema* infections in preparation for almond pollination. Colonies are placed in orchards during the bloom in January-February and subsequently moved north on trucks in spring to pollinate cherries, apples, pears, cranberries, blackberries, alfalfa, onion seed, canola, carrot and several other crops in Oregon and Washington. Some of these colonies continue their migratory treks eastward in summer to produce honey in the Dakotas and Montana.

If *N. ceranae* truly is a more persistent and virulent pest of honey bees than *N. apis*, understanding its seasonal distribution, prevalence, and intensity year round in both stationary and migratory colonies is vital to effectively manage it. Furthermore, comparing seasonal distributions in two different kinds of beekeeping operation could indicate factors that are similar and contrasting between the two types of beekeeping. The focus of the current study was to track *Nosema* in Pacific Northwestern stationary and migratory colonies to gain a clearer understanding of the presence and distribution of *Nosema* throughout the year.

Materials and Methods

Sample sources

Twenty stationary colonies located on the WSU campus in Pullman, WA (WA stationary) and a group of colonies imbedded in a large migratory beekeeping operation (WA migratory) were sampled monthly between April 2008 and May 2009. Stationary colonies were not chemically treated for pests or parasites, while the migratory colonies were treated according

to the usual practices of the beekeeper, including spring and fall treatments for mite and *Nosema* control.

Additional samples submitted to the WSU honey bee diagnostic lab from stationary and migratory apiaries in the PNW (PNW stationary and migratory – Table 1) were tested for the *Nosema* intensity and, if present, identified to species. These colonies were sampled only once, providing a snapshot of the current parasite load of the colony.

Approximately 300 adult worker bees were collected into 70% ethanol from the inner hive cover and outermost frames of the top box of the colony. Sampling in this location is common and results in a composite sample comprised of adult bees of varying ages and *Nosema* infection levels.

Spore quantification

Abdomens from 50 bees from each colony sample were macerated in 50 ml of distilled water using a mortar and pestle to release the spores from the midgut into the suspension. The suspension was then centrifuged and the pellet containing the *Nosema* spores was re-suspended in 50 ml of distilled water in a beaker and a magnetic stir bar was used to maintain thorough mixing. Ten µl of the solution was pipetted onto a hemacytometer which standardized the volume of liquid in a sample to approximate the average number of spores (Cantwell 1970). Spores were observed and counted under light microscopy at 400x magnification to estimate the number of spores per bee. Subsamples of 1ml per colony were kept for DNA extraction, polymerase chain reaction (PCR), and restriction digestion for species identification.

Molecular identification of *Nosema* spp.

Nosema DNA was extracted according to Higes et al. (2006) using a High Pure PCR template Preparation Kit (No. 179682 Roche Diagnostic). Primers, ssu-res R1 and F1, from Klee et al. (2007), were used to amplify a portion of the 16S small subunit (SSU) rRNA gene. This sequence is highly conserved in *Nosema* (Higes et al. 2006). PCR products were electophoresed to ensure amplification of the *Nosema* DNA, then stained with ethidium bromide. Identification of species, *N. ceranae* and *N. apis* (Figs. 1 and 2), relied on digestion of the amplified DNA with restriction enzymes Pac1 and Nde1, respectively, which yielded different fragment lengths for *N. ceranae* and *N. apis* (Klee et al. 2007) (Fig. 1).

Results

DNA analysis

Samples that yielded a definitive *Nosema* species determination are listed in Table 1. Many more samples were analyzed, but species could not be determined beyond the generic level or DNA was not present at all. *Nosema ceranae* was the predominant species identified in the samples analyzed, present in all months sampled, and the only species identified in stationary apiaries (WA stationary and PNW stationary). Two samples from a migratory beekeeper in WA tested positive for a mixed infection of *N. ceranae* and *N. apis* (Table 1). Additionally, a sample from Oregon was submitted without location data and tested positive for *N. apis*, although it was not possible to know whether the source was from a migratory or stationary beekeeper.

Monthly sampling

Figure 3 depicts the intensity (mean monthly *N. ceranae* infection levels) and monthly proportion of infected colonies across all colonies in the WA stationary apiary. The highest intensity and prevalence in the stationary colonies occurred during April-May of 2008 and 2009.

In May 2008, 95% of the colonies were infected with *N. ceranae* at a mean intensity of $6.3x10^6$ spores per bee. Subsequently, the intensity and proportion of colonies infected decreased naturally (no chemical controls were used) throughout the summer, falling to their lowest level in August and September with only 6.0% of the colonies infected at a mean intensity of $2.0x10^5$. By May 2009, 100% of these colonies were infected at the highest intensity recorded, $1.6x10^7$ spores per bee. Both intensity and the proportion of colonies infected with *N. ceranae* followed a similar trend in the WA stationary apiary (Fig. 3).

Figure 4 depicts the monthly infection levels and proportion of colonies infected in the WA migratory operation. Peak intensity ($1.5x10^6$ spores per bee) and proportion of colonies infected with *Nosema* (100%) occurred in February 2008, earlier than in the WA stationary apiary. The lowest intensity was $2.0x10^4$ spores per bee in August 2008, and September had the lowest proportion of colonies infected (8.0%).

The peak intensity level was 10 times lower in the migratory colonies (Fig. 4) than in the stationary colonies (Fig. 3). Colonies in the migratory operation received spring and fall treatments of fumagillin, which could have accounted for this lower level of infection. An antifungal agent produced by the fungus, *Aspergillus fumigatus*; fumagillin interferes with the vegetative growth of *Nosema* inside the epithelial cells of the honey bee midgut, though has no known activity against intact spores in the midgut or environment.

Discussion

Molecular data indicated that the dominant species of *Nosema* in the PNW colonies sampled was *N. ceranae*. It remains unclear how long *N. ceranae* has been present in the region and to what extent it has outcompeted or displaced *N. apis.* Although *N. apis* was not widely

detected in the current study, it may persist at low levels or in specific areas or times of the year in the region.

The Washington stationary apiary was sampled monthly throughout the winter, when *Nosema* levels were at their highest, while most migratory and other PNW stationary colonies were not sampled monthly. More extensive sampling is needed throughout the year, including the winter, to confirm the status of *N. apis* in the region and throughout the range of *A. mellifera*.

There appeared to be a seasonal pattern to colony level *N. ceranae* intensity levels and proportion of colonies infected in the stationary WA colonies. Both factors increased over the winter and declined during the summer months (Fig. 2). These finding are comparable to those previously recorded for *N. apis* (Farrar 1944; Jaycox 1962; Duoll and Cellier 1961; L'Arrivee 1963; Taber and Lee 1973; El-Shemy and Pickard 1989).

A similar pattern was observed in the migratory colonies (Fig. 3), though the peak intensity and proportion of bees infected occurred earlier, in February 2009, which in California is very similar to Washington in April-May. The lack of increase in intensity in the fall, and the overall lower intensity levels in the migratory colonies, could have been due to the fall application of fumagillin for the control of *Nosema,* or the lack of prolonged cold temperatures (during overwintering in California). *Nosema* infection in both treated and untreated colonies showed decreases in intensity through the summer and a general increase through the winter.

Despite apparent seasonal changes, *N. ceranae* was detected in all months sampled both in the WSU stationary apiary and in the migratory colonies (Table 1, Figs. 2 and 3) which was similar to the findings of Martin-Hernandez et al. (2007). Persisting throughout the year is one of the serious concerns associated with *N. ceranae,* i.e., that it does not decrease to undetectable levels after winter buildup as does *N. apis*. This persistence at low levels throughout the season

could permit *N. ceranae* to increase rapidly to detrimental levels over the winter or provide a competitive advantage over *N. apis.*

The reasons for the decline of *N. ceranae* through the summer months could be related to several factors. As the colony develops through the spring, long-lived, highly-infected overwintering bees die, replaced by shorter-lived summer bees. These summer bees live only a matter of weeks as opposed to the months that winter bees persist, which may not allow enough time for the parasite to reach injurious levels inside the midgut. Additionally, "cleansing flights" to defecate outside the hive are taken, thus ending continued accumulation of fecal deposits inside the hive. Fresh pollen and nectar are also collected and eaten at this time, improving the nutritional status and overall health of the colony.

Colonies unable to completely purge themselves of the parasite (as may be the case with *N. ceranae*) could be adversely affected by the decreased longevity and premature loss of infected workers throughout the season (Mayack and Naug 2009; Woyciechowski & Moron 2009; Kralj and Fuchs 2010), though Amdam and Omholt (2003) found that around 80% loss of foragers needed to occur to significantly reduce the age of onset of foraging in a colony. As the queen increases egg production to replenish the workforce, further nutritional stress is placed on the colony. Such colonies are forced to enter the upcoming fall and winter in an infected and weakened state that will only decline further as the winter progresses. Depending on the level of infestation of *V. destructor* and other pests and pathogens, the amount and quality of stored food and the *Nosema* load that is carried into the winter, colonies that otherwise seem healthy could be on a path toward winter and spring dwindling.

Analyzing DNA from archived honey bees could add to our knowledge of the history of *N. ceranae* infection in the PNW and elsewhere, and could potentially show the progression from

N. apis to *N. ceranae* as the primary microsporidian parasitizing PNW honey bees. Researchers have already identified *N. ceranae* in samples dating to the 1990s in the U.S. (Chen et al. 2008), and its initial introduction likely precedes that date. Additionally, these data could be used to study shifts in seasonality of *Nosema* associated with the appearance of *N. ceranae*, if they exist.

Since its implication in CCD in 2007 (Cox-Foster et al. 2007), much work has been done on *N. ceranae* to understand its singular and combined effects on honey bee colony health (Higes et al. 2007a; Alaux et al. 2009; Mayack and Naug 2009; Kralj and Fuchs 2010). vanEnglesdorp et al. (2009) analyzed 61 variables including parasite and pathogen loads, physiological status, and pesticide levels and found that no single measure was the likely cause of colony collapse. Instead, colonies appearing with signs of CCD proved to have a broader array of pathogens at higher loads. These findings underscore the importance of first understanding the basic biology and distribution of various pathogens and parasites in honey bees to predict when they cause colony weakness and mortality. Additionally, research studying the effects and outcomes of additive and synergistic interactions among colony stressors is needed to better understand their role in colony health.

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Location	County	Date	n tested	*Nosema* spp.	beekeeping type
WA stationary	Whitman Co., WA	4/10/2008	3	*N. ceranae*	stationary
	Whitman Co., WA	5/27/2008	7	*N. ceranae*	stationary
	Whitman Co., WA	6/13/2008	1	*N. ceranae*	stationary
	Whitman Co., WA	6/27/2008	8	*N. ceranae*	stationary
	Whitman Co., WA	7/16/2008	8	*N. ceranae*	stationary
	Whitman Co., WA	7/28/2008	6	*N. ceranae*	stationary
	Whitman Co., WA	8/13/2008	5	*N. ceranae*	stationary
	Whitman Co., WA	10/30/2008	1	*N. ceranae*	stationary
	Whitman Co., WA	11/30/2008	2	*N. ceranae*	stationary
	Whitman Co., WA	1/7/2009	4	*N. ceranae*	stationary
	Whitman Co., WA	1/28/2009	10	*N. ceranae*	stationary
	Whitman Co., WA	2/28/2009	5	*N. ceranae*	stationary
	Whitman Co., WA	3/28/2009	3	*N. ceranae*	stationary
WA migratory	Stanislaus Co., CA	2/1/2008	2	*N. ceranae*	migratory
	Stanislaus Co., CA	3/1/2008	1	*N. ceranae*	migratory
	Yakima Co, WA	4/2/2008	1	*N. ceranae*	migratory
	Grant Co., WA, ND	7/31/2008	1	*N. ceranae/N. apis*	migratory
	Grant Co., WA, ND	8/31/2008	1	*N. ceranae/N. apis*	migratory
	Yakima Co, WA	10/31/2008	2	*N. ceranae*	migratory
	Stanislaus Co., CA	3/1/2009	1	*N. ceranae*	migratory
	Stanislaus Co., CA	3/7/2009	4	*N. ceranae*	migratory
	Stanislaus Co., CA	3/22/2009	1	*N. ceranae*	migratory
PNW other	Clallam Co., WA	5/27/2008	4	*N. ceranae*	stationary
(stationary &	Pierce Co. WA	6/13/2008	1	*N. ceranae*	stationary
migratory)	Clallam Co., WA	7/1/2008	1	*N. ceranae*	stationary
	Payette Co., ID	2/1/2009	1	*N. ceranae*	migratory
	Skagit Co., WA	2/19/2009	1	*N. ceranae*	stationary
	Grande Prairie, Co, AB	2/25/2009	9	*N. ceranae*	stationary
	Stanislaus Co., CA	3/13/2009	1	*N. ceranae*	migratory
	Fresno Co., CA	3/20/2009	1	*N. ceranae*	migratory
	Skagit Co., WA	4/4/2009	1	*N. ceranae*	stationary
	Skagit Co., WA	4/11/2009	1	*N. ceranae*	stationary
	Pierce Co. WA	4/16/2009	2	*N. ceranae*	stationary
	Pierce Co. WA	4/22/2009	1	*N. ceranae*	stationary
	Snohomish Co., WA	4/26/2009	1	*N. ceranae*	stationary
	Payette Co., ID	5/7/2009	1	*N. ceranae*	migratory
	Snohomish Co., WA	5/18/2009	1	*N. ceranae*	stationary
	Snohomish Co., WA	5/19/2009	1	*N. ceranae*	stationary
	Kitsap Co., WA	6/2/2009	1	*N. ceranae*	stationary
	Spokane Co., WA	6/12/2009	1	*N. ceranae*	stationary
	Kitsap Co., WA	6/18/2009	1	*N. ceranae*	stationary
	Canyon Co., ID	7/1/2009	1	*N. ceranae*	migratory
	Oregon	09/2009	1	*N. apis*	-
	Oregon	09/2009	1	*N. ceranae*	-
	Oregon	09/2009	1	*N. ceranae*	-

Table 1. Summary of samples tested for *Nosema* identification.

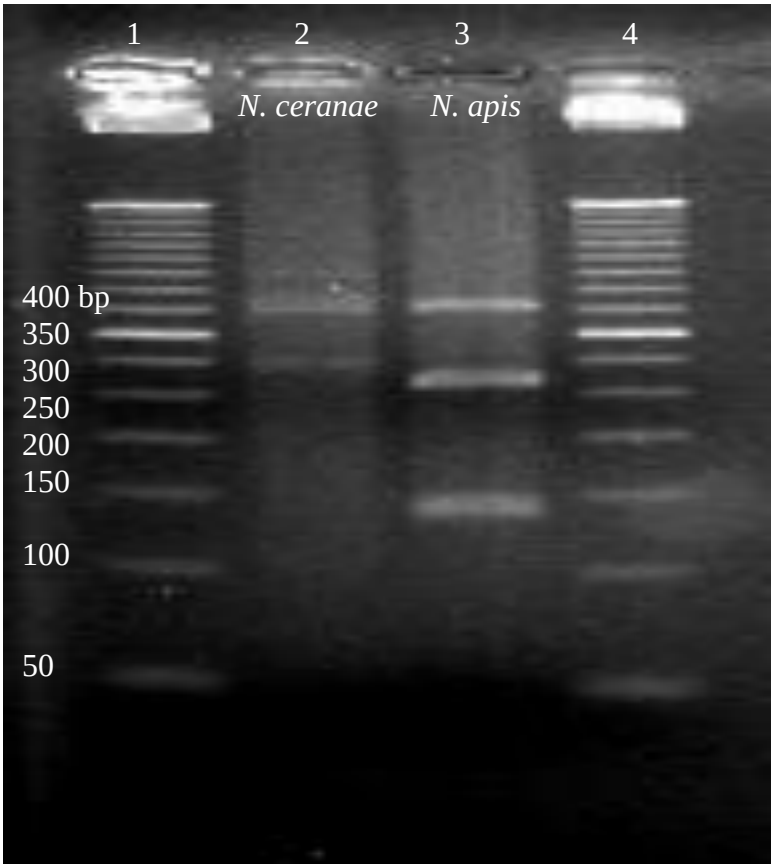

Fig. 1. Gel showing DNA extracts of *Nosema ceranae* (lane 2) and *Nosema apis* (lane 3) after restriction digestion. Some uncut *Nosema* DNA is still visible, approximately 400 bp in length in lanes two and three. Lanes one and four are 50 bp ladders. The highlighted bands in the ladders correspond to 350 bp. The *N. ceranae* DNA was cut by the restriction enzyme Pac1 to yield fragments of approximately 293 and 103 bp in lengths (lane 2). The *N. apis* DNA was cut by Nde1 to yield fragments of approximately 266 and 136 bp in length (lane 3).

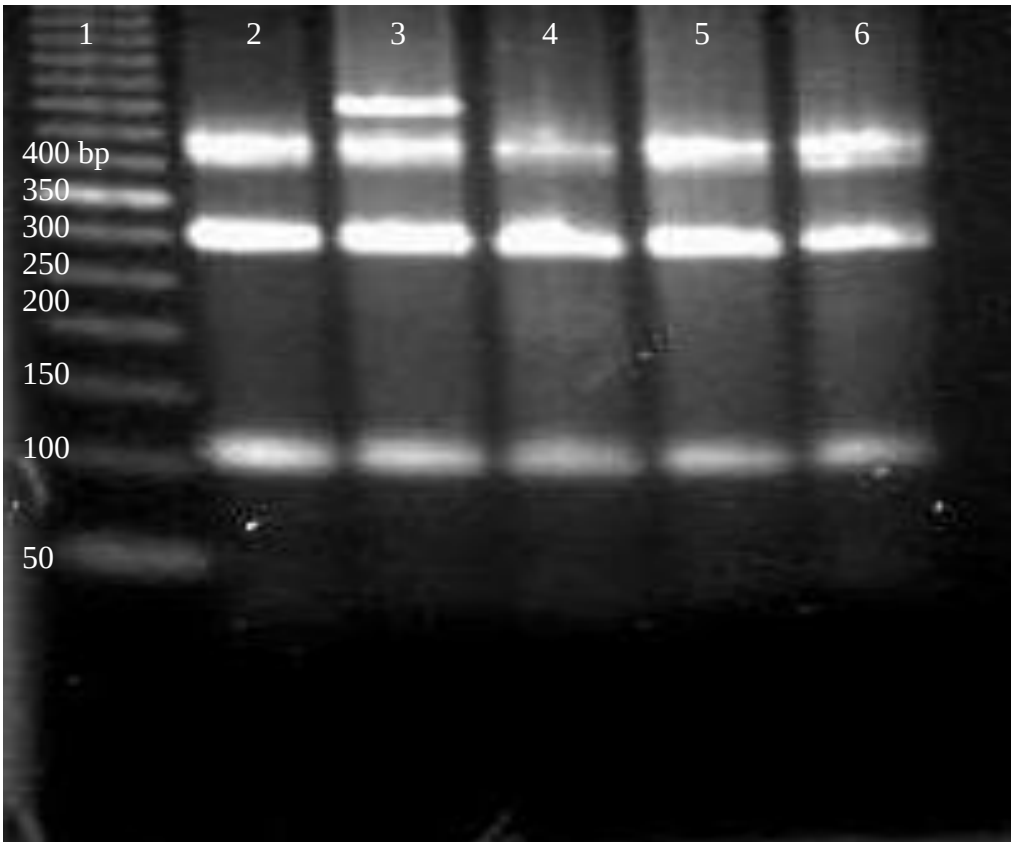

Fig. 2. Stationary apiary samples testing positive for the presence of *N. ceranae*. Lane one is the 50 bp ladder. Uncut *Nosema* DNA remains at 400 bp. Bands present in lanes 2-6 at 293 bp and 103 bp correspond to *N. ceranae*.

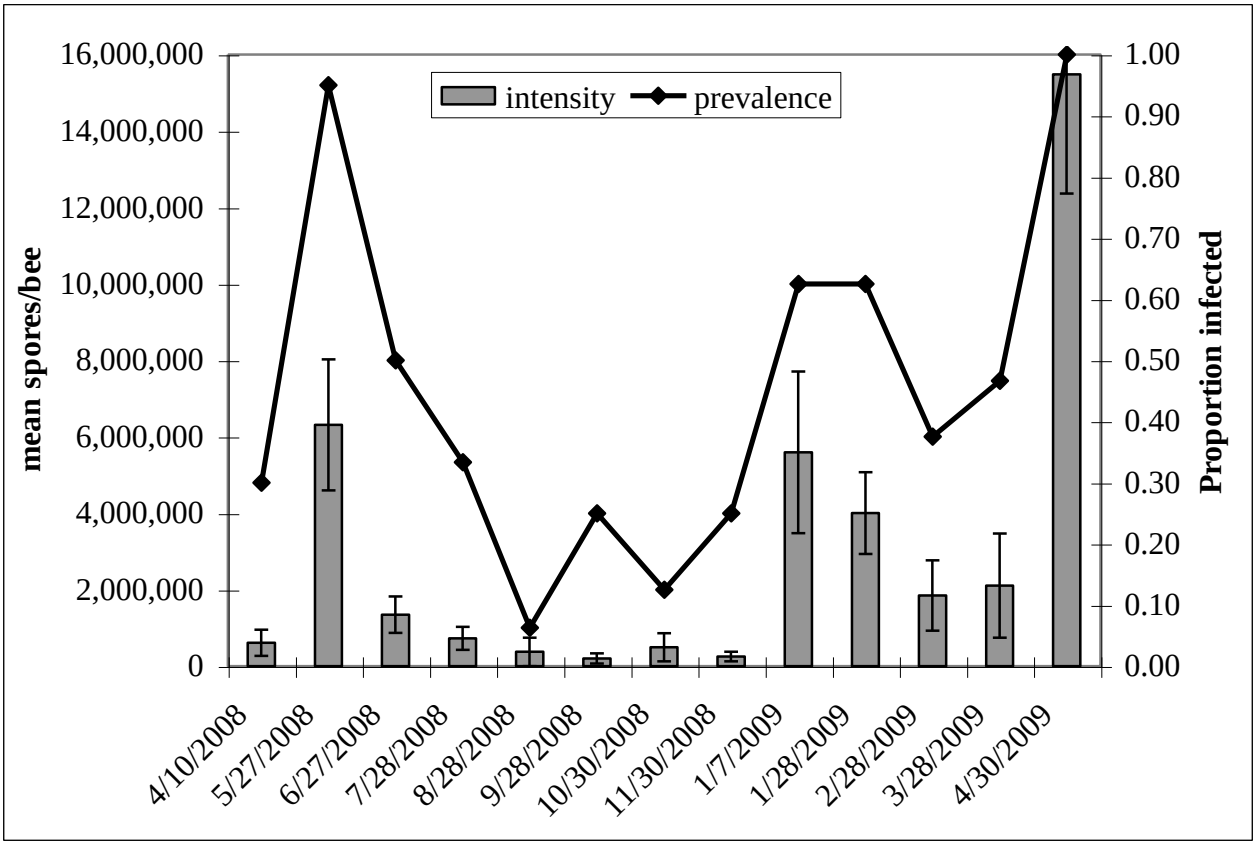

Fig. 3. Monthly intensity (mean spores per bee across all colonies) and prevalence (proportion of colonies infected) in the WA stationary colonies.

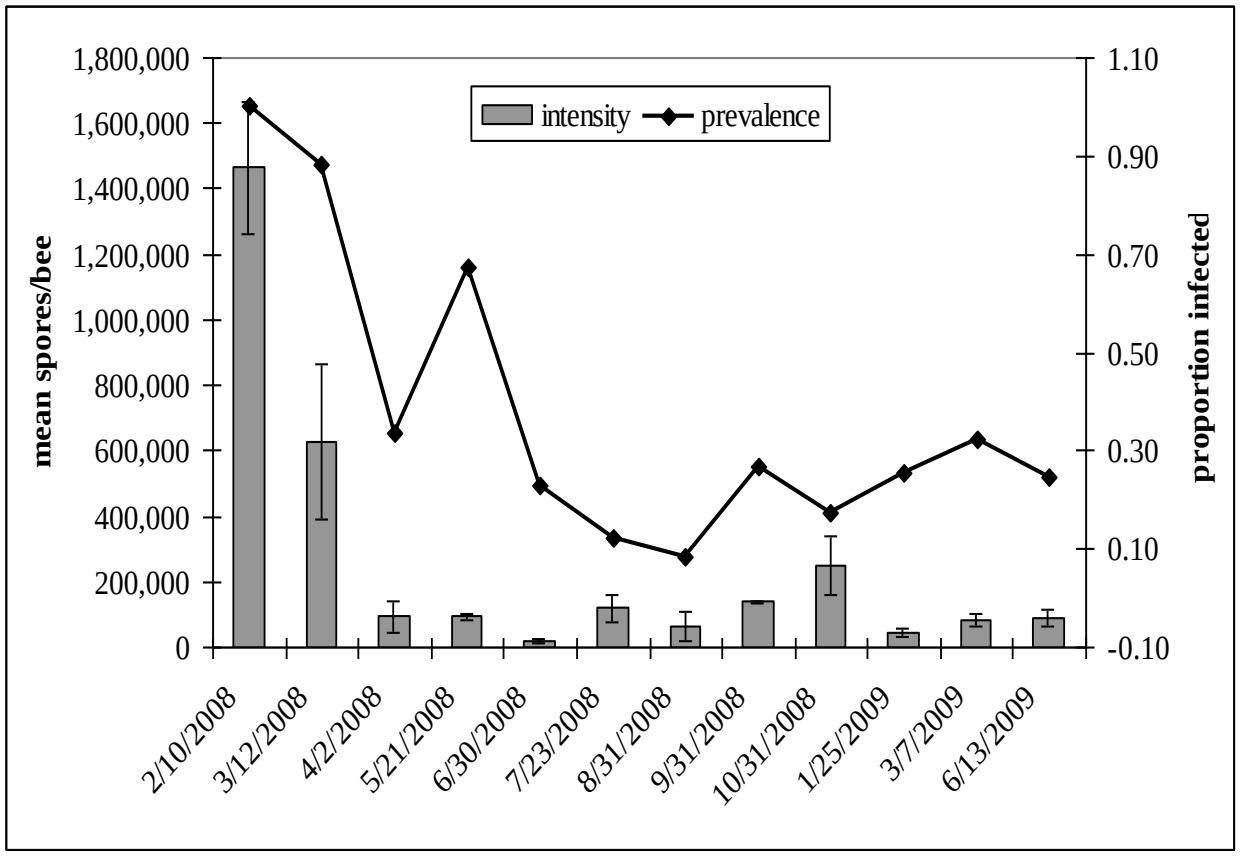

Fig. 4. Monthly intensity (mean spores per bee across all colonies) and prevalence (proportion of colonies infected) in the WA migratory colonies.

Distribution of *Nosema ceranae* (Fries) in age cohorts of the western honey bee (*Apis mellifera* L.)

ABSTRACT

Nosema ceranae intensity (mean spores per bee) and prevalence (proportion of bees infected in a sample) were analyzed in honey bees of known ages. Sealed brood combs from five colonies were incubated, emerging bees were marked with paint, released back into their colonies of origin, and recaptured at emergence (0-3 days old), as house bees (8-11 days old), and as foragers (22-25 days old). Fifty bees from each of the five colonies were processed individually at each collection date for the intensity and prevalence of *Nosema ceranae* infection. Using PCR and specific primers to differentiate *Nosema* species, *N. ceranae* was found to be the only species present during the experiment. At each collection age (emergence, house, forager) an additional sample from the inner hive cover (background bees = BG) of each colony was collected to compare the *N. ceranae* results of this sampling method, widely used for *Nosema* spore quantification, to the samples comprised of marked bees of known ages. No newly emerged bees exhibited infection with *N. ceranae.* One house bee out of the 250 individuals analyzed (prevalence = 0.4%) tested positive for *Nosema ceranae*, at an infection level of $3.35x10^6$ spores. Infection levels were not statistically different between the newly emerged (mean = 0 spores/bee) and house bees (mean = $1.34x10^4$ spores/bee) (P=0.99). Foragers exhibited the highest prevalence (8.3%) and infection intensity (mean = $2.382x10^6$ spores/bee), with a range of $0 – 8.72x10^7$ spores in individual bees. The average infection level across all foragers was significantly higher than that of newly emerged bees (P=0.01) and house bees (P=0.01). Implications of these findings are discussed.

1. Introduction

Microsporidia that infect honey bees are intracellular parasites of the midgut epithelia. Of the two species known to infect *Apis mellifera,* one, *Nosema apis* Zander, has a long association with overwintering losses and spring dwindling of honey bee colonies in temperate climates (Farrar 1942, Farrar 1944). *Nosema ceranae* Fries was not discovered until 1996 in China, parasitizing the Eastern honey bee, *Apis cerana* Fabricius. Since that time, several studies have identified the parasite in populations of *Apis mellifera* at various locations throughout the world (Klee et al. 2007, Paxton et al. 2007, Calderon et al. 2008, Chen et al. 2008, Williams et al. 2008, Giersch et al. 2009, Higes et al. 2009), and it has been implicated as a potential factor in collapsing colonies (Cox-Foster et al. 2007, Higes et al. 2007).

In previous studies on *Nosema apis*, many individual bees showed no positive signs of *N. apis* in the tissues of the midgut, while other individuals exhibited exceptionally high levels of *N. apis* (El-Shemy and Pickard 1989). Indeed, researchers have found that sampling individuals and groups of bees at various places both inside and outside the hive yield significantly different estimates of *N. apis* infection levels (L'Arrivee 1963a, L'Arrivee 1963b, El-Shemy and Pickard 1989). These discrepancies may be further exacerbated by the time of day and year of sampling (Duoll 1965, Moeller 1956). Differences in *N. apis* prevalence and intensity are apparently due to the distributed, but predictable, ages of bees in a sample collected from a specific site in or outside the colony at a particular time.

Less is known regarding the variation in *N. ceranae* levels as bees age. There is some suggestion that *N. ceranae* exhibits different infection patterns than *N. apis*, including a higher virulence (Higes et al. 2007) and more persistent presence in the hive throughout the year

(Martin-Hernandez et al. 2007). However, recent evidence suggests that there may be a similar distribution of *N. ceranae*-infected bees (compared to *N. apis*-infected bees) in a colony, with infection levels higher in foraging-age bees than in younger bees that yet remain inside the colony (Meana 2010).

Phylogenetic analysis of the relationship between the Microsporidia suggests that the two species may be more unrelated than previously assumed (Chen et al. 2009), and therefore life histories and host-pathogen interactions should be viewed as potentially distinct between the two species. In that case it is clearly important to understand how differences between *Nosema* species could be manifested at the individual bee level. The main objective of this study was to investigate changes in *N. ceranae* infection levels by analyzing individual bees over time.

2. Materials and methods

Five colonies naturally infected with *N. ceranae* were chosen from a single apiary based on a similar number of frames of bees and brood. Prior to, during, and after the experiment, samples were taken for DNA analysis. Specific primers were used to amplilfy a sequence of the 16S rRNA region of *Nosema* DNA (Klee et al. 2007). Restriction enzyme digestion with Pac1 and Nde1, followed by gel electrophoresis was used to verify the *Nosema* sp. as *N. ceranae* (M. Smart unpublished data).

One full frame of capped brood from each colony was removed and incubated at 34° C. Screens were inserted over the brood area to confine newly emerging adult bees to their frame of emergence. Frames were checked every 8-12 hours for adult bee emergence. Emerged bees were marked with enamel paint on the thorax and placed in one of five colony-specific holding cages at 27° C and 80% RH, and provisioned with water and 1:1 sugar syrup solution.

Emerged adults were maintained in cages until a minimum of 300 bees had emerged from each of the five brood frames. Fifty bees were initially collected (20 Aug 2008) for spore quantification to establish a baseline infection level for newly emerged adults. All remaining marked bees were returned to their colonies of origin for later recapture and analysis.

Fifty marked bees were collected from each colony at 8-11 days of age. Honey bees exhibit age polyethism, or age-based division of labor, and bees of this age typically reside in the hive near the brood area, caring for developing brood via feeding and cleaning behaviors. Another fifty painted bees were collected at 22-25 days of age. Bees at this age have typically already made the transition to becoming foragers outside of the hive. Additionally, during each collection of 50 marked bees, another sample of 50 bees was taken from the inner hive cover (= background bees (BG)) of each colony on each sample date. The background bees were used to compare the marked bees of known age to a sample of what we hypothesize to be a cross-sectional representation of various ages in the colony.

All collected bees, including BG bees, were processed individually by maceration of the abdomen in 1 ml of distilled water (adapted from Cantwell 1970). Ten μl of the homogenate were then pipetted onto the hemacytometer and spores per bee were quantified according to Cantwell 1970. One-way ANOVAs were conducted using SAS v. 9.2 to analyze the data.

3. Results

The three different age groups of bees (newly emerged, house bees, forager bees) yielded very different profiles for *N. ceranae* infection levels (spores per bee). Newly-emerged adult bees never tested positive for *N. ceranae* infection (Table 1). Of the 250 individual 8-11 day old house bees tested, one individual (1/250 = 0.4%) had an infection level of 3.35×10^6, while the

remaining 249 individuals did not exhibit any *N. ceranae* spores. The average infection level of house bees was therefore $1.34x10^4$ spores per bee (Fig. 1).

Foragers (age 22-25 days old) had the highest proportion of individuals testing positive for *N. ceranae* (20/240 = 8.3%) (Table 1) and the highest average level of infection ($2.38x10^6$) (Fig. 1). The range of individual forager bee *N. ceranae* infection levels was quite broad, ranging from $0 – 8.72x10^7$ spores per individual bee. *Nosema ceranae* infection levels were not significantly different between the newly emerged and hive bees (P=0.99) (Fig. 1). However, the mean infection level of forager bees was significantly higher than either the newly emerged (P=0.01) or hive bees (P=0.01) (Fig. 1).

Both the mean infection level (Fig. 2) and the prevalence of bees infected in a sample (Fig. 3) increased with the age of the marked bees sampled. The mean infection level in the background bees did not differ across sampling date, while at the same time the percentage of infected bees did vary (Figs. 2 and 3). Infection levels in the background bees remained relatively level, but were different from the marked bees at all three sample dates (Fig. 2).

Infection levels in Table 1 are differentiated by the mean number of spores in infected bees (NSI) compared to the mean number of spores in all bees per sample (NSS). The mean NSI was always much higher than NSS due to the majority of bees in any given sample being free of *N. ceranae* spores.

The relationship between the percentage of infected bees in a sample and infection level is displayed in Fig. 4. These were highly correlated ($r^2 = 0.75$) based on our data, especially when the percentage of infected bees was less than 0.15.

4. Discussion

Understanding patterns of *N. ceranae* infection in individual bees related to age provides a clearer view of the dynamics occurring inside the hive. As demonstrated previously with *N. apis* (Pickard and El-Shemy 1989) and in the current study on *N. ceranae*, honey bees exhibit a pattern of drastic increases in infection level and percentage of individuals infected with increasing ages of bees in the sample. This increase occurs some time during the time between house bee and forager bee (Fig. 1).

Using mean spore infection rates across all bees in a given sample, even in the more greatly infected forager bee group, gives a skewed view of *N. ceranae* distributions in the colony (Table 1). Certain individuals in the colony harbor the majority of spores and are thus likely to contribute to continued disease transmission of *N. ceranae* within the hive. However, the precise mechanism of transmission of *N. ceranae* spores from one bee to the next under natural conditions remains unclear.

Our ability to accurately detect how many infected individuals are actually present in a sample is hampered by the fact that composite samples are typically used to estimate mean infection levels in a colony. Infection levels in composite samples of background bees were significantly different from the levels in the age cohorts on all sample dates. However, the infection levels in the background bees were somewhat intermediary between the house bees and forager bee levels. (Fig. 2) These findings suggest that bees collected from the inner hive cover are composed of a variety of old and young bees and, depending on the goals of the sampler, may provide a better whole colony mean infection level than sampling just foragers.

Our results (Fig. 4) showed a correlation between infection levels in composite samples and the percentage of infected bees in the sample, at least at low to moderate levels. As the percentage

of bees infected and infection levels rise in a colony, more individuals are at risk of contamination, especially over the winter. Further investigation of this relationship could help improve our ability to predict the affects of rising *N. ceranae* infections on honey bee colony health.

Considering the decline in immune function that occurs as bees age (Amdam et al. 2005), the pattern of increasing *N. ceranae* infections in older age bees makes sense and may be an important clue to better understanding the transmission and control of *N. ceranae*. The transition from nurse to foraging tasks, that typically occurs 18-28 days after adult emergence in honey bees, is associated with an increase in risky behavior, including guarding and foraging roles (Amdam et al. 2005). Additionally, Mayack and Naug (2009) hypothesized that energetic stress due to *N. ceranae* infection led to precocious and risky foraging. Precocious foraging has a negative effect on colonies, decreasing worker longevity and productivity.

Normal adult bees undergoing the nurse to forager transition exhibit a rapid decline in immune function, accompanying an increase in JH that induces hemocyte death (Amdam et al. 2004). Hemocytes have many important functions in the cellular immune response, including: phagocytosis, encapsulation, nodulation, wound repair and the production of antibacterial peptides (Amdam et al. 2005). Furthermore, Antunez et al. (2009) found that in response to infection with *N. apis*, honey bee humoral immune response is affected, causing the honey bee antimicrobial peptides abaecin, defensin, and hymenoptaecin to become quickly activated. After infection with *N. ceranae*, transcription of these peptides was significantly suppressed, suggesting differential susceptibility of the immune response of honey bees to the two species of *Nosema*.

Nosema ceranae-infected honey bees in a colony are not static in time and space. They forage, share food, and move around inside and outside the colony, coming into contact with nestmates and individuals from other colonies. Why some bees become infected relatively young, while others live out their entire life without becoming infected with *N. ceranae* is an important question yet to be answered, and what actually happens to the highly infected foragers is just beginning to be understood. Kralj and Fuchs (2010) showed that a larger proportion of *N. ceranae*-infected individuals departed from colonies than returned. Further, infected foragers released at distances from the hive took longer to return and did so in smaller numbers than uninfected foragers. These results, in which infected individuals were apparently lost from the colony, were similar to findings by the same researchers studying the effects of *Varroa destructor* Anderson and Trueman (2000) mites on honey bee flight behavior, and could reflect a common response of honey bees to high parasite loads (Kralj and Fuchs 2006). The loss of these highly parasitized foragers may benefit the colony by decreasing the overall parasite load.

Worldwide colony losses have yet to be attributed to any single factor, as demonstrated by a recent study in which 61 factors potentially contributing to Colony Collapse Disorder (CCD) were analyzed, including *N. ceranae* infection (vanEnglesdorp et al. 2009). No single factor was found to be able to distinguish CCD from healthy colonies (*Ibid.*, 2009). Understanding the relationship between colony level infections and percentage of bees infected, and the impact of other pests, parasites, and stressors could help more quickly diagnose the severity of *N. ceranae* infections and risk of colony loss.

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Colony #	Date	Age group	n sampled	n infected	% infected	NSI (x10^6)	NSS (x10^6)
1	20-Aug	NE	50	0	0	0.00	0.00
	28-Aug	house bees	50	1	2	3.35	0.07
	11-Sep	foragers	50	6	12	19.65	2.36
	20, 28 Aug; 11 Sep	BG	150	19	13	13.07	1.66
2	20-Aug	NE	50	0	0	0.00	0.00
	28-Aug	house bees	50	0	0	0.00	0.00
	11-Sep	foragers	40	7	18	32.98	5.77
	20, 28 Aug; 11 Sep	BG	150	3	2	19.22	0.38
3	20-Aug	NE	50	0	0	0.00	0.00
	28-Aug	house bees	50	0	0	0.00	0.00
	11-Sep	foragers	50	1	2	27.50	0.55
	20, 28 Aug; 11 Sep	BG	150	6	4	23.72	0.95
4	20-Aug	NE	50	0	0	0.00	0.00
	28-Aug	house bees	50	0	0	0.00	0.00
	11-Sep	foragers	50	2	4	51.38	2.06
	20, 28 Aug; 11 Sep	BG	150	1	1	1.15	0.01
5	20-Aug	NE	50	0	0	0.00	0.00
	28-Aug	house bees	50	0	0	0.00	0.00
	11-Sep	foragers	50	4	8	14.70	1.18
	20, 28 Aug; 11 Sep	BG	150	4	3	25.10	0.67

Table 1: Summary of *N. ceranae* infection levels and percent of individual bees infected with *N. ceranae* in each of five colonies. **NE** = newly emerged bees, **BG** = background bees collected from the inner hive cover at the same time as each age group (NE, house bees, forager bees), **NSI** = mean number of spores/infected bee, **NSS** = mean number of spores/sampled bee (composite sample).

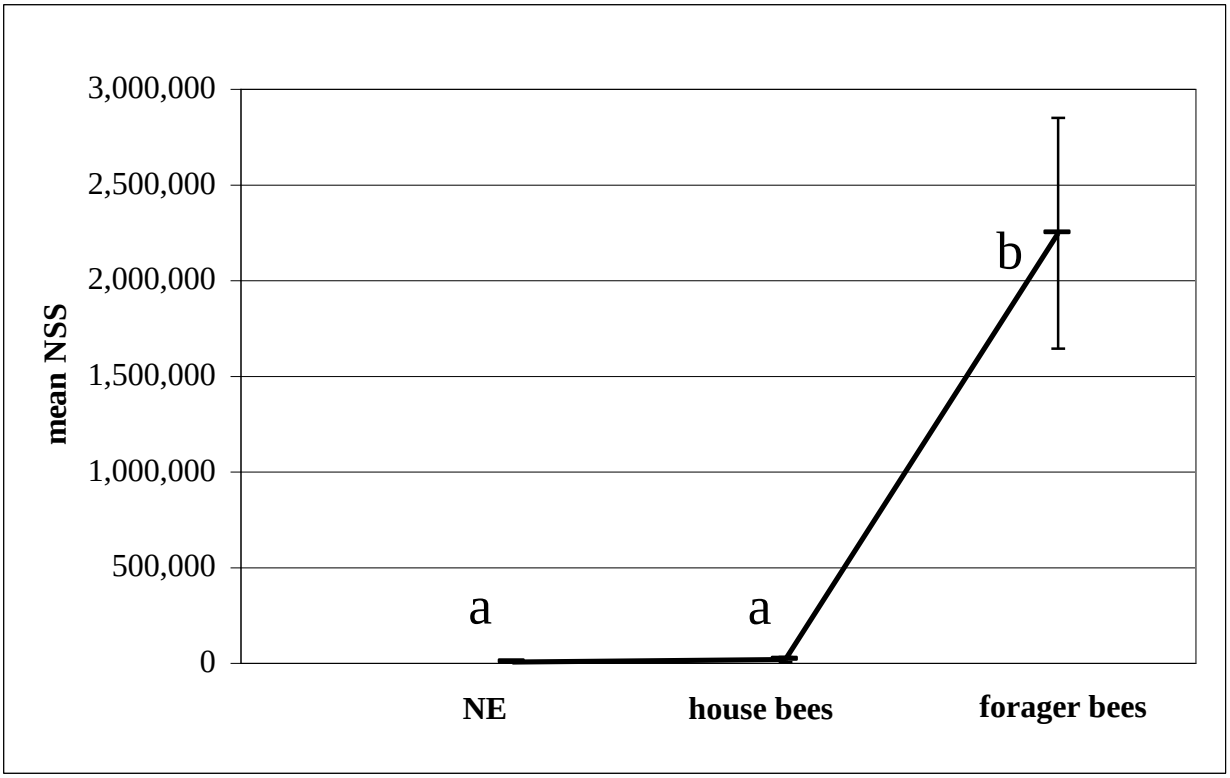

Fig. 1. Mean *N. ceranae* infection levels across all bees sampled at each age group,
NSS = mean number of spores per sampled bee in composite sample, **NE** = newly emerged bees.

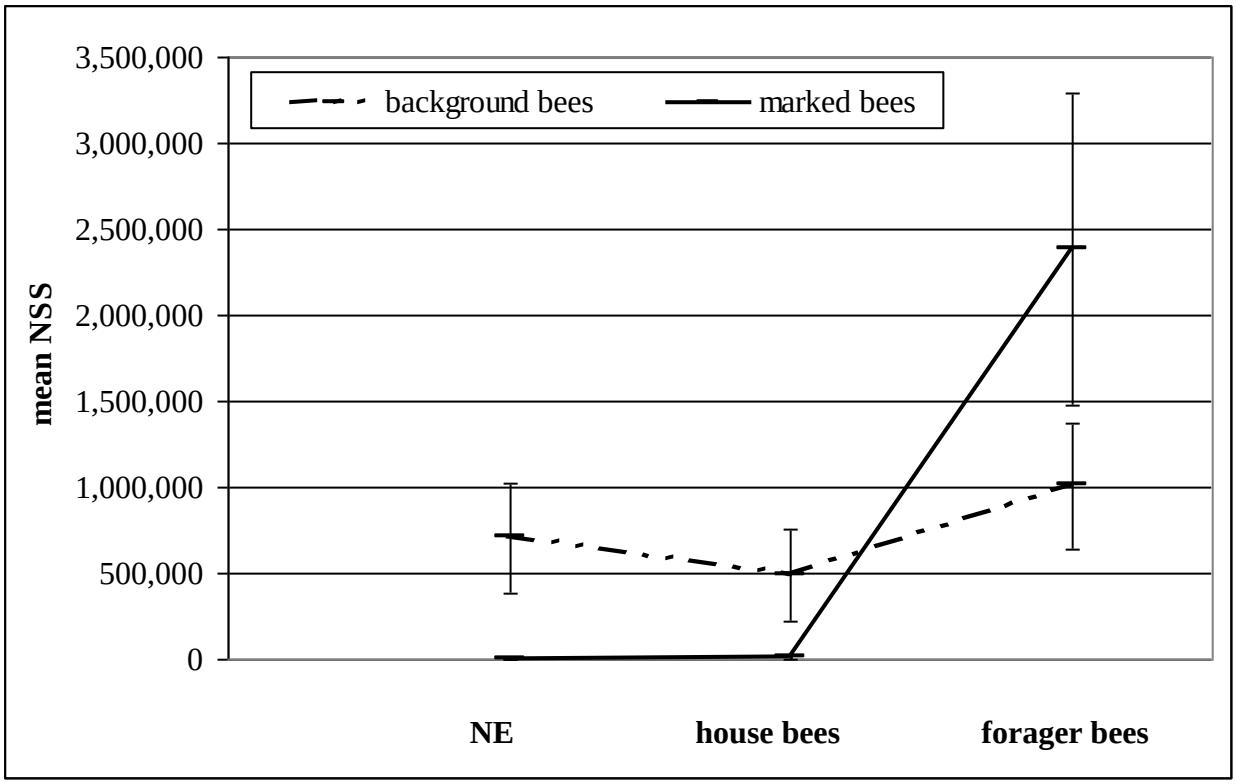

Fig. 2: Mean *N. ceranae* infection levels of background bees and each age group sampled. **NSS** = mean number of spores per bee in composite sample **NE** = newly emerged bees.

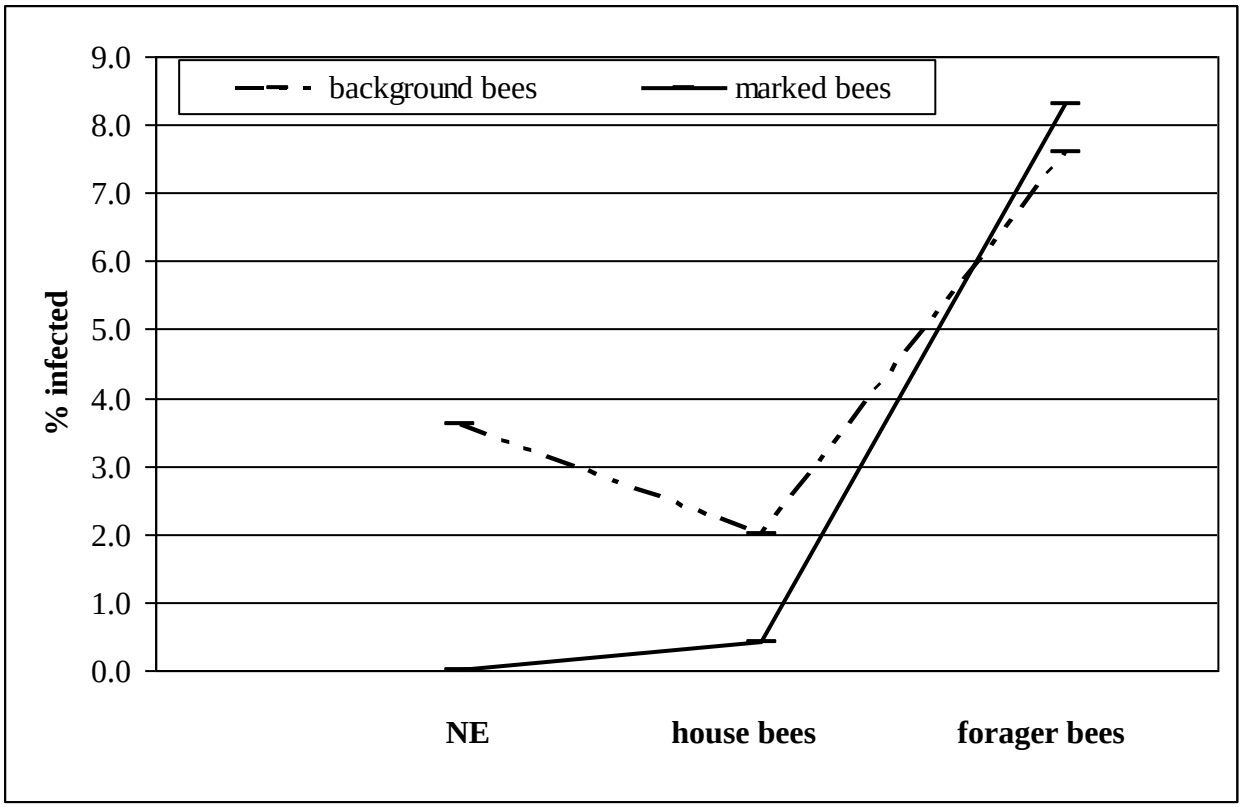

Fig. 3: Percentage of individual bees infected at each age cohort and in the background bee samples, **NE** = newly emerged bees.

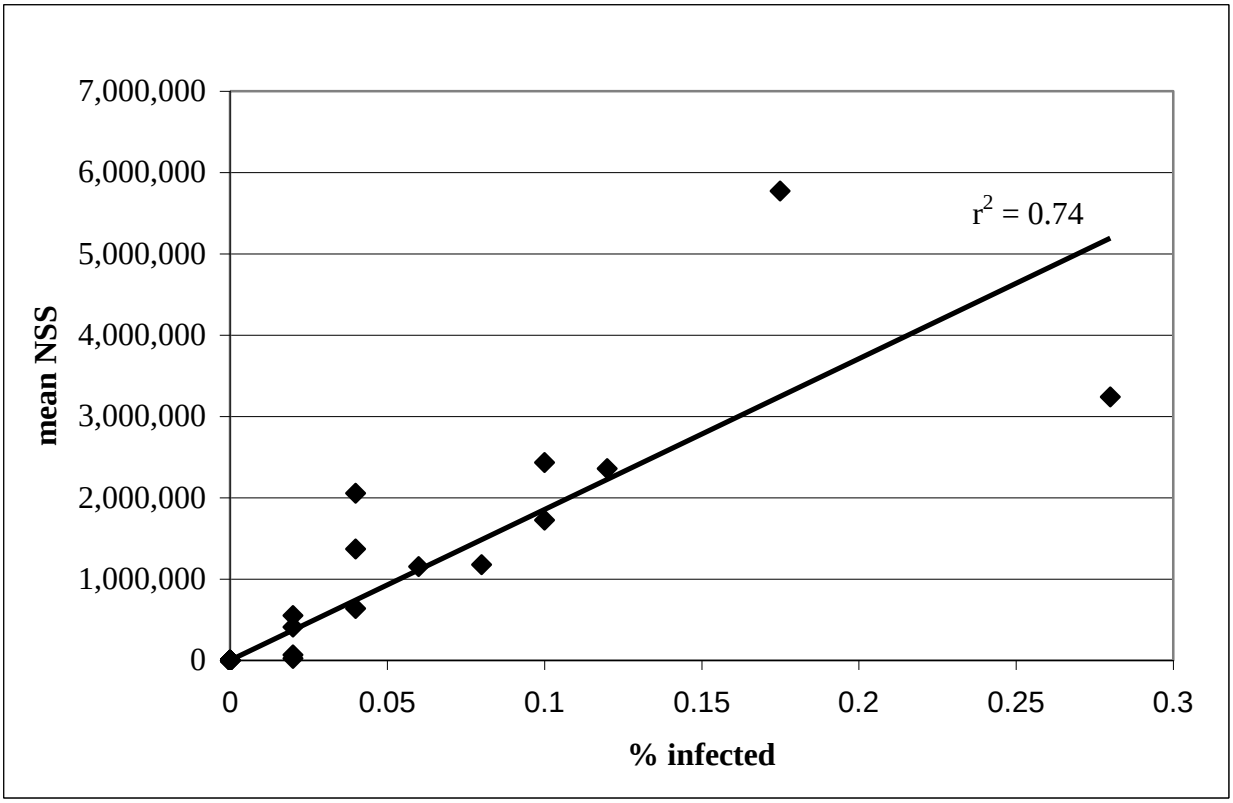

Fig. 4: Percentage of infected bees per sample vs. *N. ceranae* infection level across each fifty bee composite sample (NSS). **NSS** = mean number of spores per bee in composite sample.

Artificial inoculation of field honey bee (*Apis mellifera* L.) colonies with *Nosema ceranae* (Fries) and co-infection with *Varroa destructor* (Anderson and Trueman) mites in the Pacific Northwest

ABSTRACT

Forty colonies located in a single apiary were sampled weekly between May 2009-September 2009 (19 weeks) to detect *Nosema ceranae* (mean spores per bee), *Varroa destructor* (mites per 100 bees), and monthly for *Acarapis woodi* (Rennie) (percent infestation). Groups of 10 colonies each received one of the following treatments: miticide/weekly inoculation with *N. ceranae* spores, no miticide/weekly inoculation with *N. ceranae* spores, miticide/ no *N. ceranae* spore inoculation, or no miticide/no *N. ceranae* spore inoculation. Formic acid (Mite-Away II^{TM}) was applied to half of the colonies at the beginning of the experiment. The number of frames of bees and brood between the beginning and end of the experiment, and the amount of honey produced were measured as indicators of colony strength and production, respectively. No significant differences in strength or production were found between colonies in the treatments. Data were analyzed to detect interactions between *N. ceranae* and *V. destructor* (*A. woodi* was detected at very low levels throughout the study). Results indicated that interaction did occur between *N. ceranae* by week and *V. destructor* by week, but not between the two parasites. The potential implications of co-infections of parasites in honey bees are discussed.

Introduction

The microsporidian parasite *Nosema ceranae*, described by Fries et al. (1996), originally infected the eastern honey bee, *Apis cerana* F., but is now known to occur in populations of the western honey bee (*Apis mellifera* L.) throughout much of its range (Higes et al. 2006, Huang et al. 2007, Klee et al. 2007, Paxton et al. 2007, Calderon et al. 2008, Chen et al. 2008, Williams et al. 2008, Giersch et al. 2009, Higes et al. 2009). Recent studies of *N. ceranae* have shown it to exhibit a higher virulence (Higes et al. 2007, Paxton et al. 2007) and to have a greater persistence year round in honey bee colonies than *Nosema apis* Zander (Martin-Hernandez et al. 2007).

Another honey bee parasite, *Varroa destructor* Anderson and Trueman (2000), is the most destructive pest of honey bee colonies worldwide, reproducing in brood cells and parasitizing both immature and adult bees. *Varroa destructor* levels tend to build through the summer when the queen is laying up to 2,000 eggs per day and ample brood is present to parasitize. Left untreated, high mite infestations can kill or severely weaken colonies going into winter and have been found to be a primary cause of winter mortality (Guzman-Novoa et al. 2010).

Mites can affect the physiology of worker bees in infested colonies, decreasing hemolymph vitellogenin titers and the proportion of normal hemocytes present in newly emerged, 5-day old, and 30-day old bees (Amdam et al. 2004). Honey bees with depleted levels of vitellogenin have fewer protein reserves to access during the pollen-limited overwintering period inside the colony and, therefore, could reduce longevity and/or increase susceptibility to pathogens and parasites over the winter due to the lower number of hemocytes. Furthermore, *V. destructor* is known to be associated with the prevalence of certain honey bee viruses (Nordstrom 2003, Chen et al. 2004, Tentcheva et al. 2004) both through the direct transmission

of viruses to brood and through the suppression of the honey bee immune system (Shen et al. 2005, Yang and Cox-Foster 2005). The effects of high levels of *V. destructor* infestation potentially allows viruses and other microbes (Yang and Cox-Foster 2007) to replicate more readily in colonies highly infested with *V. destructor*.

Nosema ceranae has been shown to have a partial immuno-supressive effect on the transcription of honey bee antimicrobial peptide genes associated with the humoral immune response, including abaecin, defensin, and hymenoptaecin, compared to control bees and bees infected with *N. apis* (Antunez et al. 2009). Glucose dehydrogenase, involved in encapsulation during the insect cellular immune response to fungal invasion, was significantly reduced, as was vitellogenin, seven days after the inoculation of honey bees with *N. ceranae* (*Ibid.*, 2009), which could contribute to increased susceptibility to pathogens and decreased longevity in adult honey bees highly infected with *N. ceranae*.

Additionally, energetic stress is evident in *N. ceranae*-infected honey bees by their increased responsiveness to sucrose during proboscis extension response trials (Mayack and Naug 2009). This vigorous drive to feed, coupled with abnormally low levels of vitellogenin (Antunez et al. 2009), could cause *Nosema*-infected worker bees to undergo the transition to forager bee earlier in life, to forage at more risky times, and to experience decreased longevity as a result (Woyciechowski and Kozlowski 1998).

As described by Amdam et al. (2004), simulated winter bees reared in colonies with high mite infestations exhibited decreased protein reserves and fewer hemocytes over the winter. *Nosema ceranae* levels rise over the winter, further suppressing the immune system and nutritionally stressing bees that enter the winter in this condition. Both parasites have similar affects on their hosts and could be expected to interact with each other and other pathogens to

reduce colony strength and to increase mortality. Given the importance of the presence of honey bees with ample stores of protein and healthy immune systems going into the winter, we hypothesized that colonies with elevated levels of V. *destructor* and *N. ceranae* would exhibit differential survival, showing signs of weakening and collapsing in the fall, and likely not surviving through the winter as a result.

The goals of this study were to: (1) maintain elevated levels of *N. ceranae* in colonies via weekly artificial inoculations with spores, and (2) measure the effects of elevated levels of each parasite, and the co-infective affects of both parasites at high levels on production, strength, and overwintering success.

Materials and Methods

Treatments

Experimental design was a completely randomized design with a two-way treatment structure and repeated measures pairing added or natural *N. ceranae* with miticide or no miticide. Four treatments were applied to 10 colonies each as follows: (1) addition of approximately $5.0x10^7$ *N. ceranae* spores in 10 ml sugar syrup per week paired with a spring application of formic acid for mite control; (2) addition of 10 ml of sugar syrup paired with a spring application of formic acid for mite control; (3) addition of $5.0x10^7$ *N. ceranae* spores in 10 ml sugar syrup per week with no mite treatment; (4) 10 ml of sugar syrup and no mite control.

Forty single deep colonies of varying strength were ranked according to their initial *Varroa* levels (mites/100 bees) and *N. ceranae* levels (mean spores/bee). Colonies were then assigned treatments 1-4 to ensure that all four treatments contained colonies across all mite and *N. ceranae* levels. Colonies were then randomly assigned positions in the apiary. Colonies receiving miticide had formic acid pads (Mite-Away II™, Mann Lake Ltd.) placed on wooden

frames at the top of the colonies for a period of 21 days beginning in week 2, per label recommendation.

Every week during the experiment, a sample of approximately 300 bees was taken from the inner hive cover and outermost frames of the top box from each of the 40 colonies. Samples were then processed weekly to quantify *N. ceranae* infection levels (mean spores/bee according to Cantwell 1970, using 50 bees) and *Varroa* levels (mites/100 bees after 60 minutes in an orbital shake to dislodge mites) and monthly for tracheal mite (*A. woodi*) load (percent infestation). *Varroa* mites were monitored (as described above) and levels surpassing treatment thresholds were noted (Strange and Sheppard 2001). Bees were sampled again in the spring to determine the level of *N. ceranae* infection and *Varroa* infestation after the overwintering period.

Spore preparation

Spring colonies containing bees with high levels of *N. ceranae* spores (approximately $3.0x10^7$ spores per bee) were sacrificed and whole dead bees were maintained in a cooler at 4° C. Every week, 100 bees were macerated in 100 ml dH_2O and the mean number of spores per bee in the sample was quantified on a hemacytometer under 400x magnification. Once the average number of spores was quantified, the 100 ml solution was diluted in 1:1 (weight to volume) sucrose solution to bring the total concentration to $5.0x10^7$ spores/10 ml. Using a 50 ml syringe, 10 ml of the solution were expelled from the syringe across the top bars of the bottom box of each of the twenty colonies receiving *N. ceranae* inoculum every week beginning in week 2. The other 20 colonies received 10 ml of 1:1 sucrose solution alone as a control.

Results

Table one displays the mean mite infestations and *N. ceranae* infection levels in colonies in all weeks. *Varroa* levels in colonies receiving miticide were significantly lower (ANOVA, $P\leq$

0.05) in all weeks after the formic acid was applied (week 2). The overall mean infection level was higher in colonies with added *N. ceranae* spores (ANOVA, $P< 0.05$). However, due to the interaction of *N. ceranae* by week, *N. ceranae* levels were significantly higher (ANOVA, $P \leq 0.05$) in colonies with added *N. ceranae* during only two weeks of sampling, week 6 (ANOVA, $P= 0.02$) and week 7 (ANOVA, $P= 0.05$).

Only two colonies survived through the winter, one colony from treatment 1 and another from treatment 3. Live bees were sampled from these two colonies and *N. ceranae* infection and V. *destructor* infestations were quantified. *Varroa* mites and *Nosema* spores were not present on/in these bees. Dead bees from colonies that died over the winter were collected to determine *N. ceranae* levels, the means of which are shown in Table 1.

Figure one depicts the average intensity of *N. ceranae* infection in colonies receiving inoculation with *N. ceranae* spores compared to colonies that did not receive additional *N. ceranae*. Wide variation in infection levels existed both within and between treatments, as evidenced by the large error bars in the figure. There were no significant differences in *N. ceranae* levels between treatments at the outset of the experiment ($P> 0.05$). Colonies inoculated with *N. ceranae* maintained generally higher average infection levels (Figure 1) further into the experiment than did naturally infected colonies. However, intensity of *N. ceranae* infection was depleted across all treatments by the end of the experiment when no differences were measured ($P> 0.05$).

Mean *Varroa* infestations between colonies treated and untreated with formic acid in the spring are displayed in Figure 2. P-values were significant for all weeks between miticide treatments after week 2 ($P\leq 0.05$), when the formic acid was applied (Figure 1). Mites in all colonies steadily increased as the experiment progressed, but began to increase dramatically,

particularly in the untreated colonies (Figure 2), after week 14. Significant differences remained ($P \leq 0.05$) between treatments despite this increase, although mite levels had begun to reach injurious levels in colonies in all treatments by week 12.

Colonies were tested for the presence and percent infestation of tracheal mites each month (Figure 3). *Acarapis woodi* was not detected in either of the first two sample dates (weeks 2 and 6). Colonies in treatment 3 had the highest percent infestation (1.5%), however, this level of *A. woodi* infestation was not significantly different (ANOVA, $P > 0.05$) from the other treatments on that date, or any other dates.

Honey production is depicted in Figure 4 across *Nosema* and mite treatments. There were no differences (ANOVA, $P > 0.05$) between the amount of honey produced in colonies based on treatment. Similarly, differences in colony strength (Figure 5), measured as the change in the number of frames of bees and brood, were not significant between treatments (ANOVA, $P > 0.05$). However, trends did exist in which colonies receiving miticide had a positive change in strength, while those receiving no miticide had a negative change (Figure 5), and this factor may be significant at higher sample sizes.

Discussion

In this study we attempted to maintain *N. ceranae* infections at elevated levels by artificially adding spores to colonies every week to study the interactive affects of *N. ceranae* and *V. destructor*. The overall *N. ceranae* infection levels in these inoculated colonies was higher than in uninoculated colonies; however, these higher levels were not sufficiently high to cause significantly greater colony weakness or mortality.

Methods used here to collect and preserve spores (by maintaining whole dead infected bees in a cooler for use each week) may have been insufficient to maintain viable spores for the

duration of the experiment. Recent studies have shown a reduced viability of *N. ceranae* spores under freezing temperatures (Fries 2009), but a greater tolerance of desiccation and high temperatures (Fenoy et al. 2009, Martin-Hernandez et al. 2009), than *N. apis*. Improvements in the preservation of spores for use in inoculation experiments are needed to study the interplay that may occur when two or more stressors simultaneously parasitize both individual bees and colonies.

Varroa destructor treatment proved to significantly affect the level of mite infestation from week 3 onward, due to the efficacy of the formic acid application in the spring. However, all colonies, including those treated with formic acid, had reached the economic injury level for *Varroa* fall treatment beginning in week 12 (Strange and Sheppard 2001). Fall treatment for mites occurred in October, though the damage done by the mites was already apparent and likely irreversible. Many bees with deformed wings and dead brood were observed in these colonies, along with phoretic mites on adult bees, indicating very high levels of mite infestation and the likely occurrence of viruses acting to significantly weaken winter bees. As a result of the high mite infestation, overwintering survival was poor, reaching 100% mortality in two of the four treatments with just one colony each surviving to the spring in the other two treatments (treatments 1 and 3).

Nosema ceranae treatment only significantly affected colony levels of the parasite in two weeks of the study (weeks 6 and 7), though infection levels in these colonies were generally higher and persisted longer into the study than in untreated colonies. Additionally, there were no significant differences in spore levels between *N. ceranae* treatments in the spring when dead bees were tested (Table 1).

Results indicate that in the current study, *V. destructor* was likely the primary cause of colony decline in the late fall and over the winter, similar to the results found by Guzman-Novoa et al. (2010). *Nosema ceranae* levels were in decline by the end of the study regardless of treatment and this may reflect either a lack of viable spores from the infected source bees, or part of the larger general trend of *N. ceranae* infection declines during this time of the year in the region (M. Smart, unpublished data).

The lack of difference in colony production (kg honey per treatment) was likely due to mite levels remaining below injurious levels in all colonies at the time of the nectar flow. *Nosema ceranae* levels at this time, while generally higher, were not high enough to have deleterious effects, nor would *N. ceranae* be expected to weaken or kill colonies at the height of a nectar flow. The lack of difference in strength (number of frames of bees and brood) was likely due to the high levels of *Varroa* mites across all treatments by the end of the experiment, when the final strength measurement was taken. Colonies were already in a state of decline due to the mites, so differences between the beginning and end were not as pronounced as would otherwise be expected.

A recent mutilifactorial analysis of healthy and collapsed colonies by vanEnglesdorp et al. (2009) reported that no single factor was predictive of colony mortality, but rather multiple factors such as the presence of several different pathogens at "elevated levels" existed in colonies exhibiting signs of colony collapse. Another study found that interactions between *N. ceranae* and a systemic neonicotinoid insecticide occurred and significantly weakened colonies (Alaux et al. 2009). Studies such as these underscore the importance of testing and understanding the effects of multiple stressors acting on honey bee colonies in various locations and times of the year to improve honey bee colony health.

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	V. destuctor mites/100 bees, mean ± s.e.				*N. ceranae* spores/bee, mean ($x10^5$) ± s.e. ($x10^5$)			
Treatment	1	2	3	4	1	2	3	4
Week 1	3.1±0.8	3.5±0.7	2.1±0.4	2.4±0.7	4.60±2.13	7.30±3.69	3.50±0.98	4.40±0.99
2	4.5±0.8	3.7±0.5	4.1±0.6	3.0±0.3	4.00±1.73	2.45±0.94	7.25±2.69	6.80±1.36
3	1.0±0.7	0.8±0.2	3.6±0.8	2.8±0.6	5.30±1.78	7.10±1.79	5.30±2.03	3.45±1.18
4	1.0±0.5	2.3±0.7	3.1±0.7	3.1±0.5	4.00±1.59	3.45±1.67	3.05±1.22	2.05±2.05
5	1.0±0.3	1.7±0.4	2.7±0.4	3.9±0.9	6.00±2.68	2.80±1.67	3.45±2.62	2.65±1.13
6	0.6±0.2	2.1±0.7	4.9±1.0	4.1±1.0	6.45±2.63	1.40±0.71	4.40±2.69	0.15±0.15
7	1.0±0.3	1.4±0.5	4.6±0.9	3.4±0.7	6.20±2.85	1.60±0.57	3.65±1.82	1.25±0.86
8	1.4±0.6	2.0±0.6	3.7±0.6	2.4±0.5	3.00±2.24	1.30±0.76	11.6±6.13	1.15±0.74
9	1.4±0.4	2.5±0.4	5.0±0.8	4.6±0.9	3.83±1.95	0.35±0.21	5.80±3.02	3.15±2.16
10	1.6±0.5	3.3±0.8	7.5±1.3	6.3±1.1	0.05±0.05	0.45±0.40	9.30±9.19	0.40±0.40
11	2.6±0.8	4.5±1.1	14.0±2.7	10.7±2.3	0.39±0.39	0	4.75±4.75	0.20±0.20
12	4.0±1.4	6.2±1.5	10.2±2.0	7.9±1.8	0.05±0.05	0	1.25±1.25	0.60±0.40
13	4.1±1.3	6.0±1.4	11.1±1.3	10.3±2.3	1.11±0.78	0	9.50±9.17	1.05±1.05
14	5.7±1.7	5.7±1.4	9.6±1.8	10.4±1.7	0	0	8.55±4.85	0.20±0.20
15	7.2±1.8	9.2±1.7	17.4±1.8	13.9±2.9	0.39±0.39	0.05±0.05	3.70±2.47	0
16	6.5±1.6	10.0±1.9	20.9±2.7	16.2±3.9	.078±0.52	0.35±0.35	0.45±0.30	0
17	8.8±2.2	18.5±5.0	28.0±3.9	33.2±8.3	1.00±0.68	0	1.70±1.65	0.80±0.64
18	13.8±2.5	25.3±4.3	36.6±6.2	39.3±9.5	1.67±1.67	0.15±0.15	0.95±0.68	0.50±0.50
19	24.6±5.2	33.0±8.0	42.1±6.1	44.2±7.6	0.56±0.56	0	0.90±0.90	2.40±2.40
Spring	0±0	-	0±0	-	16.5±8.49	14.3±4.76	10.1±5.51	10.3±9.26

Table 1. Weekly mean *V. destructor* and *N. ceranae* levels. Two live colonies (treatment 1 and 3) remained on 3/15/2010 and were tested for *V. destructor*. These live colonies and dead bees from the other colonies were used to estimate *N. ceranae* infections in the spring.

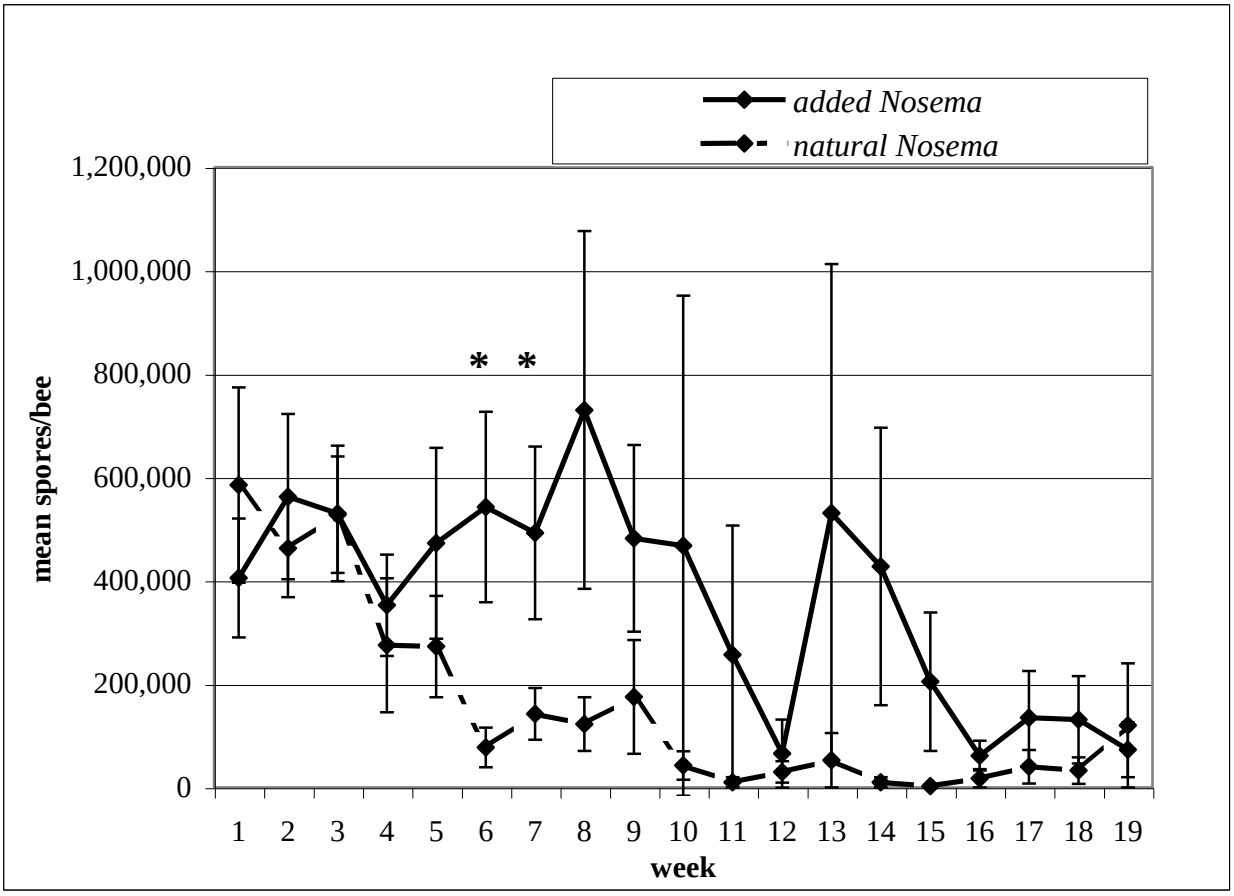

Figure 1. Mean weekly intensity of *N. ceranae* infection in colonies with added vs. natural (not added) *N. ceranae* spores. Addition of *N. ceranae* spores to half of the colonies began in week 2. *denotes significant difference ($P \leq 0.05$) in *N. ceranae* levels between treatments.

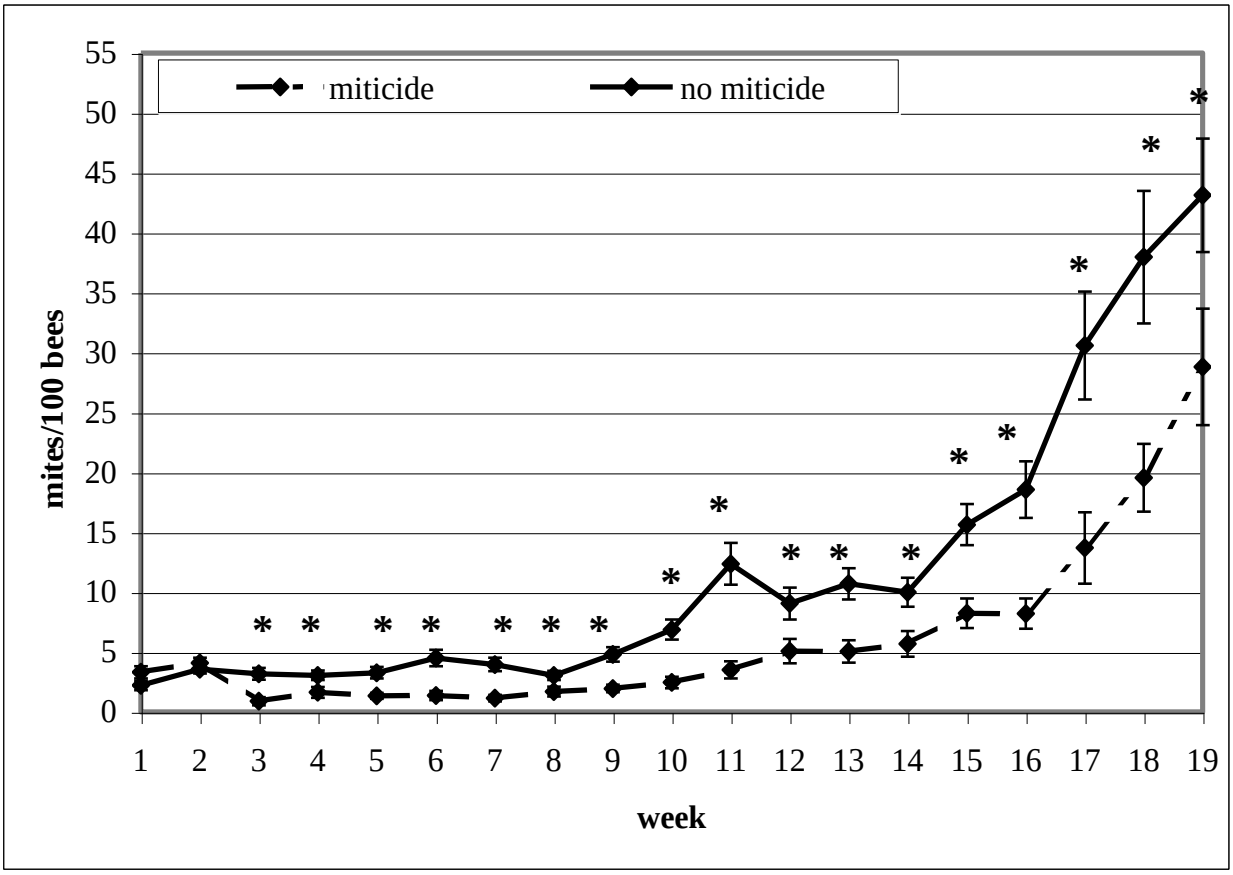

Figure 2. Mean weekly levels of *V. destructor* in colonies treated and untreated with miticide (formic acid). Formic acid pads were placed inside half of the colonies on week 2 and removed on week 5. *denotes significant difference ($P \leq 0.05$) in *V. destructor* level between treatments.

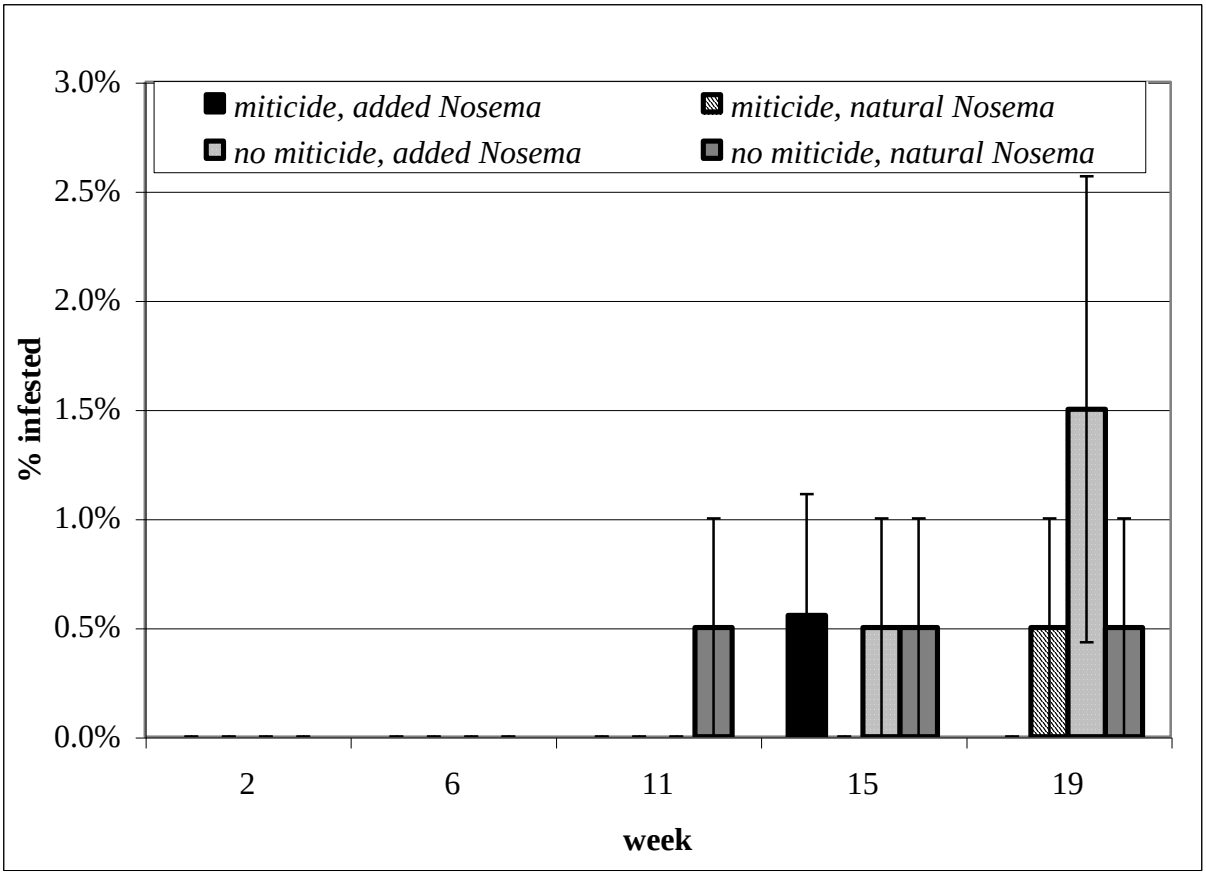

Figure 3. Mean monthly percent of bees infected with *A. woodi* in colonies in each treatment, (P> 0.05).

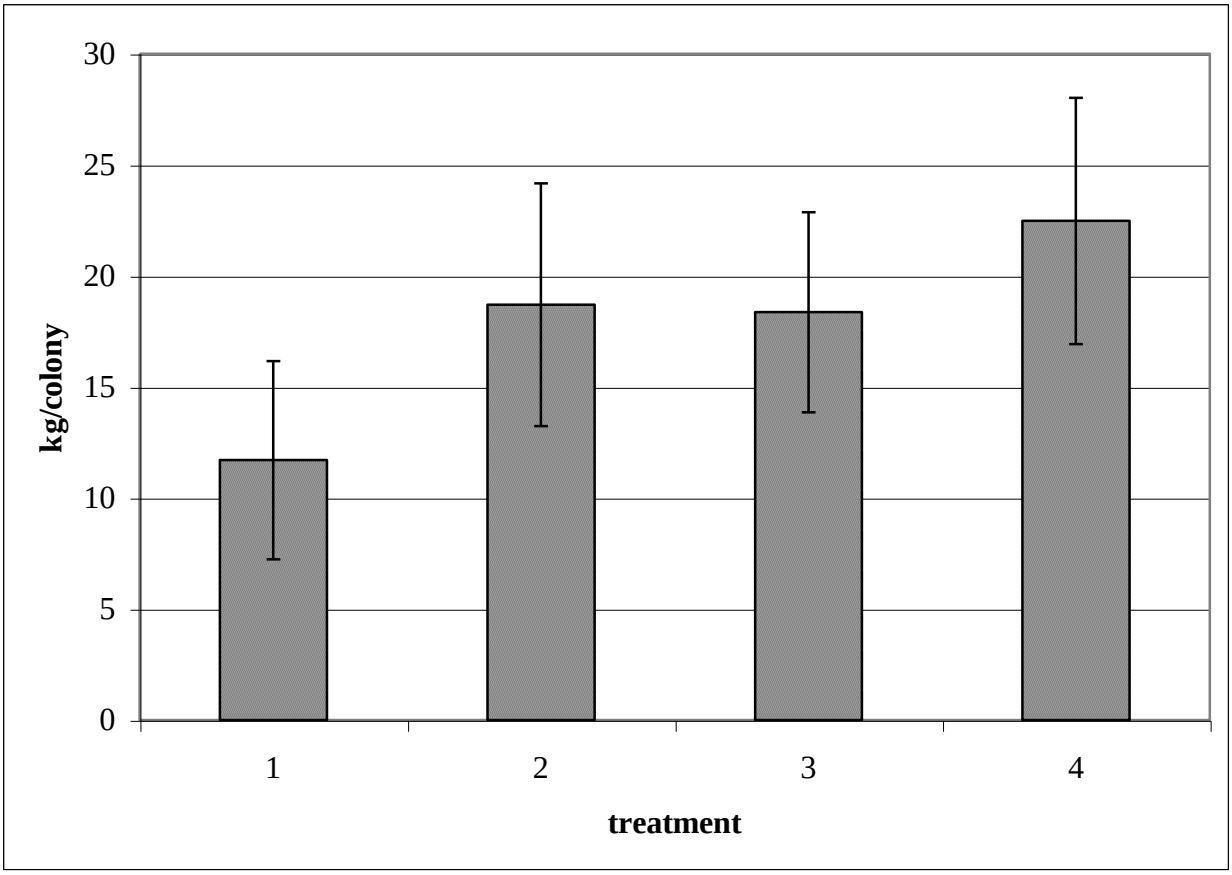

Figure 4. Mean kg of honey produced in colonies at each treatment, (P> 0.05).

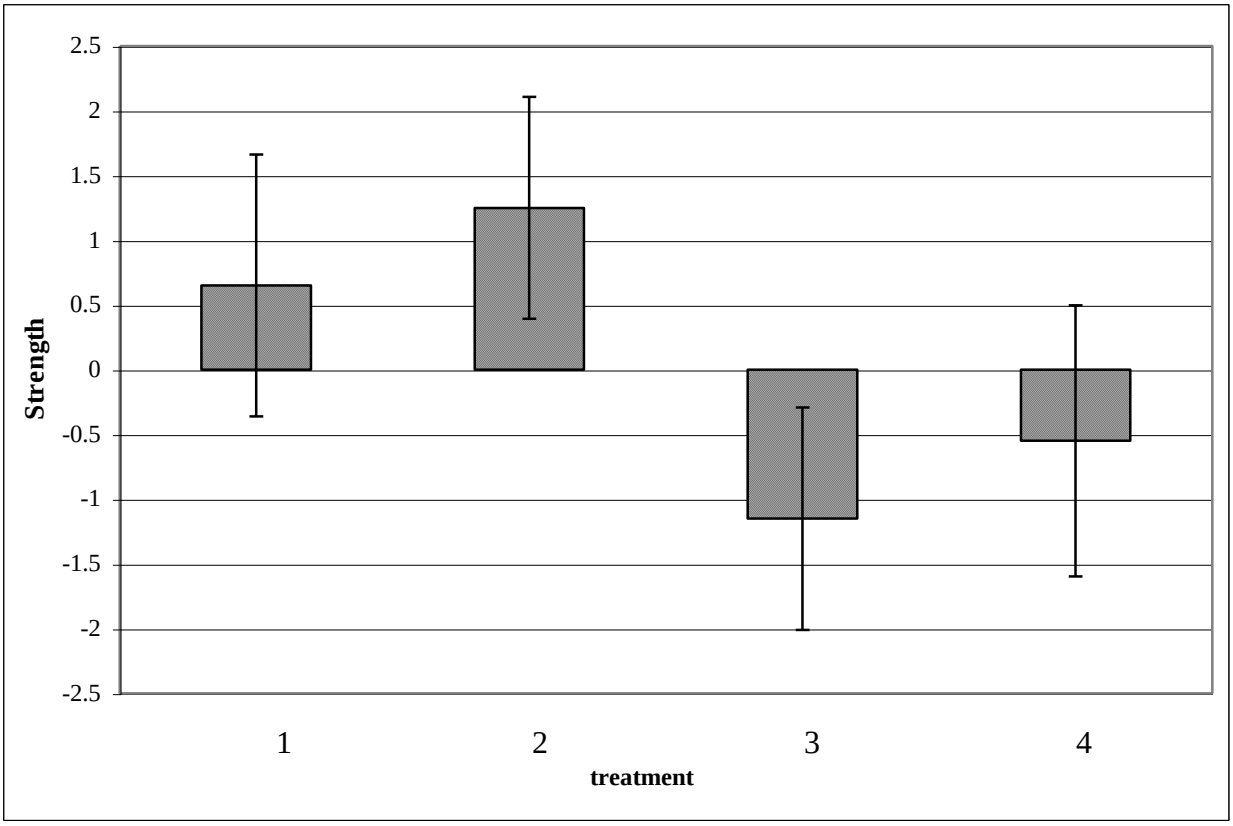

Figure 6. Change in colony strength between treatments. Strength is the mean total number of frames of bees and brood at the end – beginning, (P> 0.05).