Nosema ceranae: A sweet surprise? Investigating the viability and infectivity of the honey bee (*Apis mellifera* L.) parasite *N. ceranae*

by

Courtney Ivy MacInnis

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Ecology

Department of Biological Sciences

University of Alberta

Abstract

Nosema disease is a prominent malady among adult honey bees (*Apis mellifera* L.), caused by the microsporidian parasites *Nosema apis* and *N. ceranae*. The biology of *N. apis* is well understood, as this parasite was first described over a century ago. Unlike *N. apis*, *N. ceranae* is an emerging parasite of the honey bee, and consequently, we do not yet understand how long spores of this parasite survive in honey bee colonies, or how they are transmitted among bees. We investigated the viability and infectivity of the infectious (spore) stage of *N. ceranae* in substrates associated with honey bee colonies after exposure to 20, 33, -12, and -20°C, over various time intervals. Spores stored in honey and sugar syrup survived considerably longer than those stored in water or on wax comb, with low loss in viability at freezing temperatures for up to one year. Honey and sugar syrup appear to provide a reservoir of viable and infective spores that can initiate or perpetuate *N. ceranae* infections in honey bee colonies. This study provides information that may help enhance current management recommendations for apiculturalists.

Dedication

To my mom and dad, Donna and Ian MacInnis. For every bonfire, boat ride, bake-a-thon, and night at Nagagami I've missed over the last three and a half years. For every time you've packed me up and dropped me off at an airport, this is for you. You are my sunshine.

Acknowledgments

First and foremost, I would like to express my gratitude to my graduate advisors, Dr. Stephen F. Pernal, and Dr. B. Andrew Keddie. Steve, though often busier than what I would consider humanly possible, has always humoured me by inviting me into his office to ask questions each time I've stopped at his half-open door asking "have you got five?", knowing that I would almost always require more than five minutes of his time. Steve has provided numerous edits and suggestions not only to this thesis, but nearly every proposal and oral presentation I've worked on over the course of my program, for which I am forever grateful. Thanks for taking a chance on me four years ago, I certainly owe all of my success to you.

Andy Keddie has certainly fostered my critical thinking skills, and is the reason I ask "why" so often. His enthusiasm for his work is contagious, and I like to think it's rubbed off on me. Andy has always encouraged me to do "more", and he has also provided numerous edits to this thesis, for which I am thankful. I would also like to thank Dr. Jessamyn Manson, who served as a member of my supervisory committee, and provided advice to me on numerous topics on numerous occasions.

Thanks also go out to various members of the Pernal Bee Lab, past and present, many of whom are more like family to me than lab mates. Abdullah (Father *Nosema*), for imparting all his cage experiment knowledge onto me. Amanda, for her help with staining techniques. Amara, for showing me the ropes in the North (molecular biology) Lab. Elena, for always making sure my beekeeping skills are up to snuff. Jamie, for assisting with all my cage experiments, and Marta and Renata for providing constructive feedback on many applications and presentations.

Thanks to the National Bee Diagnostic Centre for providing me with'round-the - clock access to their fluorescence microscope. I might still be counting spores if it weren't for you guys. Patricia and Jamie, thanks for always providing an ear when I needed to babble about *N. ceranae* for hours on end. Emily, thanks for always listening to me after I've said, "I'm leaving after this glass of water" ...five refills later.

Thanks also to my partner in crime, and office mate, Amanda (Weevilbee). Thank-you for your encouragement and friendship, and of course, for putting up with all of my terrible puns. My graduate school experience would have been much less fun without you!

I would like to thank Agriculture and Agri-Food Canada, and the University of Alberta for providing funding for this project.

To all of the influential people in my life, many of whom reside in, or come from a small town in northern Ontario, thanks for teaching me about community, and that it's all right to be different. Your altruism is inspiring, and I wouldn't be here without you!

Lastly, to all of the honey bees that unknowingly sacrificed themselves for this project, I wish I could properly repay you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
ANOVA	Analysis of Variance
CCD	Colony Collapse Disorder
d.p.i.	Days Post Inoculation
DAPI	4’ 6-Diamidino-2-phenylindole
FBS	Fetal Bovine Serum
HSD	Honest Significant Differences
ID	Infectious Dose
NEB	Newly-Emerged Bee
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
p.i.	Post Inoculation
PI	Propidium Iodide
PTP	Polar Tube Protein
RH	Relative Humidity
RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal RNA
sdH_2O	Sterile Distilled Water
ssrDNA	Small Subunit Ribosomal DNA
ssurRNA	Small Subunit Ribosomal RNA

Chapter 1: Literature Review

1.1 The Microsporidia

The term "microsporidia" is used to describe a group of obligate, intracellular, eukaryotic parasites that are capable of infecting a variety of terrestrial and aquatic hosts. The first identification of a microsporidian parasite occurred in 1857 (Nägeli 1857). Näegeli identified the causative agent of pébrine or "pepper disease" in *Bombyx mori* L. as *Nosema bombycis* (Näegeli); the parasite was ravaging the European silk industry at the time (Nägeli 1857). Since the identification of *N. bombycis* in 1857, microsporidians have negatively impacted several other industries, most notably the beekeeping (Becnel and Andreadis 1999) and fisheries (Shaw and Kent 1999) industries. The microsporidia are also widely recognized within the medical field due to their association with immunocompromised individuals, particularly those suffering from human immunodeficiency virus or acquired immune deficiency syndrome (Didier and Weiss 2012).

There are presently more than 1, 200 identified species of microsporidia classified into 200 genera (Becnel et al. 2014). The microsporidia are currently phylogenetically classified based on small subunit ribosomal DNA (ssrDNA) sequences (Vossbrinck and Debrunner-Vossbrinck 2005, Vossbrinck et al. 2014); five clades have emerged as a result of this classification. Vossbrinck and Debrunner-Vossbrinck (2005) further separated these five clades into three classes: Marinosporidia, Aquasporidia, and Terrosporidia, based upon host habitat. Vossbrinck et al. (2014) indicated that while

there is a strong correlation between host habitat and taxonomic grouping, overlap does occur in several classes.

Molecular phylogenetic analysis of protein encoding genes for α and β-tubulin (Baldauf et al. 2000, Keeling et al. 2000), tubulin (Baldauf et al. 2000), elongation factor-1α (Baldauf et al. 2000), and TATA box binding protein (Fast et al. 1999) as well as re-analysis of large subunit rRNA (Van de Peer et al. 2000) have revealed that microsporidia are eukaryotic in origin with a fungal relationship. Several genes encoding mitochondrial heat shock protein 70 (Germot et al. 1997, Hirt et al. 1997, Peyretaillade et al. 1998), and the largest subunit of RNA polymerase II (Hirt et al. 1999) have also confirmed this relationship. Sequencing of the *Encephalitozoon cuniculi* Levaditi et al. 1923 genome extended the evidence of fungal origin, placing microsporidia as a sister group of fungi (Katinka et al. 2001). James et al. (2013) and Letcher et al. (2013) have classfied the Microsporidia as a member of the Phylum Cryptomycota, in the basal fungi, along with *Rozella* spp. Cornu and *Aphelidea* Karpov et al. 2014.

1.2 Morphology of the Microsporidian Spore

The Microsporidia are unicellular eukaryotic parasites that possess a number of unique features. The infectious (spore) stage of the parasite, is environmentally resistant, and is the only stage of the parasite capable of surviving outside of the host (Vávra and Larsson 2014). Because of its environmental sturdiness and ease of visualization, the spore is often used for diagnostic purposes (Cali and Takvorian 2014). Microsporidian spores range from 1-40 μm in length, and are typically oval to pyriform in shape, although rod-like (*Resiomeria odonatae* Larsson) and round (*Coccospora micrococcus*

Léger and Hesse) spores do occur (Vávra and Larsson 2014). Microsporidian spores possess a thick wall comprised of three layers: an electron dense exospore, a thick chitinous endospore, and a plasma membrane (Cali and Takvorian 2014). This wall encompasses the organelles and other components interior to the spore, including the nuclei, posterior vacuole, polarplast and the infection apparatus, the polar filament. Under conducive conditions (e.g., correct ion concentration, pH, temperature), the polar filament everts and injects the infectious sporoplasm into a host cell, initiating the reproductive process (James et al. 2006, Vávra and Larsson 2014).

1.3 Transmission of Microsporidia

Microsporidian spores infect new, susceptible hosts via one of two routes: vertical or horizontal transmission (Dunn and Smith 2001). Vertical transmission is characterized by low virulence (Dunn and Smith 2001); it involves the passage of the parasite from parent to progeny, often transovarially in invertebrates (Fine 1975). It is through this mechanism that *Amblyospora* spp. Hazard and Oldacre 1975 and *Thelohania* spp. Henneguy 1892 maintain populations within their mosquito hosts (Kellen et al. 1966, Andreadis and Hall 1979). In mammals, vertical transmission via a transplacental mechanism has been observed (Baneux and Pognan 2003). Horizontal transmission is the more common route of transmission utilized by the microsporidia (Stentiford and Dunn 2014), which in invertebrates often involves the ingestion of spores from the environment, followed by infection of the midgut (De Graaf et al. 1994a, Vávra and Larsson 2014). It is likely to select for high parasite replication and virulence, as

transmission to a new, susceptible host often relies on ingestion of the parasite (Ebert and Herre 1996).

1.4 Microsporidian Life Cycle

The microsporidian life cycle can be divided into three separate stages, a vegetative reproductive stage (merogony), a spore-producing stage (sporogony), and an infectious (spore) stage (Vávra and Larsson 2014). Microsporidian reproduction occurs via binary fission, schizogony or plasmotomy; the method is dependent upon the species (Vávra and Larsson 2014). Here an overview of reproduction via binary fission is described, as it is common among *Nosema* spp. (Cali and Takvorian 2014), and is the method by which *Nosema apis* Zander and *Nosema ceranae* Fries et al. 1996 reproduce (Fries 1993, Fries et al. 1996).

Binary fission in microsporidia has been well-described in several previous studies, including: Fries (1993), Fries et al. (1996), Vávra and Larsson (1999), Gisder et al. (2011) and Cali and Takvorian (2014). Briefly, when a microsporidian spore has reached its target tissue (e.g. a host midgut), where conditions are ideal, germination is triggered resulting in the eversion of the polar filament (Figure 1). If the everted filament punctures an appropriate cell, the infective sporoplasm (diplokaryotic in *N. apis* and *N. ceranae*) passes through and enters the host cell to commence reproduction (Figure 2). This process is the beginning of merogony; shortly after infection the sporoplasm matures into a meront, which then produces daughter cells through nuclear fission. In some species of microsporidia (e.g. *Nosema* spp.) several rounds of merogony take place; two are known to occur in *N. apis* (Gray et al. 1969; Fries 1993) and at least two in

N. ceranae (Gisder et al. 2011). The end of merogony and beginning of sporogony is delineated by the deposition of electron-dense material onto the plasma membrane of the daughter meronts, now referred to as sporonts. The sporonts divide once, producing two sporoblasts which, after polarization occurs, are referred to as spores (Larsson 1986). These mature, infective spores are then released from the original infected host (e.g. via feces, or from a cadaver) in order for the parasite to infect a new host (Cali and Takvorian 2014).

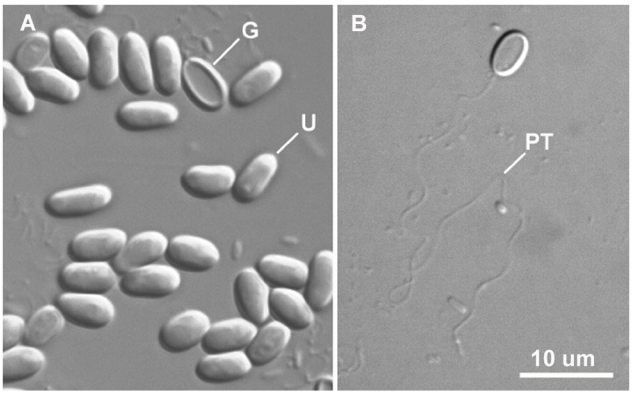

Figure 1. Light micrograph of *Antonospora locustae* Canning 1953 with pressure-induced polar tube eversion. A) Germinated (G) and ungerminated (U) spores. B) Germinated spore where the polar filament (PT) has everted and is visible.[1]

[1]Figure reprinted from Keeling, P. (2009) Five questions about microsporidia. PLoS Pathog **5**(9), e1000489.

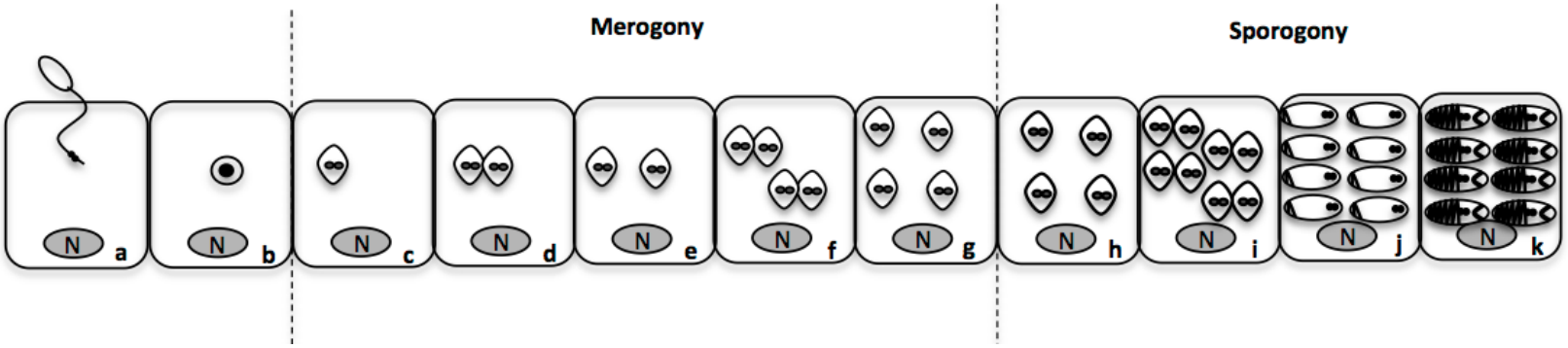

Figure 2 *N. ceranae* life cycle. a) A mature spore germinates, its polar filament punctures a honey bee midgut epithelial cell, and it injects infective sporoplasm. b) Sporoplasm within a honey bee midgut epithelial cell. c) The sporoplasm matures into a meront. d) The meront undergoes binary fission, producing paired meronts. e-g) Another round of merogony. h) Daughter meronts mature into sporonts. i) Sporonts will divide once. j) Sporonts mature into sporoblasts. k) Sporoblasts mature, and are now referred to as spores.[2]

[2]Figure adapted from Gisder S., Möckel, N., Linde, A., Genersch, E. (2011) A cell culture model for *Nosema ceranae* and *Nosema apis* allows new insights into the life cycle of these important honey bee-pathogenic microsporidia. Env Microbiol **13**, 404-413.

1.5 The Genus Nosema

Most entomophathogenic microsporidia occur within the genus *Nosema*. One of the most specious genera, it is comprised of over 150 species infecting at least 12 orders of insects, especially members of Diptera and Lepidoptera (Becnel and Andreadis 2014). As *Nosema* spp. are parasitic, infected hosts are often negatively impacted; depending on the host species, this can either help or hinder various industries (Becnel and Andreadis 2014). For example, *Nosema pyraustae* Paillot can be used as a biological control agent to manage populations of *Ostrinia nubilalis* Hübner (European Corn Borer), a serious pest of maïze (Lewis 1978, Lewis and Lynch 1978). This microsporidian positively impacts the agriculture sector, while *N. apis* and *N. ceranae* parasitize the western honey bee (*Apis mellifera* L.), negatively impacting agriculture, and the beekeeping industry (Fries 1993, Higes et al. 2013a).

Nosema spp. sustain a Class I host-parasite interfacial relationship with their hosts, whereby the parasite plasmalemma maintains direct contact with the host-cell cytoplasm (Cali and Owen 1988). In general, *Nosema* spp. remain diplokaryotic and diplosporoblastic throughout their lifecycles (Sprague 1978, Cali and Takvorian 2014) although there are exceptions e.g., *Nosema locustae* Canning 1953 (Canning 1953). *Nosema* spp. infections can be transmitted vertically (e.g., *Nosema granulosis* Terry et al. 1999 in *Gammarus duebeni* Liljeborg) (Terry et al. 1999), horizontally (e.g., *N. apis* in *A. mellifera* (Bailey 1955)), or both vertically and horizontally (e.g., *N. bombi* Fantham and Porter in *Bombus* spp. Latreille) (Rutrecht and Brown 2008). Host tissues infected by *Nosema* spp. vary with species, while tissue tropism is common, strict tissue tropism

within the genus is rare, although it does occur. It was previously thought that *N. apis* and *N. ceranae* could infect the hypopharyngeal, salivary, mandibular and venom glands of the honey bee, and that *N. ceranae* could also parasitize the Malpighian tubules and fat body in addition to the midgut tissues (Chen et al. 2009, Copley and Jabaji 2011). Nevertheless, it is now understood that both parasites do, in fact, only infect the midgut portion of the honey bee digestive tract (Fries 1993, Fries et al. 1996, Huang and Solter 2013).

1.6 The Western Honey bee: Apis mellifera

The western honey bee, *A. mellifera*, is the world's most intensively managed pollinator required for the pollination of many fruit, vegetable, and high value cash crops. Klein et al. (2007) suggest that the honey bee is responsible for pollinating one third of the world's food crops. Its pollination services contribute an estimated $15 billion annually to U.S. agriculture (Calderone 2012) and $3-4 billion annually to Canadian agriculture. In Canada, this includes North America's supply of hybrid canola seed (Darrach and Page 2016). Unfortunately, the honey bee is susceptible to a variety of health threats, including a diverse group of parasites and pathogens (Pernal and Clay 2013). The abnormally high levels of honey bee colony losses experienced by Europe, North America, and several other regions over the last decade (Martín-Hernández et al. 2007, vanEngelsdorp et al. 2007, vanEngelsdorp et al. 2008, vanEngelsdorp et al. 2009, Currie et al. 2010, Genersch et al. 2010, Nguyen et al. 2010, vanEngelsdorp et al. 2011, van der Zee et al. 2012), have highlighted the importance of these pollinators, and led to

an era of research striving to improve management techniques for pathogens and overall honey bee health.

1.7 Nosema apis

Nosema disease is a prominent malady among adult honey bees caused by *N. apis* and *N. ceranae*. *N. apis* is a microsporidian parasite of the honey bee midgut first reported as causing disease in adult honey bees in 1857 (Dönhoff and Leuckart 1857). It was not until 1909, however, that the parasite was named and classified by Enoch Zander (Zander 1909). Given that *N. apis* has been observed in *A. mellifera* for over a century, there is a vast body of literature surrounding this host-parasite relationship. For a more extensive review of this literature, the reader is directed to Fries (1993).

Nosema apis is transmitted via a fecal-oral route (Bailey 1955) with contaminated comb providing the primary source of spores (Bailey 1981). Fries (1993) also states that robbing of a weak, *N. apis*-infected colony also spreads the parasite as the stores often contain spores. Once ingested by an adult honey bee, infective spores reach the midgut, germinate and reproduce (Fries 1993).

Gross pathology of a *N. apis* infection often includes: dysentery, crawling bees, queen supersedure (Farrar 1947, Bailey 1955, Bailey 1967, Bailey 1981) and a reduction in honey bee lifespan. Infection with *N. apis* at the colony level is often associated with reduced honey production (Farrar 1947, Cantwell and Shimaunki 1969, Fries et al. 1984), and increased winter mortality (Farrar 1942). Levels of *N. apis* are highest in the spring when winter bees that have been confined to the colony have not yet been replaced by young bees (Bailey 1955). The levels of *N. apis* remain low over the summer, and begin to increase in the fall (Bailey 1955).

Fumagilin-B® is the only registered chemotherapeutic available to treat infections caused by *N. apis*. This antibiotic was first isolated from the fungus *Aspergillus fumigatus* Fresenius by Hanson and Eble (1949). In the early 1950's, it was discovered that fumagillin was effective at suppressing *N. apis* infections in caged honey bees (Katznelson and Jamieson 1952, Bailey 1953). Acetic acid fumigation and heat treatment of *N. apis*-contaminated comb were also found to be effective methods for reducing *N. apis* infections in honey bee colonies (Bailey 1957, Shimanuki and Cantwell 1969). Until recently, it was thought that *N. apis* was the only microsporidian parasite capable of causing infections in adult honey bees (Fries 1997).

1.8 Nosema ceranae

1.8.1 Discovery and Distribution

Nosema ceranae was first described by Fries et al. (1996) in honey bees obtained from managed colonies of *Apis cerana* Fabricius south of Beijing, China. In 1997, Fries et al. established that *N. ceranae* was infective for *A. mellifera* in the laboratory (Fries 1997), but it was not until 2005 that the parasite was detected in populations of *A. mellifera*, first in Taiwan (Huang et al. 2007) and shortly thereafter in Spain (Higes et al. 2006). Samples of *A. mellifera* screened for the presence of *N. ceranae* have now revealed that the parasite has been present in populations of *A. mellifera* for much longer than a decade, as early as 1975 (Klee et al. 2007, Paxton et al. 2007, Chen et al. 2008, Williams et al. 2008a, Invernizzi et al. 2009, Currie et al. 2010, Guzmán-Novoa et al. 2011, Traver and Fell 2015). Infections likely went unnoticed as *N. ceranae* is microscopic (Paxton 2010) and of similar size to *N. apis* (Fries et al. 2006), normally requiring molecular analysis to differentiate between the two species (Martín-Hernández

et al. 2007). *Nosema ceranae* is now nearly globally distributed (Williams et al. 2014), although *N. apis* is still more prevalent in several localities including Germany, Sweden, and Norway (Gisder et al. 2010, Forsgren and Fries 2013). *Nosema ceranae* has been present in *A. mellifera* for at least 40 years (Traver and Fell 2015), but is also capable of generating infections in *B. atratus* Franklin, *B. morio* (Swederus 1787), *B. bellicosus* Smith and *B. terrestris* L. (Plischuk et al. 2009, Fürst et al. 2014), as well as *A. cerana*, *A. dorsata* Fabricius, and *A. florea* Fabricius (Chaimanee et al. 2010, Botías et al. 2012). The parasite can also infect all castes of *A. mellifera* (Fries 1997, Fries et al. 2006, Huang et al. 2007, Alaux et al. 2011, Traver and Fell 2011a). It is now considered to be the dominant species of *Nosema* infecting honey bees in many parts of the world (Chauzat et al. 2007, Paxton et al. 2007, Williams et al. 2008b, Invernizzi et al. 2009, Tapaszti et al. 2009, Stevanovic et al. 2011, Traver and Fell 2011b, Martín-Hernández et al. 2012, Emsen et al. 2016), and is considered to be more virulent than *N. apis* by some (Higes et al. 2007, Martín-Hernández et al. 2011, Williams et al. 2014), but not others (Forsgren and Fries 2010, Huang et al. 2015, Milbrath et al. 2015, Natsopoulou et al. 2016).

1.8.2 Phylogeny of N. ceranae

Fries et al. (1996) conducted the first phylogenetic analysis of *N. ceranae* based on 16S small subunit rRNA (SSUrRNA) gene sequences, and found that *N. ceranae* was more closely related to *Vairimorpha necatrix* Kramer than *N. apis*, while a subsequent study by Chen et al. (2009a) found *N. ceranae* to be more closely related to *N. bombi* Fanthem and Porter than *N. apis* using SSUrRNA sequences. Again using SSUrRNA sequences, Chen et al. (2009b) identified five separate clades of *N. ceranae* from infected *A. cerana* collected from three separate Asian locales (China, Japan, and Taiwan) and one

from *A. mellifera* collected in the United States (U.S.). The U.S. isolate was distinctly separate from the four Asian clades, which had some degree of clustering. Shafer et al. (2009) added large subunit (LS) and retained SSUrRNA to construct a phylogeny of *Nosema* spp. infecting bees. They also described a sister relationship between *N. ceranae* and *N. bombi*, and placed *N. apis* as a basal member of the group.

Previously, Tay et al. (2005) and O'Mahony et al. (2007) indicated that rRNA sequencing may not be the best method for characterizing microsporidian species because of the presence of multiple variable copies of rRNA first observed within a single spore of *N. bombi*. This polymorphism has also been observed in *N. ceranae* spores (Sagastume et al. 2011, Sagastume et al. 2014). O'Mahony et al. (2007) suggests that phylogenetic studies of microsporidia be conducted using single copy genes, or that authors first show evidence of concerted evolution within the multi-copy rRNA genes. Polar tube protein (PTP) genes (single copy genes) have recently been used to construct phylogenetic trees of *N. ceranae*, and they have been identified as reliable markers for genetic relationships at the species level (Chaimanee et al. 2011, van der Zee et al. 2014). Chaimanee et al. (2011) analyzed the relationship between isolates of *N. ceranae* obtained from *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* using both 16S rRNA and PTP gene sequences. All four isolates clustered together based on the rRNA sequences, but could be separated into three clades based upon PTP sequences.

A draft assembly of the *N. ceranae* genome has been generated using pyrosequencing (Cornman et al. 2009). This analysis has revealed that *N. ceranae* is quite different from yeast and other fungi with only a small portion of its genome devoted to transport and response to chemical stimuli, and a large fraction to growth-related

categories, likely reflecting *N. ceranae*'s parasitic nature (Fries 2010). This genome division is also consistent with a life cycle requiring rapid reproduction while being protected from the environment (Cornman et al. 2009). Using whole genome sequencing, Chen et al. (2013) identified genes and protein sequences coding for energy transport and stress response respectively, which again reflects *N. ceranae*'s parasitic nature. In addition, these sequences were more highly represented in *N. ceranae* than *N. apis*, suggesting that *N. ceranae* may have a better ability to import energy from host cells, and survive stressful situations.

1.8.3 N. ceranae Spore Morphology and Development

Nosema ceranae spores obtained from *A. mellifera* are ovocylindrical to rod-shaped (Fries et al. 1996, Chen et al. 2009), and vary in size with a length of 3.9-5.3μm and width of 2.0-2.5μm (Chen et al. 2009); they are, on average, 1μm shorter in length than *N. apis* spores (Fries 2010). *N. ceranae* spores possess a polar filament which is ~96-102nm wide (Fries et al. 1996), and has between 18-23 coils (Fries et al. 1996, Chen et al. 2009), fewer than the number of coils (>30) typically possessed by *N. apis* spores. The spores of *N. ceranae* are diplokaryotic (Fries et al. 1996, Chen et al. 2009, Gisder et al. 2011), and are surrounded by a 14-17nm thick, electron-dense exospore, and a much thicker (134-158nm) chitinous endospore, which has reduced thickness at the anterior end of the spore (Fries et al. 1996). As *N. ceranae* and *N. apis* are similar morphologically, they are often differentiated using molecular tools (Klee et al. 2007).

The midgut tissue of the honey bee is the only site of infection for *N. apis* and *N. ceranae* (Huang and Solter 2013). Spores are ingested by adult honey bees, which germinate within the midgut, if a spore's everted polar filament punctures an epithelial

cell, sporoplasm is injected and reproduction begins (Fries et al. 1996). Bailey (1972) and Fries (1988) suggest the median infectious dose (ID_{50}) for *N. apis* spores in laboratory experiments to be ~100 spores/bee. Forsgren and Fries (2010) and McGowan et al. (2016) report similar ID_{50}s for *N. ceranae* spores at 85 and 149 spores/bee respectively. In contrast, the ID_{50} reported by Huang et al. (2015) for *N. ceranae* spores ranges between 3,217-10,053 spores/bee depending upon the age of the bees at the time of infection. The variation between studies could be a result of experimental design, subspecies of *N. ceranae* or honey bee used, or differing environmental conditions.

The intracellular development of *N. ceranae* within the honey bee midgut epithelia appears to be similar to *N. apis* (Fries et al. 1996, Higes et al. 1997, Chen et al. 2009). As obligate parasites, both species rely on their hosts for energy production, reproductive stages possess mitosomes (reduced versions of mitochondria) which afford the parasites the ability to absorb ATP from host cells for reproduction (see Holt and Grozinger 2016). Approximately 3-4 days after initial infection, all developmental stages of *N. ceranae* can be found within host cells (Higes et al. 2007, Gisder et al. 2011). The presence of all developmental stages and empty spores within midgut epithelial cells indicates that intracellular germination occurs (Fries et al. 1996, Higes et al. 2007, Chen et al. 2009) as it does with *N. apis* (De Graaf et al. 1994b). *N. ceranae* infections are considered fully developed approximately 12-18 days after initial infection occurs (Forsgren and Fries 2010, Huang and Solter 2013), and produce approximately 8×10^6 spores/day (Huang and Solter 2013).

1.8.4 N. ceranae Pathology at the Individual Level

The negative impacts of infection with *N. ceranae* at the individual bee level are more immediately observable than those at the colony level. Infected queens produce above average levels of queen mandibular pheromone, which may signify a sick queen (Alaux et al. 2011). In worker bees experimentally infected with *N. apis* versus *N. ceranae*, immune system suppression has been observed in those infected with *N. ceranae* but not with *N. apis* (Antúnez et al. 2009). *Nosema ceranae* reduces apoptosis in infected honey bee ventricular epithelial cells, extending their period of growth and development (Higes et al. 2013b, Kurze et al. 2015). The parasite also induces energetic stress (Mayack and Naug 2009) and degenerates midgut tissues (Dussaubat et al. 2012).

Nosema ceranae-infected bees further experience increased sugar demand and consumption compared with uninfected or *N. apis*-infected bees (Mayack and Naug 2009, Naug and Gibbs 2009, Martín-Hernández et al. 2011). Infections with *N. ceranae* also result in reduced lifespans (Higes et al. 2007, Mayack and Naug 2009, Martín-Hernández et al. 2011, Dussaubat et al. 2012, Dussaubat et al. 2013, Goblirsch et al. 2013, Milbrath et al. 2013, Milbrath et al. 2015), and impaired flight behaviour (Dussaubat et al. 2013) but produce no measurable impact on learning or memory (Charbonneau et al. 2016). The parasite also reduces the nursing ability of young bees and leads to precocious foraging (Mayack and Naug 2009, Goblirsch et al. 2013). Synergistic effects between *Nosema* spp. and several classes of pesticides on honey bee mortality have been observed for honey bees infected with *Nosema* spp. (Alaux et al. 2009, Vidau et al. 2011, Aufauvre et al. 2012, Pettis et al. 2013), although this synergistic relationship may be dose-dependent (Pettis et al. 2013, Retschnig et al. 2014).

1.8.5 N. ceranae Pathology at the Colony Level

In contrast with *N. apis, N. ceranae* is often referred to as a "dry nosema" because dysentery is not a symptom commonly associated with latter parasite (Faucon 2005). In fact, there are very few outward, colony-level symptoms associated with *N. ceranae* infections (Fries et al. 2006, Higes et al. 2010a). Colony-level symptoms typical of a *N. apis* infection i.e., sick/crawling bees, bees with dilated abdomens, and dysentery are not associated with *N. ceranae* infections, making field diagnoses and treatment decisions difficult (Higes et al. 2010a).

At the colony level, impacts of infection with *N. ceranae* also appear to vary with geographic location. In Spain, infection with *N. ceranae* is associated with a decrease in brood rearing capacity, colony size, honey production and eventual colony collapse (Higes et al. 2008b, Higes et al. 2009, Botías et al. 2013). Colony Collapse Disorder (CCD) is a syndrome characterized by several specific symptoms, including: 1) a rapid loss of adult bees, 2) few dead bees within/surrounding the affected colony, and 3) delayed invasion of pests (Cox-Foster et al. 2007, vanEngelsdorp et al. 2009). In the U.S., descriptive analyses of CCD-affected apiaries versus healthy apiaries revealed that *N. ceranae* was only slightly more prevalent in colonies suffering from CCD (vanEngelsdorp et al. 2009), while metagenomic analyses revealed that the presence of both *Nosema* spp. in CCD-affected colonies was one of the few pathogenic variables examined that differentiated these two groups (Cox-Foster et al. 2007).

For the last decade, Canadian beekeepers have often reported *Nosema* spp. infections as factors contributing to winter colony mortality, and have treated for the infections prophylactically with Fumagilin-B® (Leboeuf et al. 2016). Long term

monitoring of honey bee colonies in Germany revealed no relationship between *N. ceranae* presence and colony mortality, with fewer than 5% of colonies monitored in the German Bee Monitoring Project classified as heavily infected with *Nosema* spp. spores (Genersch et al. 2010, Gisder et al. 2010).

The intense pathology associated with *N. ceranae* in Spain could be the result of differential susceptibility of *A. mellifera iberiensis*, the subspecies of honey bee endemic to the Iberian Peninsula, compared with other subspecies of *A. mellifera* (Paxton 2010). Other reasons purported for greater levels of colony mortality include possible increased virulence of *N. ceranae* strains in Spain compared with those found elsewhere (Williams et al. 2008a), or differences in the parasite's seasonality (Martín-Hernández et al. 2007). Martín-Hernández et al. (2007) reported an absence of seasonality associated with the parasite in Spain, while seasonal fluctuations similar to those traditionally observed with *N. apis* have been observed elsewhere (Williams et al. 2008b, Gisder et al. 2010, Williams et al. 2011, Copley and Jabaji 2012, Ibrahim et al. 2012, McGowan 2012, Traver et al. 2012), suggesting that regional differences contribute to the pathology caused by the parasite.

1.8.6 Treatment of N. ceranae

Fumagilin-B® is currently the only registered chemotherapeutic treatment registered to manage *N. ceranae* infections in North America (Williams et al. 2008b, Higes et al. 2011, Williams et al. 2011, van den Heever et al. 2015a). Its use in the European Union is permitted only under specific circumstances (Stevanovic et al. 2013). Several other compounds have been evaluated for biological activity against *N. ceranae* including thymol, resveratrol, several plant-based extracts, synthetic compounds and

bacterial metabolites (Maistrello et al. 2008, Costa et al. 2010, Porrini et al. 2010, Porrini et al. 2011, van den Heever et al. 2015a). Caged bees administered thymol or resveratrol-treated candy in one study had significantly reduced spore loads when compared to an untreated control (Maistrello et al. 2008). Costa et al. (2010) also found that caged bees fed thymol or resveratrol-treated candy or sugar syrup had reduced spore loads when compared to untreated cages. Spore loads within the treatment groups, however, gradually increased over time, with the exception of the thymol-candy group which did experience an ~30% reduction in spore load between the third and fourth (last) time points (Costa et al. 2010). Porrini et al (2010) administered surfactin S2, a bacterial metabolite produced by *Bacillus subtilus* C4 Cohn to caged *N. ceranae*-infected bees, and found that the compound was only mildly successful at reducing parasite number when compared to an untreated control. When thymol was directly compared to Fumagilin-B® at a dose concentration approximating the manufacturer's recommendation, the spore load was ~13× lower for bees in the Fumagilin-B® group while bee mortality for this group was ~1.5× higher than that of the thymol group (van den Heever et al. 2015a).

Unfortunately, Fumagilin-B®, at low concentrations, may exacerbate *N. ceranae* infections (Huang et al. 2013) and dicyclohexylamine, a salt present in the commercial formulation of Fumagilin-B® may have potential toxic side effects in adult honey bees (van den Heever et al. 2015b). In addition, Fumagilin-B® only inactivates the reproductive stages and not the infectious (spore) stage of *N. ceranae*. Therefore, when Fumagilin-B® is applied to an active *N. ceranae* infection, it suppresses the vegetative growth of the parasite rather than killing the spores. *N. ceranae* is an emerging parasite of the honey bee which does not generate any overt symptoms (Fries et al. 2006, Higes et

al. 2008b) and is an organism for which we do not yet fully understand mechanisms of transmission. Consequently, it is difficult to devise effective, economical and chemical-free strategies for beekeepers to manage *N. ceranae* infections and maintain healthy honey bees.

1.8.7 Survival of N. ceranae Spores at Different Temperatures

Several studies have examined the survival of *N. ceranae* spores under laboratory conditions and many have suggested that the organism is thermotolerant and sensitive to low temperatures (Fenoy et al. 2009, Martín-Hernández et al. 2009, Higes et al. 2010b, Sánchez Collado et al. 2014). Fries and Forsgren (2009) examined the infectivity of *N. ceranae* and *N. apis* spores in suspension after one week of exposure at 8°C (refrigerator) or -18°C (freezer). *Nosema apis* was 100% infective after exposure to -18°C while *N. ceranae* infected <20% of inoculated bees after the same exposure. Fenoy et al. (2009) measured the viability of *N. ceranae* spores in 1×PBS (phosphate-buffered saline) up to one or three months of exposure at 4, 35 or 60°C, and in RPMI (Roswell Park Memorial Institute medium), FBS (fetal bovine serum), or air dried onto glass slides after one or three weeks at -20°C. Viability was also examined for autoclaved *N. ceranae* spores, *N. ceranae* spores air dried at room temperature for up to one week, and for *N. ceranae* and *N. apis* in 1×PBS at 4°C for up to one year. For *N. ceranae* spores stored in 1×PBS and exposed to 4, 35 or 60°C, there was only a significant reduction in viability for spores stored at 4°C after 3 months. There was a significant reduction in viability for spores exposed to -20°C (frozen) in RPMI, FBS, and air dried onto glass slides. Autoclaving for 30 minutes totally inactivated *N. ceranae* spores, while spores air dried onto slides at room temperature for a week experienced no reduction in viability. After one year of

exposure to 4°C in 1×PBS, both *N. apis* and *N. ceranae* experienced an ~70% reduction in viability.

Martín-Hernández et al. (2009) inoculated *Nosema* spp.-free bees with spores of either *N. apis* or *N. ceranae* and caged and incubated the bees at either 25, 33 or 37°C. At several time points, they removed live bees to perform spore counts and histopatholigcal studies. At 33°C, the number of infected bees at each time point was similar, however *N. ceranae*-infected bees always had much higher spore counts. At 37°C, the proportion of infected bees was higher for *N. ceranae* than *N. apis* at each time point, and the spore counts were again, always higher for the *N. ceranae* group. Higes et al. (2010b) examined the differential development of both *N. ceranae* and *N. apis* infections at increasing temperature. After inoculating honey bees with either *N. ceranae* or *N. apis*, bees were caged and placed in an incubator at 33°C. At 4 d.p.i. bees were examined for parasite presence and immature stages of both parasites were visualized in the midgut epithelia of the infected bees. Remaining infected bees were then incubated at 37.2°C for 5 days. At 9 d.p.i, no immature stages of *N. apis* were observed in the *N. apis*-infected bees, only mature spores that showed signs of degeneration were seen. For the *N. ceranae*-infected bees, immature stages of the parasite as well as infective, mature spores were observed. After maintaining the cages at 37.2°C for an additional two days (11 d.p.i), there was no evidence of a microsporidian infection in *N. apis*-infected bees, while *N. ceranae* infections continued to proliferate.

Sánchez Collado et al. (2014) studied the long-term viability of *N. apis* and *N. ceranae* spores in water after exposure to several temperatures (33, 25, 4, -20°C). *Nosema ceranae* spores experienced high mortality after exposure to -20°C, and at the

last measured time point (317 d.p.i) when infectivity was evaluated, *N. ceranae* spores maintained at -20°C were significantly less infective than *N. apis* spores maintained at -20°C for the same length of time. Only one study has found evidence for cold tolerance of *N. ceranae* spores with only a 24.2% reduction in spore viability after 3 months of storage in 10% glycerol at -70°C (McGowan et al. 2016).

Despite the parasite's apparent cold sensitivity for all but laboratory cryogenic storage conditions, it persists and proliferates in cold, temperate climates as well as warmer climates, putatively displacing *N. apis* (Chauzat et al. 2007, Paxton et al. 2007, Williams et al. 2008b, Invernizzi et al. 2009, Tapaszti et al. 2009, Stevanovic et al. 2011, Traver and Fell 2011a, Martín-Hernández et al. 2012, Emsen et al. 2016). What the above studies on *N. ceranae* spore viability and infectivity illustrate is that spore survival is dependent upon the storage medium. Although infective *N. ceranae* spores have been detected in corbicular pollen (Higes et al. 2008a), and the parasite's DNA has been found in honey samples (Giersch et al. 2009), no published work currently exists on the patterns of *N. ceranae* spore viability and infectivity in media commonly associated with honey bee colonies.

1.8.8 Impetus for Research

Fumagilin-B® is the only registered chemotherapeutic available to manage *N. ceranae* infections (Williams et al. 2008b, Higes et al. 2011, Williams et al. 2011, van den Heever et al. 2015a). Fumagillin-based products have been on the market for over 60 years, and were originally designed to treat infections caused by *N. apis* (Katznelson and Jamieson 1952, Bailey 1953). Unfortunately, this product is not sporicidal (Katznelson and Jamieson 1952) and for commercial beekeepers can be quite expensive.

Here, I investigate the viability and infectivity of *N. ceranae* spores in media commonly associated with honey bee colonies (honey, pollen, water, beeswax and 2M sucrose syrup) in an effort to: 1) better understand how this parasite is transmitted, and 2) influence current management recommendations to provide beekeepers with economical, sustainable strategies to manage *N. ceranae* and improve honey bee health.

Chapter Two: Temporal Patterns of Spore Viability and Infectivity for the honey bee (Hymenoptera: Apidae) parasite *Nosema ceranae* under laboratory conditions.

2.1 Introduction

The Western honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), is the world's most intensively managed pollinator required for the pollination of many fruit, vegetable, drupe and high-value cash crops. Klein et al. (2007) suggest that the Western honey bee is responsible for pollinating one third of the world's food crops. Its pollination services contribute an estimated $15 billion annually to U.S. agriculture (Calderone 2012) and $3-4 billion annually to Canadian agriculture, the latter of which includes North America's supply of hybrid canola seed (Darrach and Page 2016). Unfortunately, the honey bee is vulnerable to a multitude of health threats including various pests and numerous parasites and pathogens (Pernal et al. 2013).

Nosema disease is a prominent malady among adult honey bees caused by the unicellular microsporidian parasites *Nosema apis* (Zander) and *N. ceranae* (Fries et al. 1996). *Nosema apis* was first described as a parasite of *A. mellifera* over a century ago (Zander 1909) and its epizootiology is well documented (Fries 1997). Unlike its congener, *N. ceranae* was originally described from the Asian honey bee (*A. cerana* Fabricius) in 1996 (Fries et al. 1996). Since its discovery, *N. ceranae* has been shown to be cross-infective, capable of generating infections in several species of *Bombus* (Plischuk et al. 2009, Fürst et al. 2014), *Apis* (Chaimanee et al. 2010, Botías et al. 2012) and all castes of *A. mellifera* (Fries 1997, Higes et al. 2006, Huang et al. 2007, Alaux et al. 2011, Traver and Fell 2011a). *Nosema ceranae* is an emergent parasite of the honey bee, it is widespread (Higes et al. 2006, Huang et al. 2007, Klee et al. 2007, Martín-

Hernández et al. 2007, Chen et al. 2008, Williams et al. 2008a, Currie et al. 2010) and is now considered to be the dominant species of *Nosema* infecting honey bees in many parts of the world (Chauzat et al. 2007, Paxton et al. 2007, Williams et al. 2008b, Invernizzi et al. 2009, Tapaszti et al. 2009, Stevanovic et al. 2011, Traver and Fell 2011b, Martín-Hernández et al. 2012, Emsen et al. 2016).

The effects of *N. ceranae* infections on individual bees have been well documented. For example, in workers, infection with *N. ceranae* decreases the lifespan and nursing ability of young bees (Higes et al. 2007, Goblirsch et al. 2013), leads to precocious foraging (Mayack and Naug 2009, Goblirsch et al. 2013), and impairs flight behaviour (Dussaubat et al. 2013). In addition, infection with *N. ceranae* suppresses the immune system (Antúnez et al. 2009), degenerates midgut tissues (Dussaubat et al. 2012), and induces energetic stress (Mayack and Naug 2009) in workers. In honey bee queens, infection with *N. ceranae* may lead to increased production of queen mandibular pheromone, which may serve as a signal for a failing or sick queens (Alaux et al. 2011). ,

Effects of *N. ceranae* on colonies of *A. mellifera* appear to vary with geographic location. Studies conducted in southwestern Europe have shown that infection with *N. ceranae* is associated with a decrease in colony size, brood rearing capacity, and honey production, and that the parasite may cause colony collapse disorder (CCD) (Higes et al. 2008b, Higes et al. 2009, Botías et al. 2013). In the U.S., CCD-affected colonies were found to exhibit only slightly higher, non-significant, prevalence and loads of *N. ceranae* than control colonies (vanEngelsdorp et al. 2009) while metagenomic analyses showed that co-infection with both *Nosema* species, rather than *N. ceranae* alone, was one of few pathogenic measures that differentiated colonies with CCD from those that were healthy

(Cox-Foster et al. 2007). In contrast, studies in western Europe have shown no relationship between *N. ceranae* presence and colony mortality (Genersch et al. 2010, Gisder et al. 2010).

Fumagilin-B® is currently the only effective, registered chemotherapeutic available to treat infections caused by *N. ceranae* (Williams et al. 2008b, Higes et al. 2011, Williams et al. 2011, van den Heever et al. 2015a). Fumagillin-based products have been on the market for over 60 years in North America, and were originally used to treat infections caused by *N. apis* (Katznelson and Jamieson 1952, Bailey 1953). At very low concentrations, Fumagilin-B® has been shown to exacerbate *N. ceranae* infections (Huang et al. 2013), and more recently, it has been demonstrated that the dicyclohexylamine salt in the commercial formulation of Fumagilin-B® may have potential toxic side effects in adult honey bees (van den Heever et al. 2015b). As *N. ceranae* is a relatively new parasite of the honey bee, and infection by the parasite does not generate any immediately overt symptoms (Faucon 2005, Fries et al. 2006, Higes et al. 2008b), our understanding of how this parasite is transmitted among individuals and colonies is incomplete, thereby precluding generation of effective disease management strategies.

Several studies have examined the survival of *N. ceranae* spores under laboratory conditions or within honey bees at high temperatures. Most have described the organism as thermotolerant and sensitive to low temperatures (Fenoy et al. 2009, Fries and Forsgren 2009, Martín-Hernández et al. 2009, Higes et al. 2010b, Sánchez Collado et al. 2014), while only one has shown evidence for cold tolerance, albeit under cryogenic storage conditions (McGowan et al. 2016). The apparent lack of environmental cold

tolerance is somewhat enigmatic as *N. ceranae* survives and proliferates in temperate climates as well as warmer climates, putatively displacing *N. apis* (Chauzat et al. 2007, Paxton et al. 2007, Williams et al. 2008b, Invernizzi et al. 2009, Tapaszti et al. 2009, Stevanovic et al. 2011, Traver and Fell 2011a, Martín-Hernández et al. 2012, Emsen et al. 2016).

Despite infective spores being detected in corbicular pollen (Higes et al. 2008a), and the microsporidian's DNA being identified in honey samples (Giersch et al. 2009), no published work currently exists on the temporal patterns of spore viability and infectivity for *N. ceranae* within matrices of honey bee colonies. Here, I investigate the viability and infectivity of *N. ceranae* spores in products commonly associated with honey bee colonies under differing environmental conditions in an effort to: 1) understand how this parasite survives and may be transmitted, and 2) influence current management recommendations to reduce the spread of the parasite and improve honey bee health.

2.2 Materials and Methods

All research was conducted at or near the vicinity of Agriculture and Agri-Food Canada's (AAFC) Beaverlodge Research Farm, in Beaverlodge, Alberta, Canada.

2.2.1 Collection, Enumeration and Identification of Nosema ceranae Spores

In order to isolate sufficient quantities of the parasite for experimentation, honey bee colonies from apiaries at the Mountain Trail Facility of Beaverlodge Research Farm (55°11'43.0"N; 119°17'57.3"W) were sampled for the presence of *N. ceranae* in May of 2014 and 2015, as well as from a cooperating beekeeper's apiary near Girouxville,

Alberta in May 2015. Samples of 100-200 forager honey bees were collected from honey-containing frames, located on the periphery of each colony, using 120 ml specimen containers (Cat # 10804-038, VWR International, Mississauga, ON). Samples were placed on crushed ice after collection and stored at -20°C until further use.

A 60 worker bee composite sample (allowing for a 5% infection to be detected with 95% certainty (Fries et al. 1984)) from each honey frame was placed into a stomacher bag (Cat # BA6040, Seward Laboratory Systems Inc., Davie, FL, USA) with 60 ml 1× PBS (pH 7.4) (1ml/bee), and macerated for 1 minute with a paddle blender (Seward Stomacher® 80 Biomaster, Seward Laboratory Systems Inc., Davie, FL, USA). Approximately 1.0 ml of the resulting macerates were aliquoted out into 1.5 ml microcentrifuge tubes (Cat # CA20901-547, VWR International, Mississauga, ON, Canada) (two aliquots per macerated sample). The macerates were used to estimate the number of spores per bee, and for DNA extraction to determine *Nosema* spp. presence via conventional polymerase chain reactions (PCR) for each sample. Macerates were stored at -20°C and retained until species identification was complete.

The number of spores per bee was estimated for each sample by first thawing and vortexing an aliquot, and then loading 6µl of the macerate onto a Z30000 Helber Counting Chamber (Hawksley, Lancing, Sussex, UK). After the spores had settled, they were enumerated under phase-contrast microscopy at 400X magnification according to Cantwell (1970), using a Nikon Eclipse Ci or Zeiss Axiostar Plus stereoscope. One aliquot was evaluated per sample.

For *Nosema* spp. identification, macerates were thawed and vortexed. A 400µl aliquot from each macerate was taken and placed into a new 1.5ml microcentrifuge tube,

which was centrifuged at 16, 873×g (Eppendorf® 5418R, VWR International, Mississauga, ON) to remove the PBS from the aliquot. DNA was extracted from the aliquots using a Qiagen DNeasy Blood and Tissue Kit (Cat # 69506, Qiagen Inc., Valencia, CA), and the concentration of the extract was measured using a spectrophotometer (Nanodrop 2000C Spectrophotometer, Thermo Scientific, Wilmington, DE, USA). Between 50-100 ng of the extracted DNA was used for amplification via conventional PCR.

A multiplex PCR method was used to amplify the 16S rRNA gene of both *N. apis* and *N. ceranae* using species-specific primers (218-MITOC for *N. ceranae* and 321-APIS for *N. apis*) (Martín-Hernández et al. 2007). Illustra™ PuReTaq Ready-To-Go™ PCR Beads were used to prepare the PCR reactions (GE Healthcare Life Sciences, Baie d'Urfé, Quebec, Canada). The freeze-dried PCR reagents were rehydrated to a final volume of 25 µl by adding sterile nuclease free water, 0.5 µl of each species-specific forward and reverse primer (0.4µM final concentration), and DNA. Reactions were then placed into an Eppendorf® Mastercycler® proS thermalcycler (VWR International, Mississauga, ON, Canada), and the resultant PCR amplicons were visualized on a 2% agarose gel stained with SYBR® Safe DNA Gel Stain (Life Technologies Inc., Burlington, ON, Canada) for 35 minutes at 110V. Macerates from samples PCR-positive for *N. ceranae* only were used to generate inocula to infect newly-eclosed *Nosema* spp.-free bees to propagate *N. ceranae* spores for experiments.

2.2.2 Propagation of N. ceranae Spores

Frames of eclosing worker bees from colonies managed by AAFC's Apiculture Program in Beaverlodge, Alberta were collected and placed in incubators (Percival Models I36NLC8, I36NLC9 and I36NL, Percival Scientific Inc., Perry, IA, USA) at 33±1.0°C and 70±5% RH. In 2014, newly-eclosed adult bees (<24 hrs, free from *Nosema spp.* infections), from all frames were collected and pooled in a 36-litre plastic tote. Groups of 100 bees were then placed into wooden hoarding cages (115×92×76 mm I.D.) where they were supplied an inoculum of ~1.5×10^5 *N. ceranae* spores/bee in 3ml 60% w/w sucrose for 24 hours. After the inoculum was consumed, the bees were provisioned on 60% sugar syrup until harvest. In 2015, plastic hoarding cages (118×98×81 mm I.D.) were used (see Fig. 5), with bees being supplied the inocula and provisioned as in 2014. In both years, 15 ml plastic centrifuge tubes (Cat # 93000-020, VWR International, Mississauga, ON, Canada) with lids modified to accommodate wire mesh or honey straining cloth were used as sugar syrup feeders.

To estimate infection intensity, 2-4 bees were removed from random subsets of cages on days 14, 16, and 17 post infection (p.i.); these were macerated and examined microscopically, as described previously, to estimate number of spores/bee. When bees were heavily infected (>2.0×10^7 spores/bee on day 16 or 17 p.i.), they were dissected to harvest the *N. ceranae* spores (see Section 2.2.3).

2.2.3 Harvesting and Purification of Nosema ceranae spores from Honey Bee Midguts

The spore purification protocol was adapted from McGowan (2012) to obtain a clean suspension and high number of viable *N. ceranae* spores without the use of chemicals. Worker honey bees heavily infected with *N. ceranae* were placed in 50mL

centrifuge tubes (Cat #89401-198, VWR International, Mississauga, ON) and chilled on crushed ice for ~15 minutes prior to dissection. Once chilled, the workers were decapitated using microscissors. Alimentary canals were removed by grasping the terminal abdominal segment with forceps, and pulling the canal out of the abdomen; canals were then placed into a sterile petri dish with sterile Type I (Nanopure) water. While viewing under a dissecting microscope, midguts were separated from the hindguts and foreguts via dissection using microscissors (i.e., one incision was made between the proventriculus and midgut, and one near the pylorus, proximal to the origin of the malpighian tubules.

Approximately 50 midguts were macerated in 5 ml of sterile Type I water using a 5ml tissue grinder (Durex® Canada, Scarborough, ON, Canada). The macerate was then filtered through a 40 μm cell strainer (Cat # 352340, Fisher Scientific, Ottawa, ON, Canada) positioned in a 50 ml centrifuge tube, and the strainer was rinsed with 5 ml of sterile type I water (4× with 500μl per rinse). This filtrate was subsequently passed through a 10 μm polyethylene/polypropylene separator (Cat # 60344, Pall Corporation, Ann Arbor, MI, USA) using vacuum filtration (Model # 2534B-01, Welch, Monroe, Louisiana, USA) and a glass filter holder assembly. The separator was rinsed with 10ml of sterile Type 1 water, and the resulting filtrate was transferred to a 50 ml centrifuge tube. A spore count was performed, as described previously, to estimate the number of spores/ml in the filtrate, which was then centrifuged at 800×g for 10 minutes. After centrifugation, the supernatant was removed, and a count performed to estimate spore loss. The pellet was resuspended in 2 ml of sterile Type I water, with the centrifugation, resuspension and counting procedure repeated. All tubes containing 2 ml of filtrate were

then combined and centrifuged at 800×g for 10 minutes. The supernatant was removed, and the pellet resuspended in the appropriate volume of water required for each experiment. After the final resuspension, a final count was performed to estimate spore loss.

2.2.4 Experimental Design

Purified *N. ceranae* spores were added to five hive matrices (honey, beeswax, bee bread, water, and 2M sucrose- a matrix used to simulate fall honey bee feed), and aliquots of each were evenly divided into four groups, with each group exposed to one of four temperature treatments (33, 20, -12, and -20°C), for a total of 20 matrix by temperature combinations. Temperature treatments at 33°C and 20°C were maintained in Percival Scientific incubators (models I36NLC8, I36NLC9), the -12°C was maintained in programmable freezer (Frigidaire model #GLFH21F8HWJ) and the -20°C temperature was maintained using a chest freezer (Kenmore model #970-C162521). Integrity of temperature profiles over time was monitored using datalogggers (Hobo TidbiT v2 Temp Logger, Cape Cod, MA, USA). The viability and *in vivo* infectivity of the treated *N. ceranae* spores was simultaneously evaluated over several time intervals specified in Table 1.

Table 1. Time intervals evaluated per matrix

Matrix	Time Intervals Evaluated (d.p.i.)
Honey	2, 7, 9, 14, 21, 28, 42, 365
Beeswax	2, 7, 9, 14, 21, 28, 35, 365
Bee Bread	2, 7, 9, 14, 21, 28, 42
Water	7, 9, 14, 21, 28, 42
2M Sucrose	7, 9, 14, 21, 28, 42

2.2.5 Inoculation of Hive Matrices and Recovery of Spores

Several aliquots of purified *N. ceranae* spore suspensions were retained for pre-treatment viability and infectivity assessment, while the remaining volumes of suspensions were used to inoculate the five hive matrices.

Over the course of the experiments, spores were recovered at each experimental time point, with five aliquots of recovered spores being prepared at each temperature by time combination, for each matrix. These recovered spore aliquots were used to assess spore viability and infectivity.

Details of spore inoculation and recovery for each matrix is described below.

2.2.5.1 Honey

Approximately 2 ml of spore suspension (containing ~1.7×10^9 *N.ceranae* spores) was added to approximately 125 ml of liquefied (primarily *B. napus* L.) honey determined to be free of *Nosema spp.* via conventional PCR (Giersch et al. 2009). Spores and honey were thoroughly mixed to ensure even suspension, 1 ml aliquots were then transferred into 180 sterile 1.5 ml microcentrifuge tubes (~$5.0.\times10^6$ spores/tube), and treated as above.

To recover spores, 500 μl of sterile Type I water was added to each aliquot and thoroughly mixed to ensure complete homogenization. The aliquot was then added to a 10 μm polypropylene/ polyethylene separator and vacuum filtered. One ml of sterile Type I water was then added to the original aliquot microcentrifuge tube and repeatedly re-pipetted (~ 15 times) to ensure all spores were recovered, with the remaining suspension mixture also added to the separator and filtered. The separator was then washed three times, each time with 1ml of sterile Type I water. The resulting filtrate (~5

ml) was collected using a 1000 µl pipette and added to a 15 ml centrifuge tube, and centrifuged at 800×g for six minutes. After centrifugation, the supernatant was removed, and 1 ml of sterile Type I water added to resuspend the pellet, which was transferred to a 1.5 ml microcentrifuge tube. The number of spores/ml was then estimated as described previously. Aliquots of recovered spores were then prepared for viability and infectivity assessments, as described in section 2.2.5.6.

2.2.5.2 Wax

A sheet of wax foundation (Alberta Honey Producers Cooperative, Spruce Grove, AB) was determined to be *Nosema* spp. free by washing both sides of the sheet with sterile Type I water, centrifuging the suspension at 1000×g, resuspending the pellet in 400 µl of 70% ethanol, and then following the DNA extraction and *Nosema* spp. identification protocol described in section 2.2.1. The wax foundation sheet was cut into 160 1cm×1cm squares, and placed onto 18mm×18mm glass coverslips (Cat # 12-541A, Fisher Scientific, Ottawa, ON, Canada), and heated gently on a hotplate to ensure the wax adhered.

Purified *N.ceranae* spores (~7.0×10^8 spores/1.7 ml sterile Type I water) were maintained in a 15 ml centrifuge tube, which was swirled continuously to ensure even suspensions while aliquoting 10 µl of inoculum (containing ~4.0×10^6 spores) onto each of the wax squares previously cooled to room temperature. Once inoculated, the squares were left to allow the inoculum to air dry onto the wax before receiving temperature treatments.

Recovering spores was performed by rinsing each wax square with 500 µl of sterile Type I water into a 50 ml centrifuge tube. The rinse water was then re-drawn into

the pipet and used to rinse the wax square another five successive times. An additional 500 µl of sterile Type I water was then added to each wax square, and was washed 30 times by drawing and expelling water from the pipette tip, with this volume finally added to the previous 500 µl in the 50 ml centrifuge tube. The resulting 1 ml of liquid was transferred to a 1ml microcentrifuge tube, which was agitated to evenly suspend the spores, and counted to estimate the number of spores/ml. Aliquots were then prepared for viability and infectivity assessments, as described in section 2.2.5.6.

2.2.5.3 Bee Bread

Frames of bee bread from *Nosema* spp.-free colonies were used for this experiment. Bee bread comprised primarily of *Taraxacum officinale* L. pollen was removed from wax broodcomb cells using sterile forceps and weighed, before 250 µl of a purified *N. ceranae* spore suspension (~3.90×10^8 spores) was added. The bee bread and inoculum were mixed well, and weighed once again before being aliquoted into bee bread cells that had been cleaned after bee bread removal (in 2014) or 1.5 ml microcentrifuge tubes (in 2015). Each aliquot was comprised of ~0.06 g of bee bread and ~3.0×10^6 *N. ceranae* spores.

To recover the temperature-exposed treated *N. ceranae* spores, the bee bread-spore mixture was removed from broodcomb cells (in 2014) using forceps and placed into 1.5 ml microcentrifuge tubes. Next (or as the initial step in 2015), 1 ml of sterile Type I water was added to each microcentrifuge tube and mixed until homogenous by repeatedly drawing and expelling the suspension with a 1000 µl pipette. This volume was transferred to a 10 µm separator, and vacuum filtered as described in section 2.2.5.1.

The microcentrifuge tube was then rinsed with an additional 1ml of sterile Type I water which was also added to the separator. The separator was rinsed 3 times with 1ml of sterile Type I water, with the filtrate collected in a 15 ml centrifuge tube. In 2015, the filtrate was passed through the separator, containing a new10μm membrane, an additional time in order to improved spore recovery. The resulting ~5 ml of filtrate was centrifuged at 800×g for 8 minutes, the supernatant removed, and the pellet resuspended in 1ml of sterile Type I water. A spore count was performed, and aliquots prepared for viability and infectivity assays as described in section 2.2.5.6.

Unfortunately, in both years of the experiment, *N. ceranae* spores could not be adequately separated from the pollen grains, thereby invalidating viability counts and precluding infectivity experiments from being performed. As such no results will be presented for the bee bread matrix.

2.2.5.4 Water

Approximately 2 ml of sterile Type I water containing ~3.64×10^8 purified *N. ceranae* spores was added to 89 ml of sterile Type I water (20°C) in a 125 ml beaker to produce 91 ml of a spore/water suspension. The beaker was swirled continuously while the suspension was added to 180 1.5 ml microcentrifuge tubes in 500 μl aliquots. The 180 aliquots were treated as above.

To assess viability and infectivity, each aliquot was gently agitated to resuspend spores and the number of spores/ml was estimated as described previously. Aliquots were then prepared for viability and infectivity assessments, as described in section 2.2.5.6.

2.2.5.5 2M Sucrose

Approximately 500 µl of purified *N. ceranae* spores was added to 80 ml of 2M sucrose. The flask containing the 2M sucrose/spore suspension was swirled continuously at 20°C and repeatedly drawn up and expelled with a 1000 µl pipette, to produce 160 500 µl aliquots containing (~4.6×10^6 spores/aliquot) in 1.5 ml microcentrifuge tubes.

To recover treated spores, 1 ml of sterile Type I water was added to each aliquot, and the liquid withdrawn and expelled with a pipette for homogenization. The number of spores/ml was estimated as described previously, and aliquots were prepared for viability and infectivity assessments as described in section 2.2.5.6.

2.2.7 Preparation of Recovered Spore Aliquots for Analysis

To assess infectivity, a volume containing ~2.2×10^6 spores was set aside from one of the five aliquots for each temperature by time combination (per matrix), if they contained greater than 3.0×10^6 spores. If all aliquots contained less than 3.0×10^6 spores, two were combined before setting aside spores for infectivity.

Aliquots destined for viability and infectivity assessments were brought to the same final volume (1 ml) before centrifugation at 800×g for six minutes. After centrifugation, the supernatant was removed from each aliquot, and the pellet resuspended in an appropriate volume of sterile Type I water for staining (ratio: 2.0×10^6 spores/20 µl of sterile Type I water) or for cage inoculation.

2.2.8 Assessing Spore Viability

The fluorescent stains 4' 6-Diamidino-2-phenylindole (DAPI) dilactate (Cat # D3571, Life Technologies Inc, Burlington, ON) and Propidium Iodide (PI) (Cat # P3566, Life Technologies Inc, Burlington, ON) were selected for this study. PI was used as the

viability stain, as it is membrane impermeant, while DAPI was used a nucleic acid counterstain. These stains were chosen as they do not overlap in excitation or emission spectra, and this dye combination can reliably differentiate living and dead spores with perceptible colours (McGowan 2012). Working stocks of both DAPI (1 mg/ml in sdH_2O) and PI (1 mg/ml in sdH_2O) were prepared and stored in a refrigerator in the dark at 4°C. Stains were added simultaneously to each aliquot (2 μl of each dye per 2.0×10^6 spores in 20 μl) destined for a viability assessment (i.e., infectivity aliquots were not stained). Stained aliquots were incubated in the dark at room temperature for 20 minutes.

After the incubation period, the aliquots were centrifuged at 800×g for six minutes. The supernatants were discarded, and the pellets resuspended in 100 μl of sterile Type I water (mixed well by pipetting and expelling). The aliquots were washed in 100 μl of sterile Type I water and centrifuged 2× at 800 x g for six minutes before being resuspended in a final volume of sterile Type I water that made the spores discernible from one another (~2.0×10^6 spores/50 μl) under the microscope.

All samples were visualized using a Fluoview FV10i fluorescent microscope (Olympus, Tokyo, JP). Prepared aliquots ready for visualization were agitated with a 10 μl pipette 30 times for homogenization prior to 6μl being taken and added to a Z30000 Helber counting chamber. The counting chamber was loaded into the FV10i, the DAPI and PI filter selected, and the total magnification set to 45×. The FV10i acquired the map image of the counting chamber, and spores in all 16 grids of the counting chamber were counted (phase contrast) and marked for stain presence: DAPI only = live spore or DAPI + PI = dead spore. If 100 spores were not present within the grid, additional areas of the

chamber were counted to bring the total number of spores as close to or above 100 as possible. Spore viability for each aliquot was calculated by using the following formula:

$$\frac{Number\ of\ spores\ stained\ with\ DAPI\ only}{Total\ number\ of\ spores\ viewed\ under\ phase\ contrast} \times 100$$

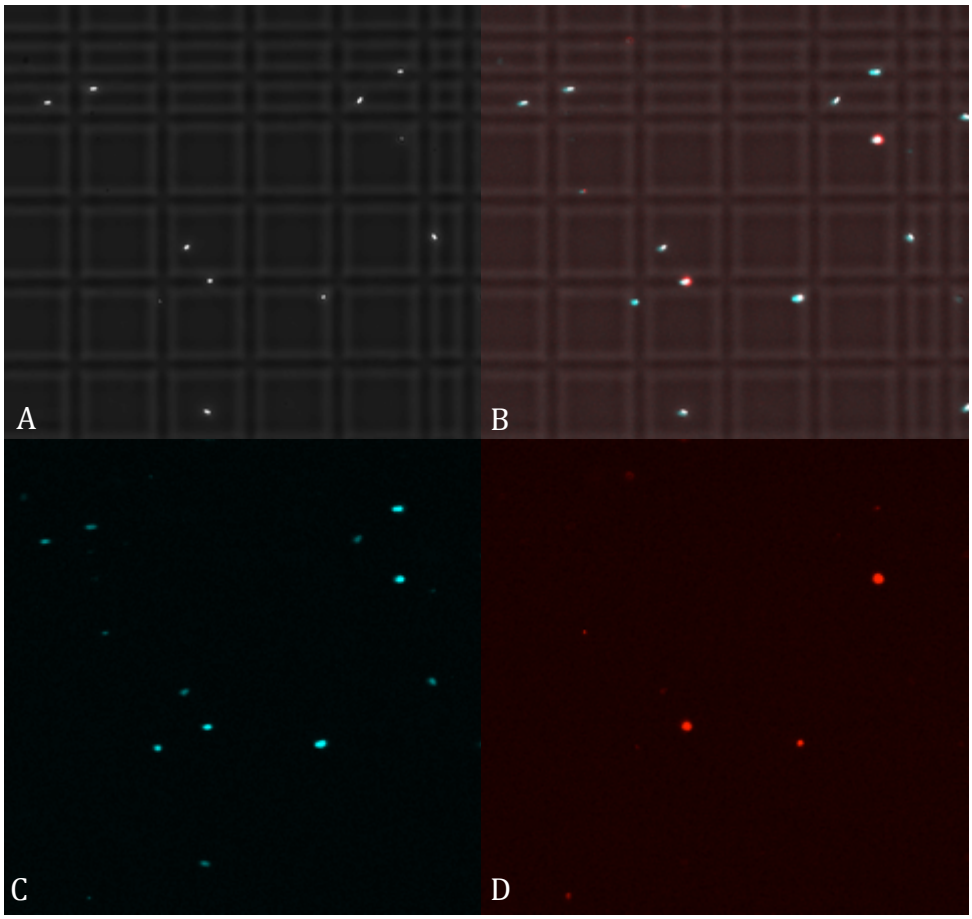

Figure 3. *N. ceranae* spores viewed under (A) phase contrast, (B) phase contrast, DAPI, and PI filters, (C) DAPI filter only, (D) PI filter only using the Fluoview FV10i.

2.2.9 Assessing Spore Infectivity

Frames of eclosing worker bees were collected from non-experimental colonies managed by the Apiculture Program at AAFC Beaverlodge. These frames were placed in frame holders, which were placed in incubators (Percival Scientific, Model I36NLC8, I36NLC9) maintained at 33 ± 1.0°C and 70 ± 5% RH. A minimum of six frames from six different colonies were maintained at any one time. Bees were collected from these frames daily, so that all newly emerged bees (NEBs) used for the infectivity assays were <24hrs old, and free of any *Nosema* spp. infection.

Infectivity aliquots were prepared from harvested spores (section 2.2.5). After centrifugation, the supernatant was discarded and the pellet resuspended in an appropriate volume of water such that when sugar syrup was added, each bee would receive ~1.0×10^5 spores in 5 µl of 50% sugar syrup.

Frames containing NEBs were brushed off into a large 36-litre plastic tote. The required number of bees for an assay was collected from this container, placed in individual feeding harnesses (Fig. 4.),adapted from the feeding system developed by Rinderer (1976), and starved for 90 minutes prior to receiving their 5 µl droplet of inoculum. The inoculum was homogenized in a 1.5 ml microcentrifuge tube with a 10 µl pipette to ensure even suspension prior to inoculating, and in between doses. The inoculum was supplied to the NEBs via a 10 µl pipette tip, with its tip cut and placed in the harness lid. The inoculum was administered to the modified pipette tip using a 10 µl pipette. After receiving the inoculum, the bees were given one hour to consume their droplets. Once the droplets were consumed, bees were confined to their harnesses for an additional 30 minutes to prevent trophallaxis once they were placed in a hoarding cage.

If bees did not consume their inoculum, they were not placed in a hoarding cage. In 2014, wooden hoarding cages were used, while in 2015, plastic hoarding cages were used (described in 2.2.1).

For each matrix, 18-25 bees were inoculated per temperature treatment at each time point. Control bees were given a dose of 5 µl of 50% (w/w) sugar syrup without *N. ceranae* spores. Once the bees were placed in hoarding cages, they were supplied with 60% (w/w) sugar syrup in a gravity feeder, and a 25% irradiated Canadian pollen patty (primarily *B. napus*) fed *ad libidum* Fig. 5. The hoarding cages were maintained in incubators at 33 ± 1.0°C and 70 ± 5% RH (as above) for two weeks. Diet was changed every 72 hours. After two weeks, all living bees were collected, bagged according to treatment group, and frozen at -20°C until they could be examined for spores.

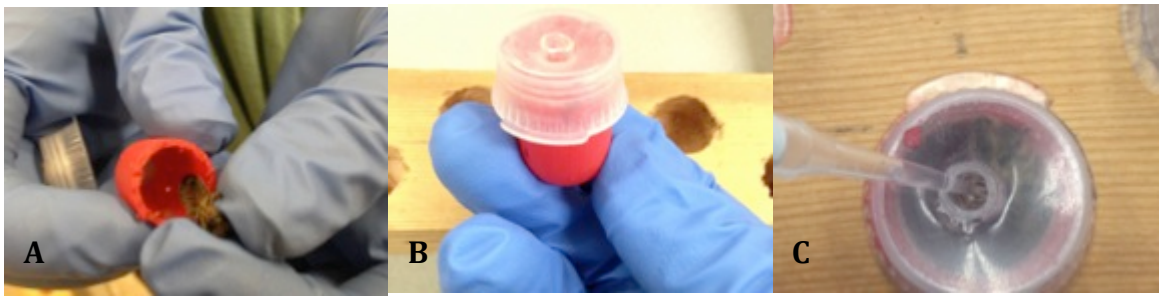

Figure 4. (A) Newly-emerged worker bee (NEB) being placed in a harness, (B) Prepared harness containing a NEB, (C) Inoculum being delivered to a harnessed NEB.

Figure 5. Inoculated bees in plastic hoarding cages for infectivity experiments.

2.2.9.1 Calculating N. ceranae Spore Infectivity

Bees that were frozen live from a given treatment group were removed from the freezer and briefly thawed (~ 5 minutes). The abdomen was removed from each and placed into its own 1.5 ml microcentrifuge tube. After adding 1 ml of 70% ethanol to each tube, each abdomen was macerated with a sterile micropestle. After maceration, the content of the microcentrifuge tube was vortexed and a spore count performed as previously described. Bees were classified as infected if at least two spores were present on the counting chamber grid. If only one spore was present, a second count was completed. If a spore was present on the grid during the second count, the bee was classified as infected. To calculate spore infectivity for each treatment group at a given time point, the following equation was used:

$$\frac{Total\ number\ infected, surviving\ bees}{Total\ number\ surviving\ bees} \times 100$$

2.2.10 Statistical Analyses

All statistical analyses were performed in Prism 6 (Graphpad, San Diego, CA) for Mac OS X. Viability data were analyzed using linear and non-linear regression analyses, where appropriate, with spore viability (mean %) as the dependent variable and length of

exposure (days) as the independent variable. For linear regression ($Y= \beta_1\chi+ \beta_0$), β_1 represents the slope of the line and β_0 represents the Y-intercept. For non-linear regression ($Y=Y_0\text{-plateau}\times e^{-k\chi}+\text{plateau}$), Y_0 represents the average Y value up to the time decay starts, plateau refers to the Y value at infinite times, and k is the rate constant (Graphpad Software Inc., 2016). Prism's curve-fitting function was used to evaluate the appropriateness of linear or non-linear regression for each dataset. After curve-fitting, a Runs test was performed on data suggested to follow a linear model to ensure that lines fit by linear regression did not deviate from the data. If the lines did not significantly deviate from the data, linear regression was used to analyze the datasets. For data suggested to be best described using non-linear models, a Replicates test was performed to assess the fit of non-linear models (curves) to the data. The curves with Replicates tests resulting in the lowest discrepancies (*F* values close to 1 with high *P*-values) were chosen to analyze each dataset. For all non-linear datasets, one-phase decay curves presented with the lowest discrepancy values, and were chosen to analyze all non-linear datasets.

For treatment responses modeled using similar regression procedures, slopes or rate constants were compared using *F*-tests to determine the overall effect of temperature on spore viability.

To examine the effects of time and temperature on spore viability in honey, water, and 2M sucrose, a two-way ANOVA was performed for each substrate with time and temperature as main effects. A two-way ANOVA was not completed for spores maintained on wax due to missing data in some time×temperature combinations. If the interaction term (time×temperature) was significant for a substrate, a one-way ANOVA

with temperature as the main effect followed by Tukey's HSD was completed at each of three (or four in the case of honey) biologically relevant time points to examine the effect of temperature on spore viability without time as an effect (Zar, 2014). One-way ANOVA with temperature as the main effect was also completed for wax viability data at three biologically relevant time points. The biologically relevant time points chosen were 7, 28 (35 for wax), 42, and 365 d.p.i. Seven d.p.i. was chosen as many laboratory-based studies examining *Nosema* spp. viability evaluate at 7 d.p.i., or begin to notice a treatment effect at that time (Malone et al. 2001, Fenoy et al. 2009, Fries and Forsgren 2009). Twenty-eight d.p.i. was chosen for comparison with other studies that terminate trials near or at that interval. (Malone et al. 2001, Fenoy et al. 2009), while 42 d.p.i. was chosen as it was the final time point for some of the assays. Finally, viability at 365 d.p.i. was evaluated, where possible, as it represented a long-term period of exposure.

Data were tested for normality and homogeneity of variance using the Brown-Forsythe test and Bartlett's test prior to performing the analyses. Only after meeting the assumptions were the ANOVAs were performed.

Estimates for 50% *N. ceranae* mortality were generated using a one-phase decay curve (previously identified as having a strong fit for non-linear datasets, see above) for non-linear datasets (Graphpad Software Inc., 2016), or extension of the linear regression line where appropriate. Infectivity data (binary response) were analyzed per matrix by time point using the chi-square test for equality of proportions followed by partitioning: the systematic collapsing of the contingency tables (four temperature treatments) into orthogonal sets of 2×2 tables and testing those treatments for statistical differences, with

Bonferroni correction at the biologically relevant time points defined previously (Sharpe, 2015).

For surviving infected bees, mean spore levels were compared using one-way ANOVA across all time points and temperature treatments with matrix as the main effect followed by Tukey's HSD after meeting the assumptions of normality (see above).

2.3 Results

2.3.1 Viability of N. ceranae spores stored in honey

Viability for spores stored at 20 and 33°C was modeled using a one-phase decay equation (Fig. 6). Viability for spores maintained at 33°C (hive temperature) and 20°C, decreased steadily over time, with viability decreasing to 0.6% and 1.0%, respectively, at 365 d.p.i. Viability functions were found to vary significantly between these two temperatures, over time ($F_{1,60}$=24.54 P<0.0001).

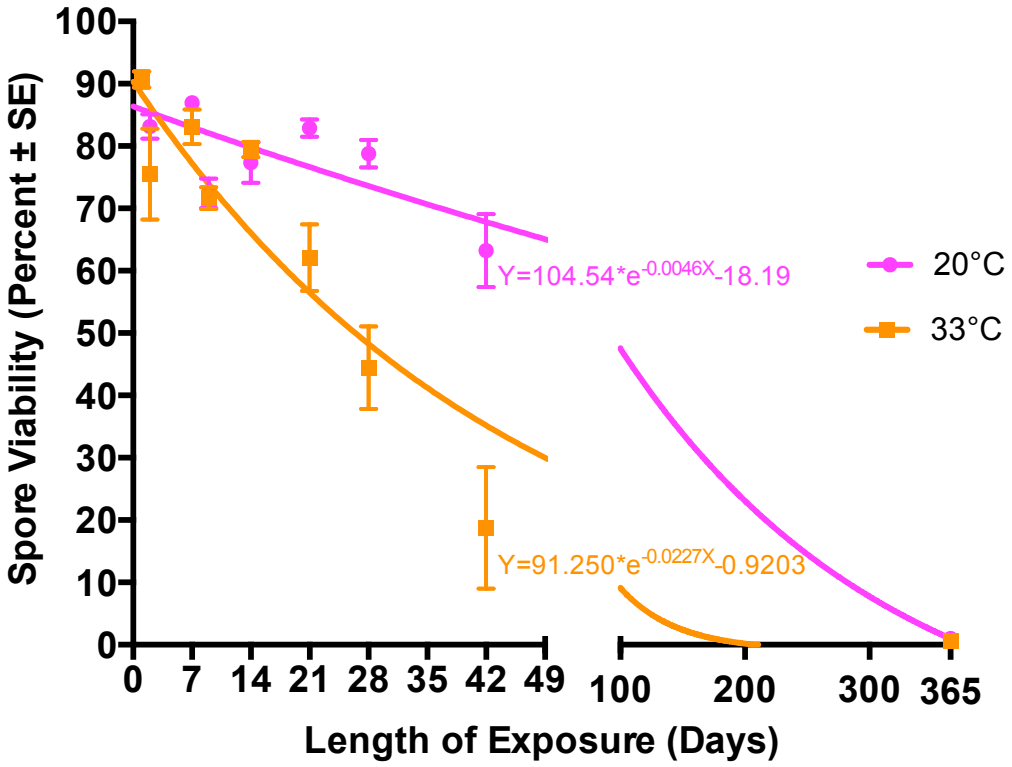

Figure 6. *N. ceranae* spore viability for spores stored in honey at 20 and 33°C. Each point represents mean spore viability(±SE) for samples analyzed at a pre-determined time interval (n=3-4 honey replicate samples per time point; also refer to Table 2).

Viability for spores maintained at -12 and -20°C was modeled using linear regression (Fig 7.). Viability decreased at a greater rate for spores kept at 33°C rather than 20°C, though there was significant difference in response between these two temperatures ($F_{1,63}$=2.715 P=0.1044). *Nosema ceranae* spores stored at -12 and -20°C maintained their initial viability throughout the duration of the experiment, while spores stored at -20°C experienced only a 5.4% decrease in viability.

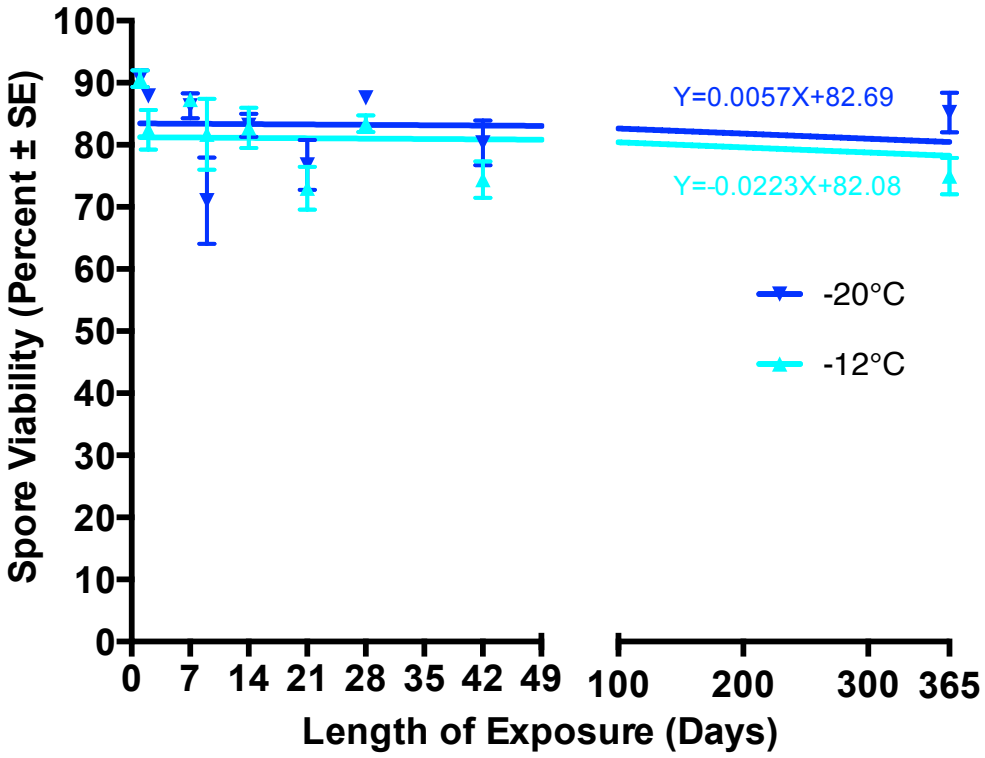

Figure 7. *N. ceranae* spore viability (±SE) for spores stored in honey at -12 and -20°C. Each point represents the mean spore viability for samples analyzed a pre-determined time interval (n=3-4 honey replicate samples per time point; also refer to Table 2).

Parametric comparisons revealed that the influence of temperature on spore survival depended on time ($F_{24,97}$=22.39 P<0.0001). This interaction was likely driven by the decline in viability experienced by spores maintained at 33 and 20 °C. To better understand the impacts of temperature treatment on spore viability without the influence of time, parametric comparisons of spore viability were made among temperatures within several biologically relevant time points. Though no differences were detected among temperature treatments at day 7 d.p.i., viability was significantly lower for spores maintained at 33°C at 28 d.p.i. and 42 d.p.i., than other temperatures (Fig 8, Table 2). Viability decreased at a greater rate for spores maintained at 33 and 20°C, and was

significantly lower than the viability maintained by spores at -12 and -20°C at 365 d.p.i. Spores maintained at -12 and -20°C experienced only minute reductions in viability over the course of the experiment.

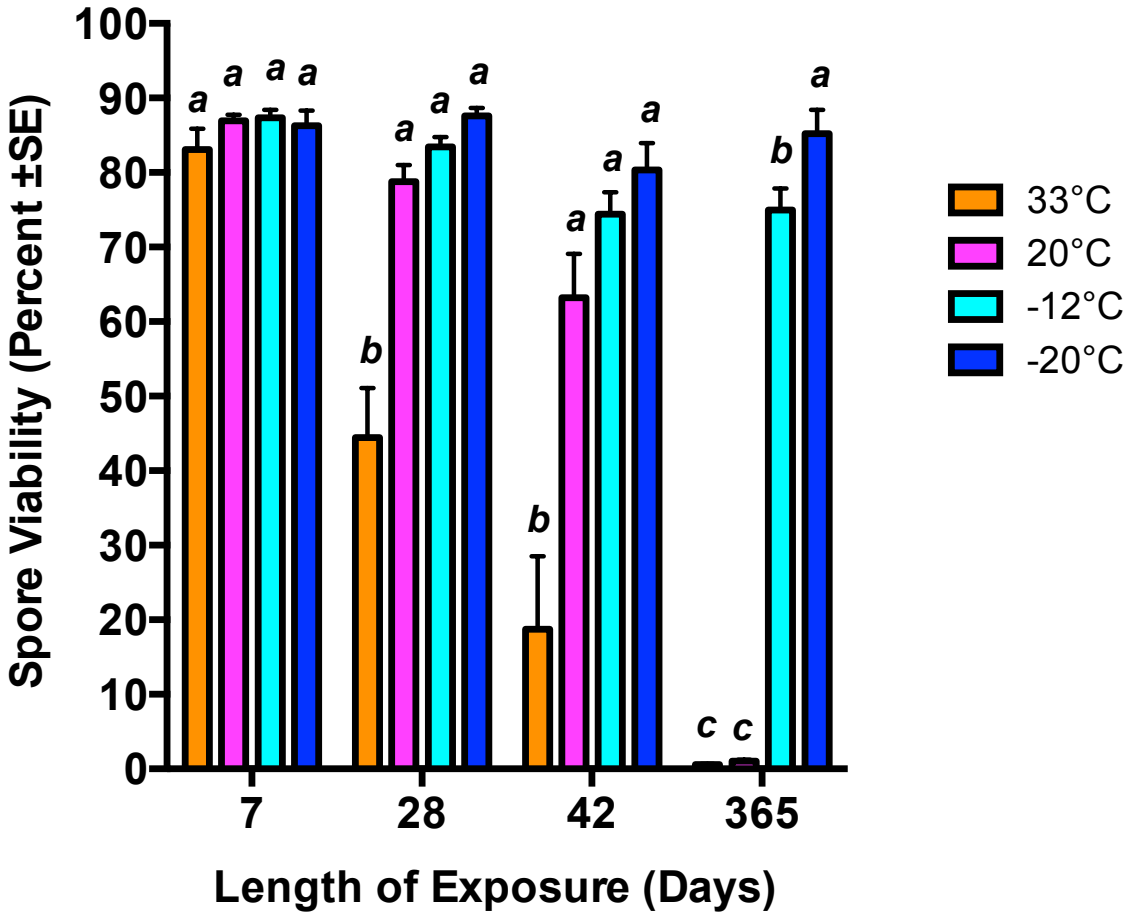

Figure 8. Mean *N. ceranae* spore viability (±SE) for spores stored in honey at 20, 33, -12, and -20°C at four biologically relevant time points. Bars with different letters indicate statistically significant differences among treatments (*P<0.05;* Tukey HSD*)* within a given time point. (Day 7 $F_{3,10}$=1.003 *P*=0.4313; Day 28 $F_{3,11}$=27.45 *P*<0.0001; Day 42 $F_{3,10}$=21.50 *P*=0.0001; Day 365 $F_{3,12}$=446.6 *P*<0.0001)

Table 2. Summary of parametric comparisons of *N. ceranae* spore survival in honey among all four treatments across four biologically relevant time points. Different letters following mean viability values denote statistically significant differences among treatments for a given time point ($P<0.05$; Tukey HSD).

Treatment	Viability (Mean % ± SE)	Replicates (n)[a]
Honey Day 7	$F_{3,10}=1.003$ $P=0.4313$	
33°C	83.4±3.2 **a**	3
20°C	87.0±0.5 **a**	4
-12°C	88.1±1.0 **a**	4
-20°C	87.3±1.8 **a**	4
Honey Day 28	$F_{3,11}=27.45$ $P<0.0001$	
33°C	49.6±5.2 **b**	4
20°C	79.2±2.2 **a**	4
-12°C	83.2±1.6 **a**	3
-20°C	87.8±1.1 **a**	4
Honey Day 42	$F_{3,10}=21.50$ $P=0.0001$	
33°C	14.1±0.1 **b**	3
20°C	64.1±6.3 **a**	3
-12°C	77.6±2.3 **a**	4
-20°C	79.2±4.6 **a**	4
Honey Day 365	$F_{3,12}=446.6$ $P<0.0001$	
33°C	0±0 **c**	4
20°C	1.5±1.5 **c**	4
-12°C	76.0±2.7 **b**	4
-20°C	85.8±3.2 **a**	4

[a] Number of spore-containing replicate honey samples analyzed per time point

2.3.2 Viability of N. ceranae spores stored on wax

Viability of *N. ceranae* spores in wax was modeled using the same one-phase decay equation as those for honey, across all temperatures evaluated. *Nosema ceranae* spores stored at 33, -12, and -20°C on wax experienced a rapid decrease in viability. Viability was less than 20% after 7 days for spores stored at 33 and -12°C, and was less than 20% for all aforementioned treatment groups at day 14 p.i. (Figs 9, 10). There was no effect of temperature on viability for spores maintained at 33 or -12°C over the course of the experiment ($F_{1,15}$=3.617 P=0.0766). Spores stored at 20°C maintained moderate viability until day 28 p.i. however viability was reduced to <10% after 1 year of storage. Because of low spore recovery, there are some missing data points among treatments at several of the time points evaluated (detailed in Table 3).

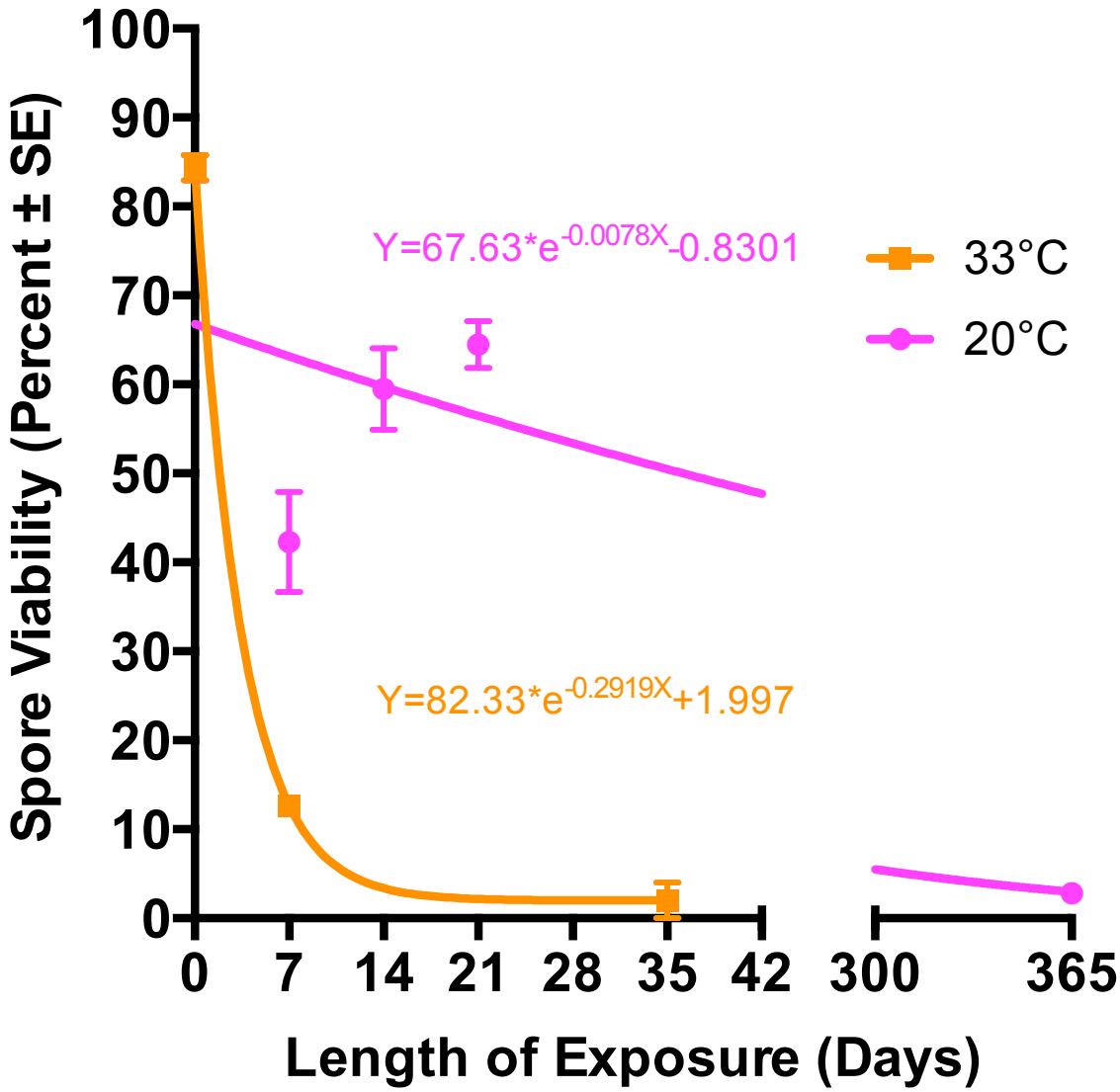

Figure 9. Mean *N. ceranae* spore viability (±SE) for spores on wax at 33 and 20°C. Each point represents the mean spore viability for samples analyzed a pre-determined time interval (n=2-4 wax replicate samples per time point; also refer to Table 3). Data points for 14, 21, 28, and 365 d.p.i. are missing for the 33°C treatment, and 28 d.p.i. for the 20°C treatment.

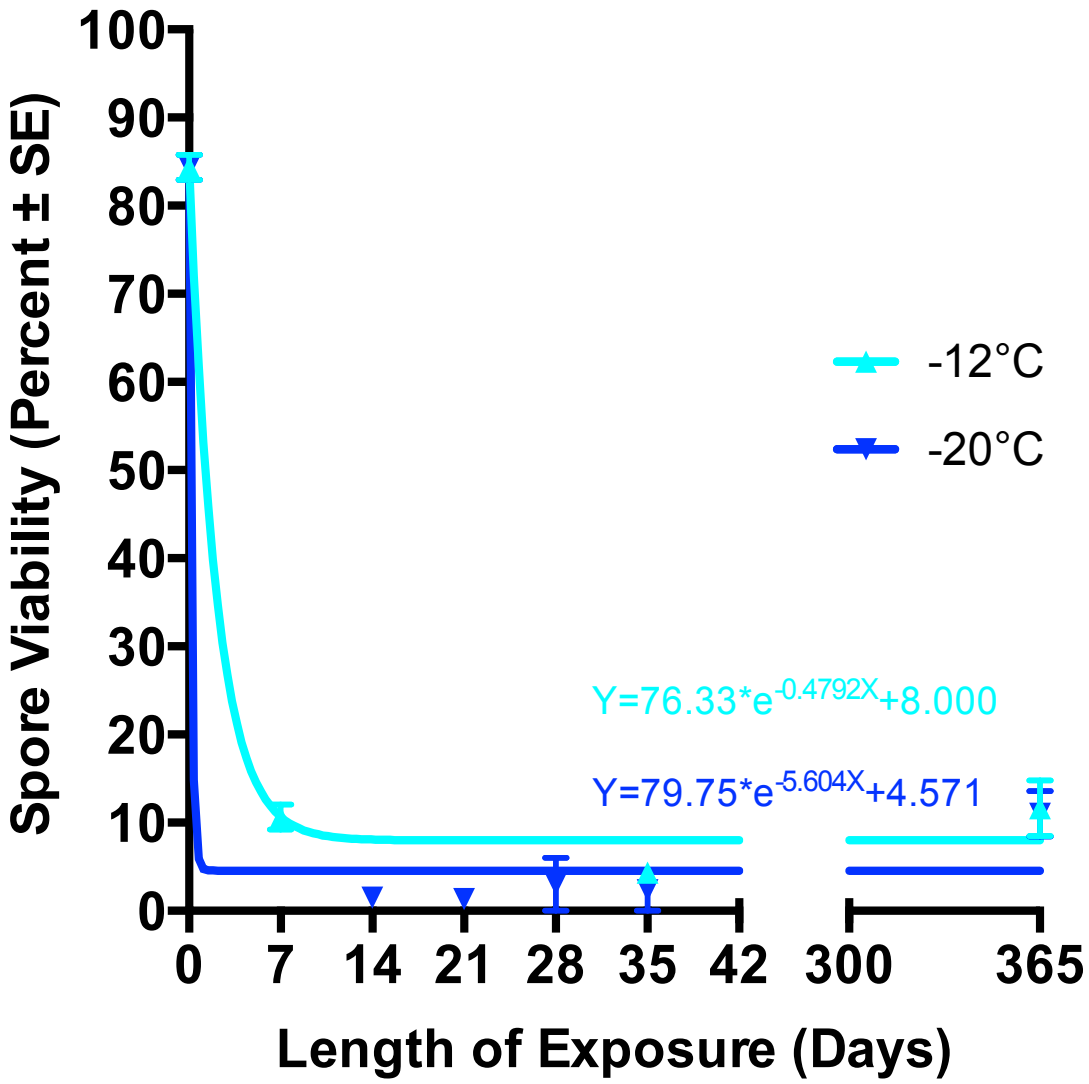

Figure 10. Mean *N. ceranae* spore viability (±SE) for spores on wax at -12 and -20°C. Each point represents the mean spore viability for samples analyzed a pre-determined time interval (n=2-4 wax replicate samples per time point; also refer to Table 3). Data points are missing for 14, 21, and 28 d.p.i. for the -12°C treatment, and 7 and 365 d.p.i. for the -20°C treatment.

Parametric comparisons among temperatures within time points showed that *N. ceranae* spores stored on wax at 20°C had significantly greater viability than spores maintained at 33 and -12°C on wax at 7 d.p.i. ($F_{2,7}$=19.57 P=0.0014) (Fig. 11, Table 3).

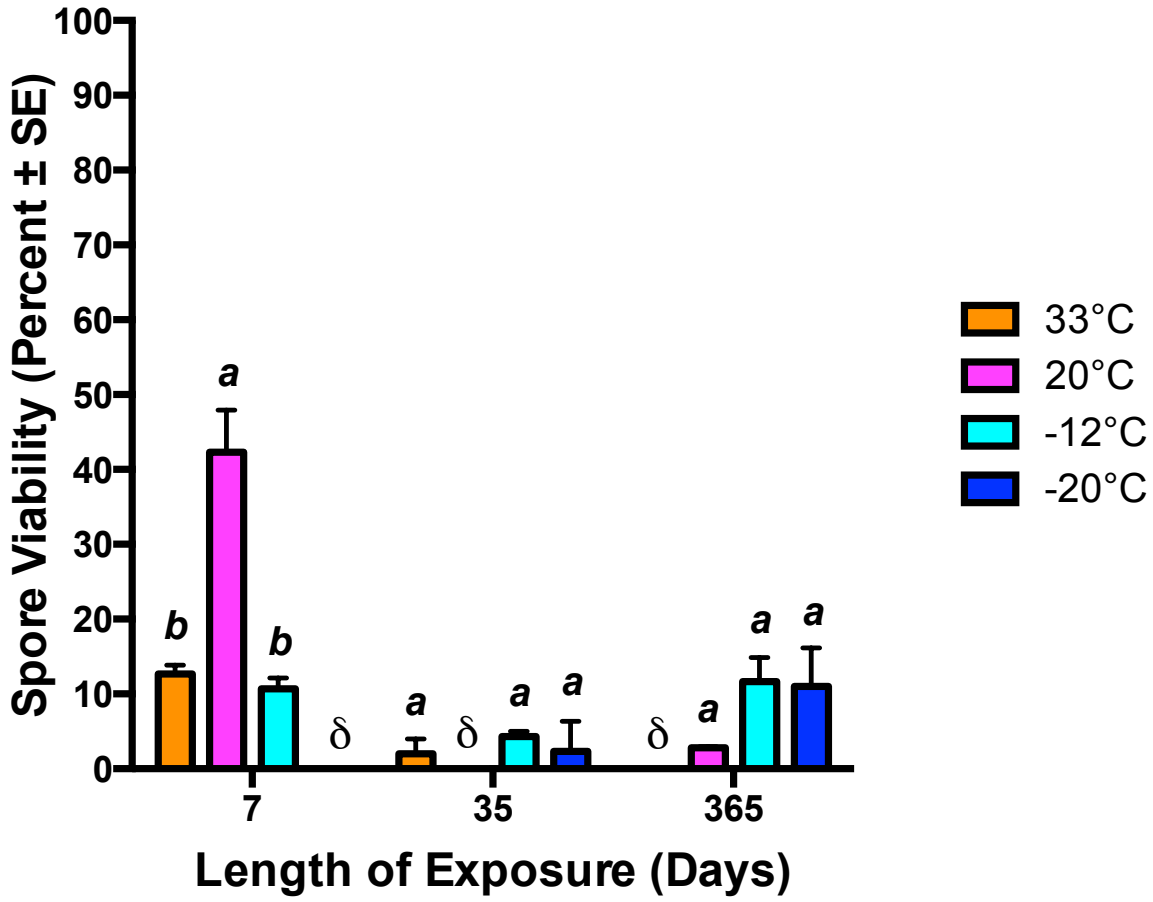

Figure 11. Mean *N. ceranae* spore viability (±SE) for spores maintained on wax at 33, 20, -12, and -20°C at three biologically relevant time points. Bars with different letters indicate statistically significant differences (*P<0.05*; Tukey HSD) among treatments for a given time point. "δ" denotes missing data missing from a treatment group, following the order: -20, 20, and 33°C at each of the given time points**.** **(**Day 7 $F_{2,7}$=19.57 P=0.0014; Day 35 $F_{2,6}$=0.4831 P=0.6389; Day 365 $F_{2,6}$=2.404 P=0.1711)

Table 3. Summary of parametric comparisons of *N. ceranae* spore survival on wax among all four treatments across three biologically relevant time points. Letters indicate statistically significant differences ($P<0.05$; Tukey HSD) among treatments for a given time point.

Treatment	Viability (Mean % ± SE)	Replicates (n)[b]
Wax Day 7	$F_{2,7}=19.57$ $P=0.0014$	
33°C	16.3±2.7 **b**	3
20°C	47.3±2.9 **a**	4
-12°C	10.9±1.7 **b**	4
-20°C	-	-
Wax Day 35	$F_{2,6}=0.4831$ $P=0.6389$	
33°C	1.1±1.1 **a**	3
20°C	-	-
-12°C	3.2±1.2 **a**	3
-20°C	1.9±1.9 **a**	3
Wax Day 365	$F_{2,6}=2.404$ $P=0.1711$	
33°C	-	-
20°C	2.8±0.2 **a**	2
-12°C	11.7±3.2 **a**	3
-20°C	11.0±2.6 **a**	4

[b] Number of spore-containing replicate beeswax samples analyzed per time point

2.3.3 Viability of N. ceranae spores stored in water

N. ceranae spore viability in water was modeled using linear regression for 33, 20 and -12°C treatments and a one-phase decay equation for -20°C.

N. ceranae spores stored at 20 and -12°C in water over the six week study period maintained significantly higher viability than spores stored at 33°C ($F_{2,72}$=6.750 P=0.0021) (Fig. 12). Spores stored at 33°C maintained high viability until day 28 p.i., then experienced an 18.0% decrease in viability from day 28 to day 42 d.p.i. Spores maintained frozen in water at -20°C endured a 53.4% reduction in viability, following a one-phase decay over the six-week exposure.

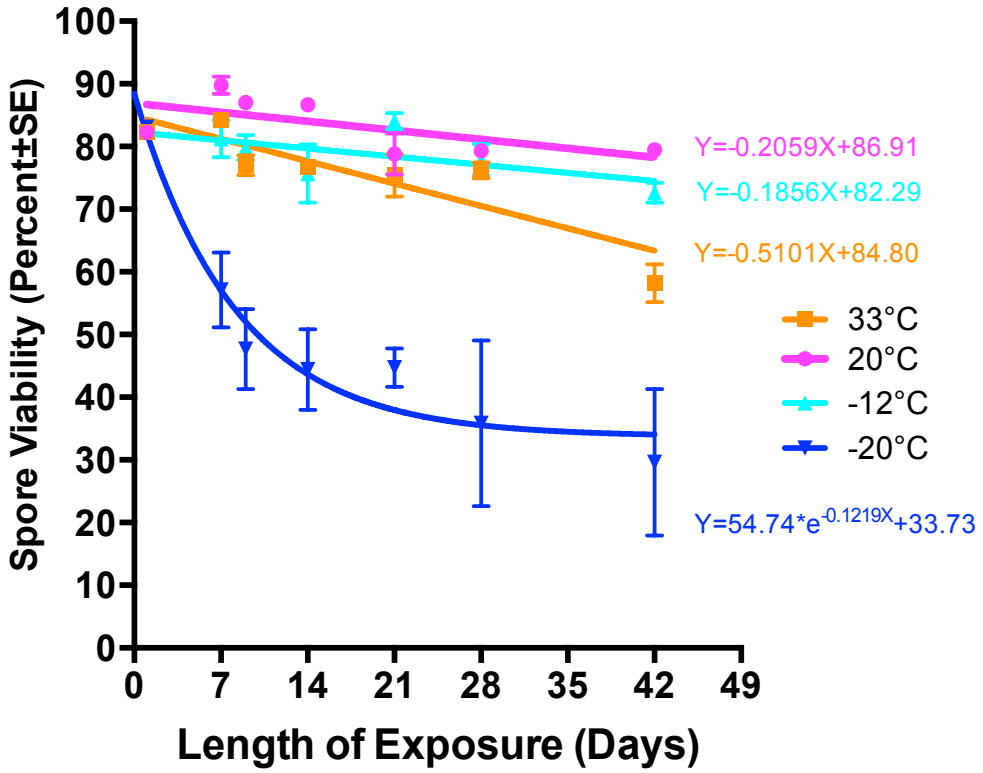

Figure 12. Mean *N. ceranae* spore viability (±SE) for spores maintained in water at 33, 20, -12 and -20°C. Each point represents the mean spore viability for samples analyzed a pre-determined time interval (n=3-4 water replicate samples per time point; also refer to Table 4).

Parametric comparisons indicated that the influence temperature on spore survival depended on time ($F_{18,69}$=8.283 P<0.0001). The time×temperature interaction was likely driven by the strong reduction in spore viability experienced by spores maintained at -20°C. To examine the impact of temperature on spore survival without the influence of time, parametric comparisons among temperatures within selected time points were completed. Viability for spores stored at -20°C was significantly lower than all remaining treatment groups at each of the biologically relevant time points. Viability for the 33°C treatment became intermediate at 42 d.p.i., the last measured time point (Fig. 13, Table 4)

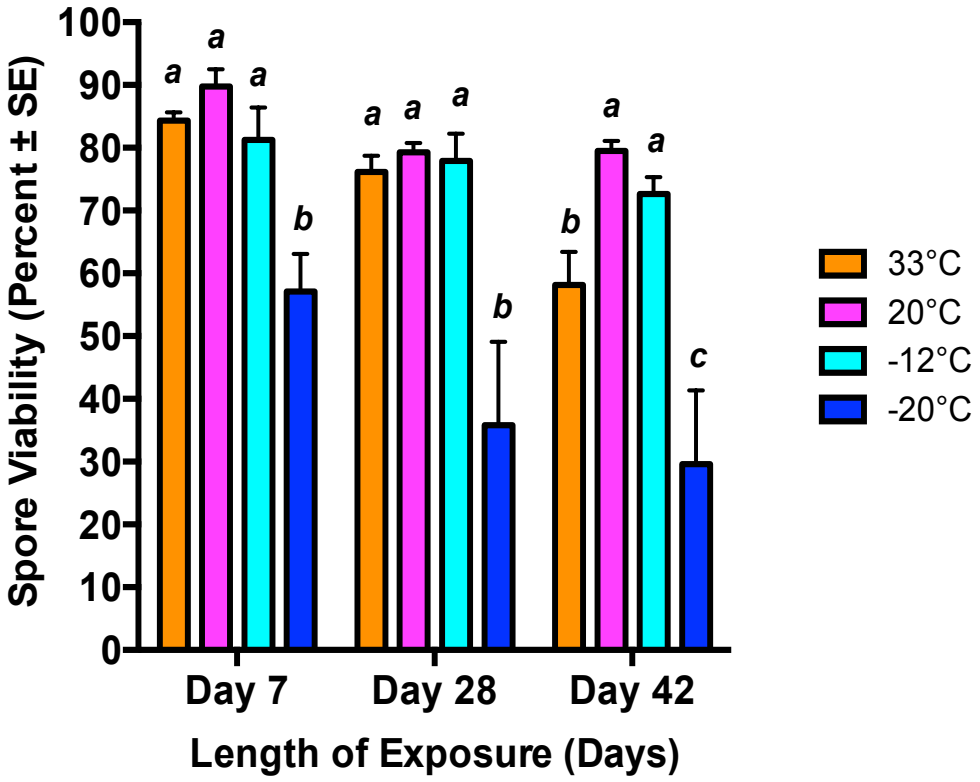

Figure 13. Mean *N. ceranae* spore viability (±SE) for spores maintained in water at 33, 20, -12, and -20°C at three biologically relevant time points. Bars with different letters indicate statistically significant differences (*P<0.05*; Tukey HSD) among treatments for a given time point. (Day 7 $F_{3,11}$=4.826 P<0.0001; Day 28 $F_{3,10}$=33.49 P<0.0001; Day 42 $F_{3,9}$=40.44 P<0.0001)

Table 4. Summary of parametric comparisons of *N. ceranae* spore survival in water among all four treatments across three biologically relevant time points. Letters indicate statistically significant differences ($P<0.05$) among treatments for a given time point.

Treatment	Viability (Mean %)	Replicates (n) [c]
Water Day 7	$F_{3,11}=4.826$ $P<0.0001$	
33°C	84.1±0.6 **a**	4
20°C	89.7±1.4 **a**	4
-12°C	81.2±3.0 **a**	3
-20°C	57.0±3.0 **b**	4
Water Day 28	$F_{3,10}=33.49$ $P<0.0001$	
33°C	76.2±1.3 **a**	4
20°C	79.3±0.7 **a**	4
-12°C	77.9±2.5 **a**	3
-20°C	35.8±7.6 **b**	3
Water Day 42	$F_{3,9}=40.44$ $P<0.0001$	
33°C	58.2±2.9 **b**	3
20°C	79.5±0.8 **a**	4
-12°C	72.7±1.5 **a**	3
-20°C	29.6±6.8 **c**	3

[c] Number of spore-containing replicate water samples analyzed per time point

2.3.4 Viability of N. ceranae spores stored in 2M sucrose

N. ceranae spore viability in sucrose solution was modeled using linear regression over all temperatures evaluated. Although *N. ceranae* spores stored in 2M sucrose at colder temperatures (-12, and -20°C) maintained slightly higher viability over the course of the experiment than spores stored at 20 or 33°C, there was no overall effect of temperature on spore viability in sugar syrup ($F_{3,107}$=2.165 P=0.0964) (Fig. 14). Across all treatment groups, spores suspended in 2M sucrose experienced only modest declines in viability over the six-week exposure (Fig. 14). On average, a loss of 18.4% viability was seen by 42 d.p.i., across all temperatures.

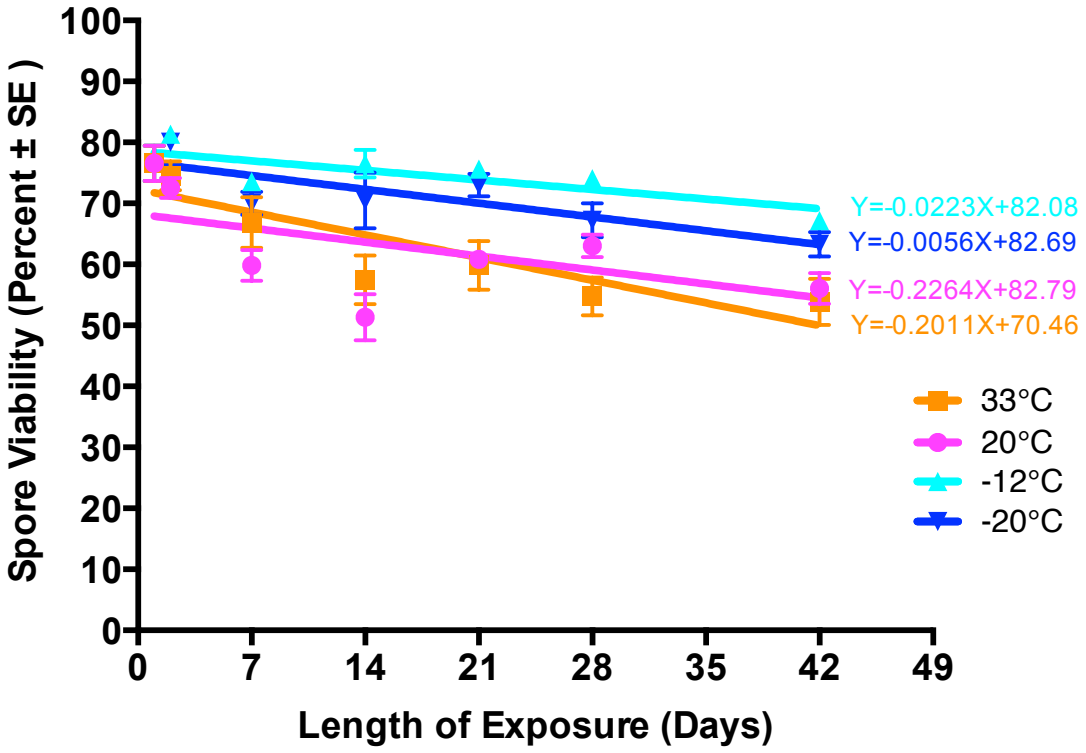

Figure 14. Mean *N. ceranae* spore viability (±SE) for spores in 2M sucrose at 33, 20, -12 and -20°C. Each point represents the mean spore viability for samples analyzed a pre-determined time interval (n=4-5) 2M sucrose replicate samples per time point; also refer to Table 5).

Parametric comparisons indicated that the impact of temperature on spore viability depended on time ($F_{18,87}$=2.311 P<0.0053). This time×temperature interaction might have been influenced by the decrease preceding the plateau in viability at 20°C. To examine the impact of temperature on spore viability in the absence of time, parametric comparisons among temperatures within time points were completed. These comparisons revealed that *N. ceranae* spores maintained in 2M sucrose at -12°C were significantly more viable than spores maintained at all other temperatures across all three of the biologically time points analyzed (Day 7 $F_{3,13}$=5.115 P=0.0149, Day 28 $F_{3,13}$=11.36 P=0.0006), with the exception of spores maintained at -20°C on day 42, which had intermediate viability at that time point ($F_{3,14}$=5.284 P=0.0120) (Fig 15, Table 5).

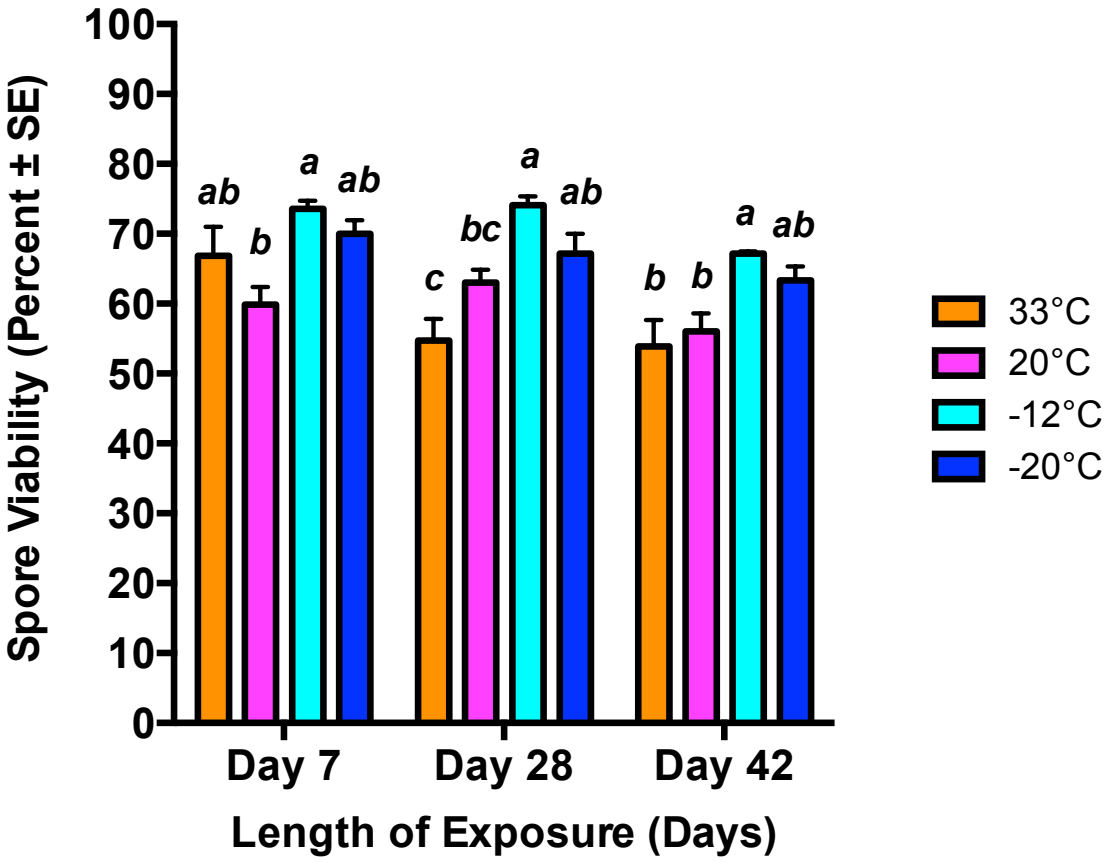

Figure 15. Mean *N. ceranae* Spore Viability (±SE) for spores maintained in 2M sucrose at 33, 20, -12, and -20°C at three biologically relevant time points. Bars with different letters indicate statistically significant differences (*P<0.05*; Tukey HSD) among treatments for a given time point. Day 7 $F_{3,13}$=5.115 *P*=0.0149; Day 28 $F_{3,13}$=11.36 *P*=0.0006; Day 42 $F_{3,14}$=5.284 *P*=0.0120)

Table 5. Summary of parametric comparisons of *N. ceranae* spore survival in 2M sucrose among all four treatments across three biologically relevant time points. Letters indicate statistically significant differences ($P<0.05$) among treatments for a given time point.

Treatment	Viability (Mean %)	Replicates (n)[d]
2M Sucrose Day 7	$F_{3,13}=5.115$ $P=0.0149$	
33°C	67.0±4.1 **ab**	4
20°C	59.8±2.5 **b**	5
-12°C	73.6±1.2 **a**	4
-20°C	70.0±1.9 **ab**	4
2M Sucrose Day 28	$F_{3,13}=11.36$ $P=0.0006$	
33°C	54.8±3.1 **c**	4
20°C	63.1±1.8 **bc**	5
-12°C	74.1±1.3 **a**	4
-20°C	67.2±2.0 **ab**	4
2M Sucrose Day 42	$F_{3,14}=5.284$ $P=0.0120$	
33°C	48.7±0.5 **b**	5
20°C	56.1±2.6 **b**	5
-12°C	67.2±0.3 **a**	4
-20°C	63.4±2.0 **ab**	4

[d] Number of spore-containing replicate water samples analyzed per time point

2.3.5 Infectivity

Overall the mean infection level in surviving bees at 14 d.p.i. was $22.9\times10^6 \pm 1.21\times10^6$. However, it was discovered that spore production was influenced by the type of matrix in which the inoculation dose was previously stored ($F_{3,727}$=82.33 P<0.0001). Bees inoculated with spores maintained in water and 2M sucrose had significantly higher spore numbers ($26.1\times10^6 \pm 2.4\times10^6$ and $50.1\times10^6 \pm 1.4\times10^6$ spores/bee respectively) than bees inoculated with spores stored in honey ($11.3\times10^6 \pm 6\times10^5$ spores/bee) or wax ($4.1\times10^6 \pm 4\times10^5$ spores/bee) (comparisons derived by Tukey's HSD).

2.3.6 Infectivity of N. ceranae spores maintained in honey

Infectivity could not be accurately modeled over time across across all treatments (Fig. 16), therefore the four treatments (33, 20, -12, and -20°C) were compared using non-parametric comparisons at the biologically relevant time points mentioned in Section 2.2.8.

N. ceranae spores maintained at 33, and -12°C in honey experienced a sharp reduction in infectivity at day 14 p.i. before experiencing a plateau at 21 d.p.i. (Fig. 16). At day 28 p.i., infectivity was significantly reduced for spores maintained at 33 and -12°C but not spores at 20 and -20°C (Day 28 χ^2 = 39.02 df=3 P<0.0001). *Nosema ceranae* spores stored at 20°C maintained moderate infectivity and viability until 42 d.p.i. (Figure 2.3.1.4), while *N. ceranae* spores stored at -20°C maintained moderate infectivity along with high viability throughout the experiment. Although *N. ceranae* spores stored in honey at -12°C maintained high viability (according to fluorescent microscopy) over the course of the experiment, low infectivity was observed at one year d.p.i. (Figs. 16, 17, Table 6).

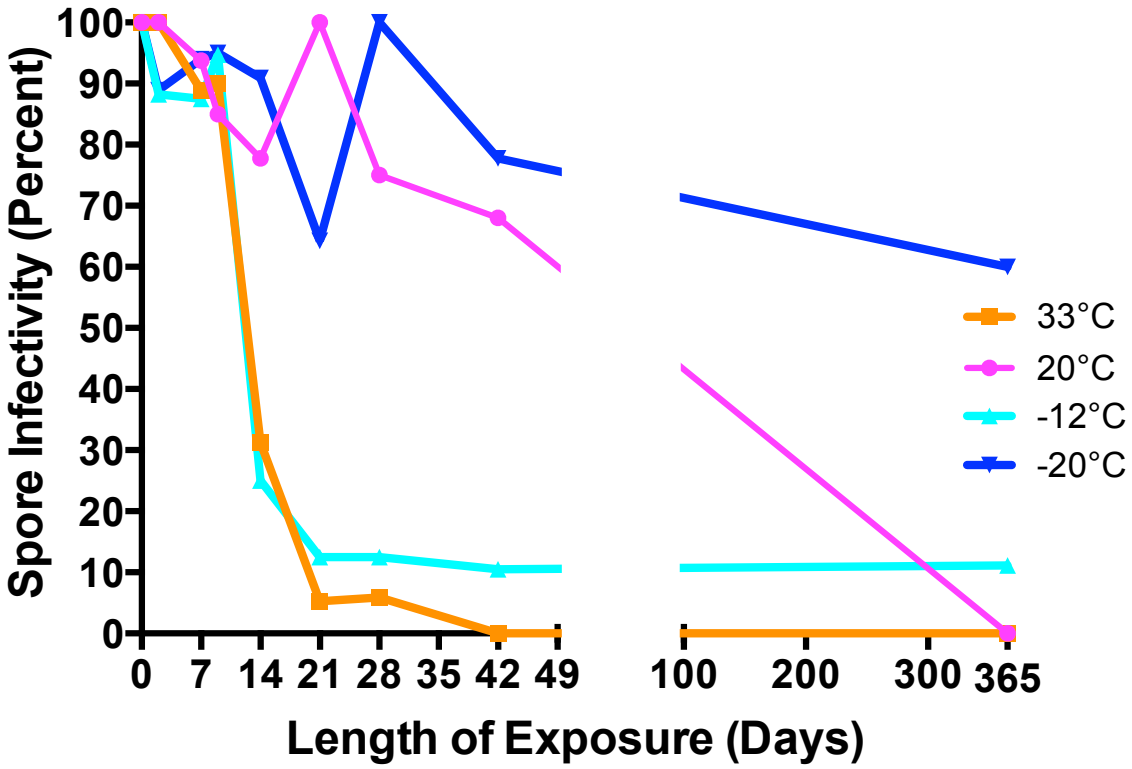

Figure 16. *N. ceranae* infectivity for spores stored in honey at 33, 20, -12, and -20°C for up to one year. Infectivity reflects the percentage of surviving, infected bees at the end of a 14-day incubation.

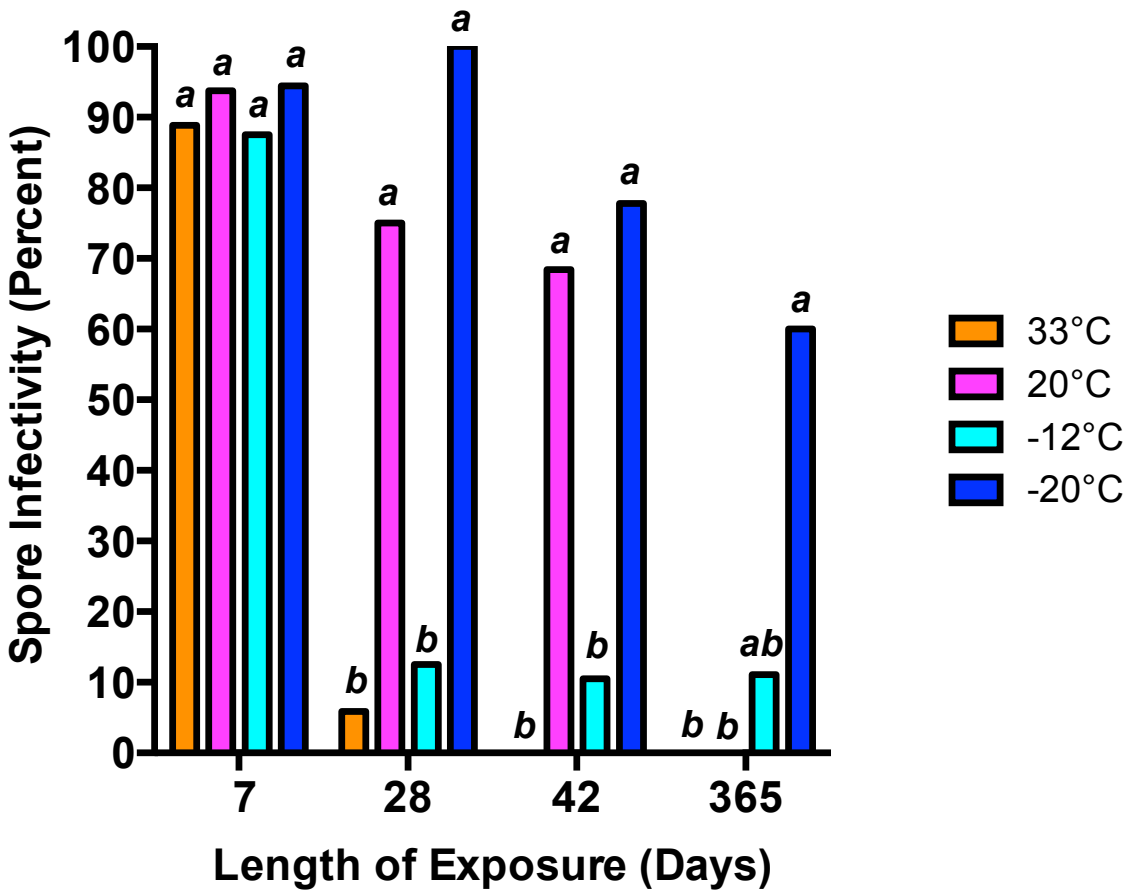

Figure 17. Mean *N. ceranae* spore infectivity for spores stored in honey at 33, 20, -12 and -20°C at four biologically relevant time points. Bars with different letters indicate statistically significant differences ($P<0.05$; Partitioning) among treatments for a given time point. (Day 7 $\chi^2(3)=0.7566$ $P=0.8598$; Day 28 $\chi^2(3)=39.02$ $P<0.0001$; Day 42 $\chi^2(3)=28.55$ $P<0.0001$; Day 365 $\chi^2(3)=12.19$ $P=0.0068$)

Table 6. Summary of χ^2 comparisons of *N. ceranae* spore infectivity for spores maintained in honey among all four treatments across four biologically relevant time points. Letters indicate significant differences ($P<0.05$) between treatments based on χ^2 test for equal proportions followed by partitioning and correction for multiple comparisons.

Treatment	Infectivity (Mean %)	Replicates (n)[e]
Honey Day 7	χ^2= 0.7566 df=3 *P*=0.8598	
33°C	89 **a**	18
20°C	94 **a**	16
-12°C	88 **a**	16
-20°C	94 **a**	18
Honey Day 28	χ^2= 39.02 df=3 *P*<0.0001	
33°C	6 **b**	19
20°C	75 **a**	20
-12°C	13 **b**	16
-20°C	100 **a**	14
Honey Day 42	χ^2= 28.55 df=3 *P*<0.0001	
33°C	0 **b**	17
20°C	68 **a**	16
-12°C	11 **b**	16
-20°C	78 **a**	14
Honey Day 365	χ^2= 12.19 df=3 *P*=0.0068	
33°C	0 **b**	9
20°C	0 **b**	6
-12°C	11 **ab**	9
-20°C	60 **a**	10

[e] Number of surviving bees inoculated with honey-treated spores at the end of a two-week incubation period

2.3.7 Infectivity of N. ceranae spores maintained on wax

As with the honey infectivity data, all treatments were compared using non-parametric comparisons at the biologically relevant time points (Fig. 19 and Table 6) mentioned in Section 2.2.8. Because of low spore recovery, and the independent nature of the aliquots, there are some discrepancies in time points analyzed between viability and infectivity data (detailed Table 3 and 7)

With exception of days 2 and 9 p.i., *N. ceranae* spores stored at 20°C maintained moderate infectivity for four weeks p.i. (Fig. 18). At day 28 p.i., spores at 20°C were significantly more infective than spores stored at -12, and -20°C (Day 28 χ^2=21.34 df=3 *P*=<0.0001, Fig. 19, Table 7). Spores stored on wax at 33, -12, and -20°C experienced a rapid decline in spore infectivity (infectivity <20% after 7 days across the three treatments, with the exception of spores stored at 33°C for 9 days (Fig. 18) while also displaying a rapid decline in viability (Fig. 9 and 10). Spores maintained on wax at -12 and -20°C did not generate infections after 9 days p.i. (Fig. 19).

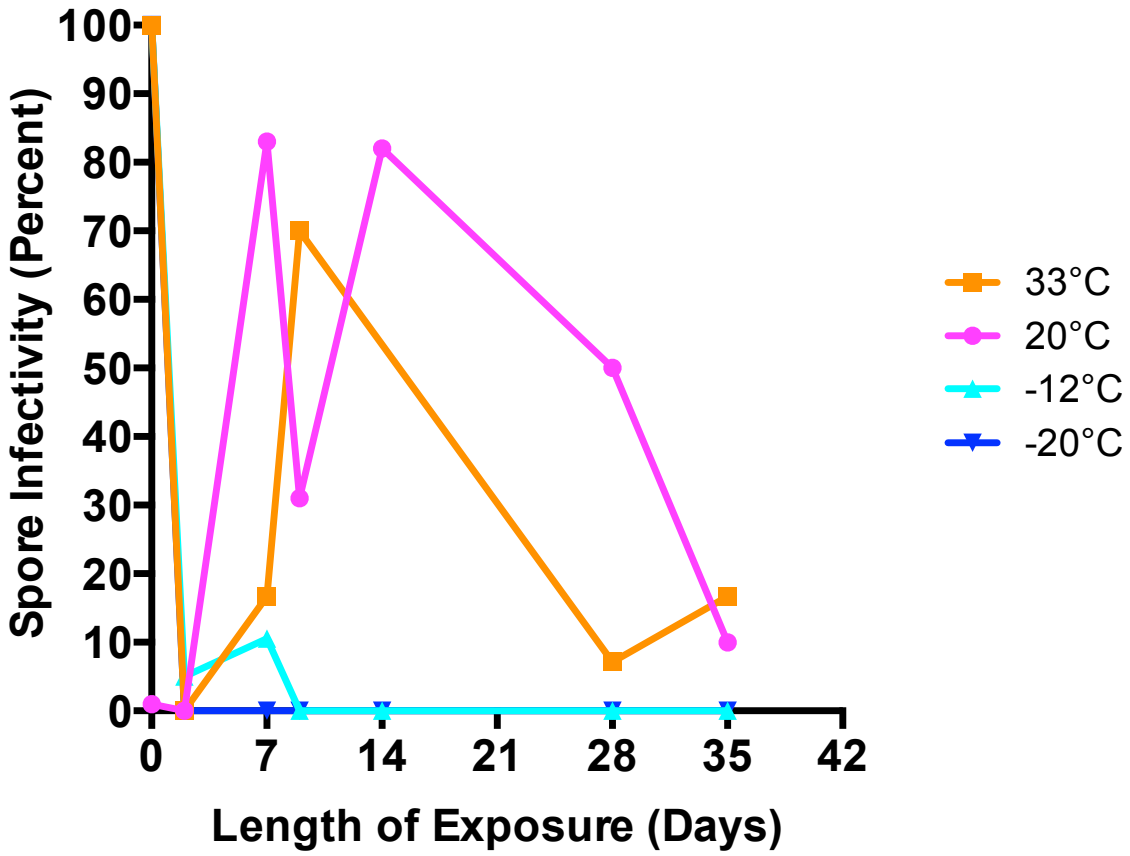

Figure 18. Mean *N. ceranae* infectivity for spores stored on wax at 33, 20, -12, and -20°C for up to five weeks. Infectivity reflects the percentage of surviving, infected bees at the end of the 14-day incubation.

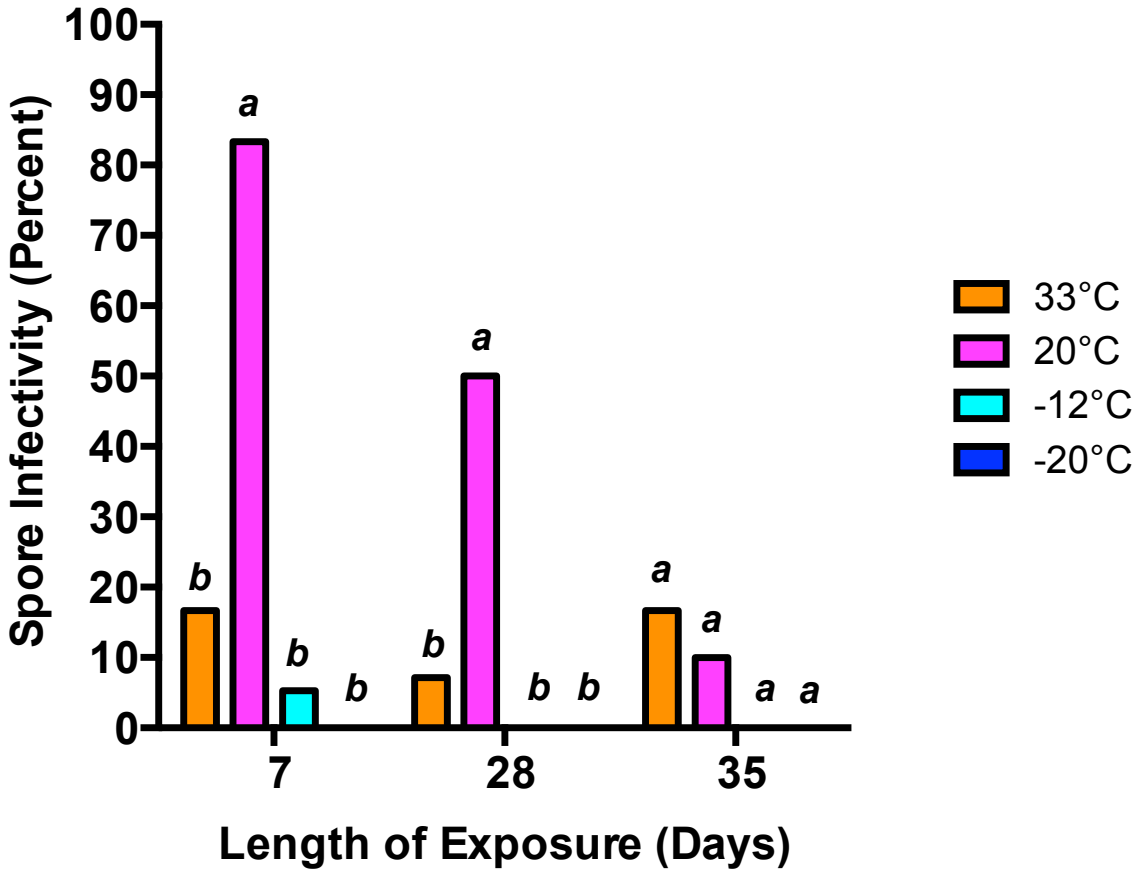

Figure 19. Mean *N. ceranae* spore infectivity for spores maintained on wax at 33, 20, -12 and -20°C at three biologically relevant time points. Bars with different letters indicate statistically significant differences (*P<0.05*; Partitioning) among treatments for a given time point. (Day 7 χ^2=36.99 *P*=<0.0001; Day 28 χ^2=21.34 *P*=<0.0001; Day 35 χ^2=4.836 *P*=0.1842)

Table 7. Summary of χ^2 comparisons of *N. ceranae* spore infectivity for spores stored on wax among all four treatments across three biologically relevant time points. Letters indicate significant differences ($P<0.05$) between treatments based on χ^2 tests for equal proportions followed by partitioning and correction for multiple comparisons.

Treatment	Infectivity (Mean %)	Replicates (n)[f]
Wax Day 7	χ^2= 36.99 df=3 P<0.0001	
33°C	17 **b**	18
20°C	83 **a**	18
-12°C	5 **b**	18
-20°C	0 **b**	16
Wax Day 28	χ^2= 21.34 df=3 P<0.0001	
33°C	7 **b**	14
20°C	50 **a**	16
-12°C	0 **b**	11
-20°C	0 **b**	19
Wax Day 35	χ^2= 4.836 df=3 P<0.1842	
33°C	17 **a**	12
20°C	10 **a**	20
-12°C	0 **a**	17
-20°C	0 **a**	15

[f] Number of surviving bees inoculated with wax-treated spores at the end of a two-week incubation period

2.3.8. Infectivity of N. ceranae spores in water

As with honey and wax infectivity, treatments were compared at the biologically relevant time points mentioned above using non-parametric comparisons.

Treatments with the exception of 20°C, experienced a sharp decline in infectivity after seven days of exposure (Fig. 20). *Nosema ceranae* spores kept in water at 20°C maintained moderate infectivity over the course of the experiment (>70% after six weeks of exposure), while exhibiting high viability (Fig. 12). Infectivity for spores stored at 33, and -12°C was reduced by ~40% over the course of the experiment, resulting in fewer than 50% of bees becoming infected at the last time point, while still displaying high (-12°C) and moderate (33°C) viability at six weeks (Fig. 12). *N. ceranae* spores maintained in water at -20°C experienced a sharp decline in infectivity at day 7 p.i., at the last time point (42 days p.i.), fewer than 10% of surviving, inoculated bees were infected. Infectivity was significantly less for spores at -20°C than spores at 20°C (Day 42 χ^2=10.73 df=3 P=0.0133, Fig. 21 and Table 8).

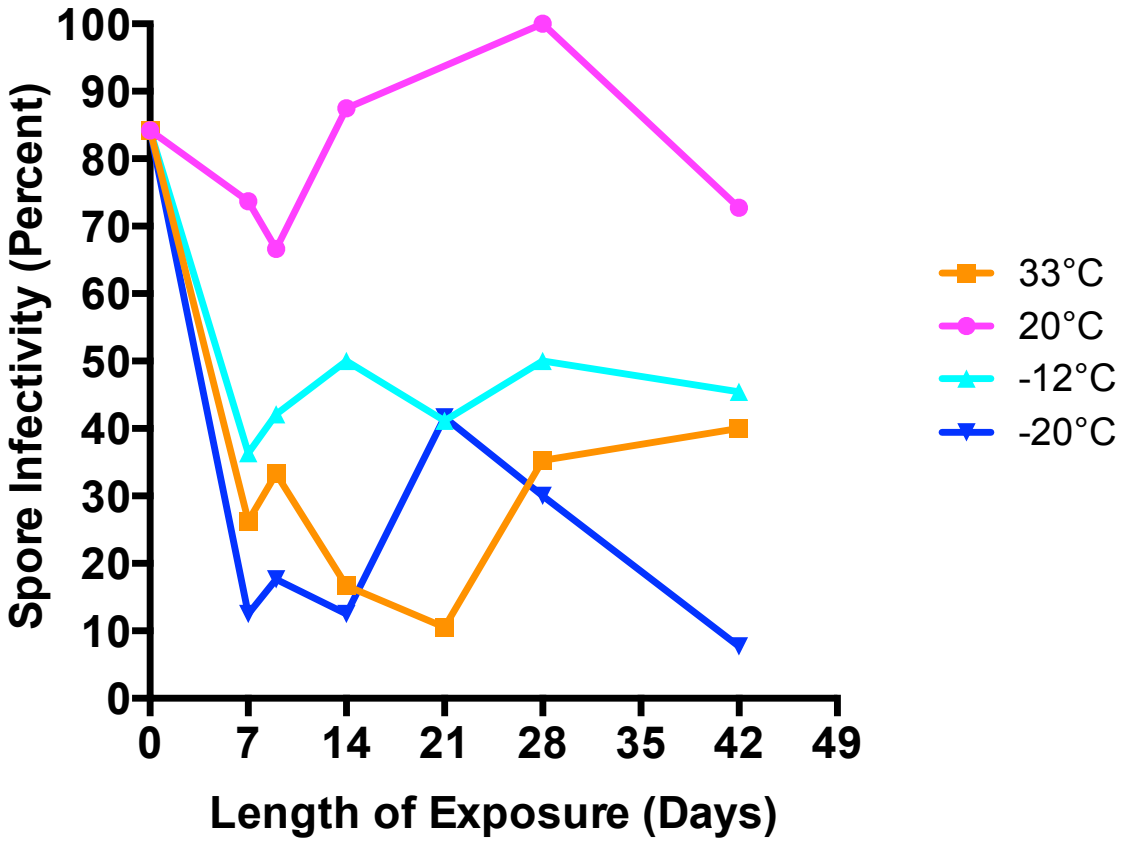

Figure 20. *N. ceranae* spore infectivity for spores maintained in water at 33, 20, -12, and -20°C for up to six weeks. Each point represents the percentage of surviving, infected honey bees at the end of a 14-day incubation period.

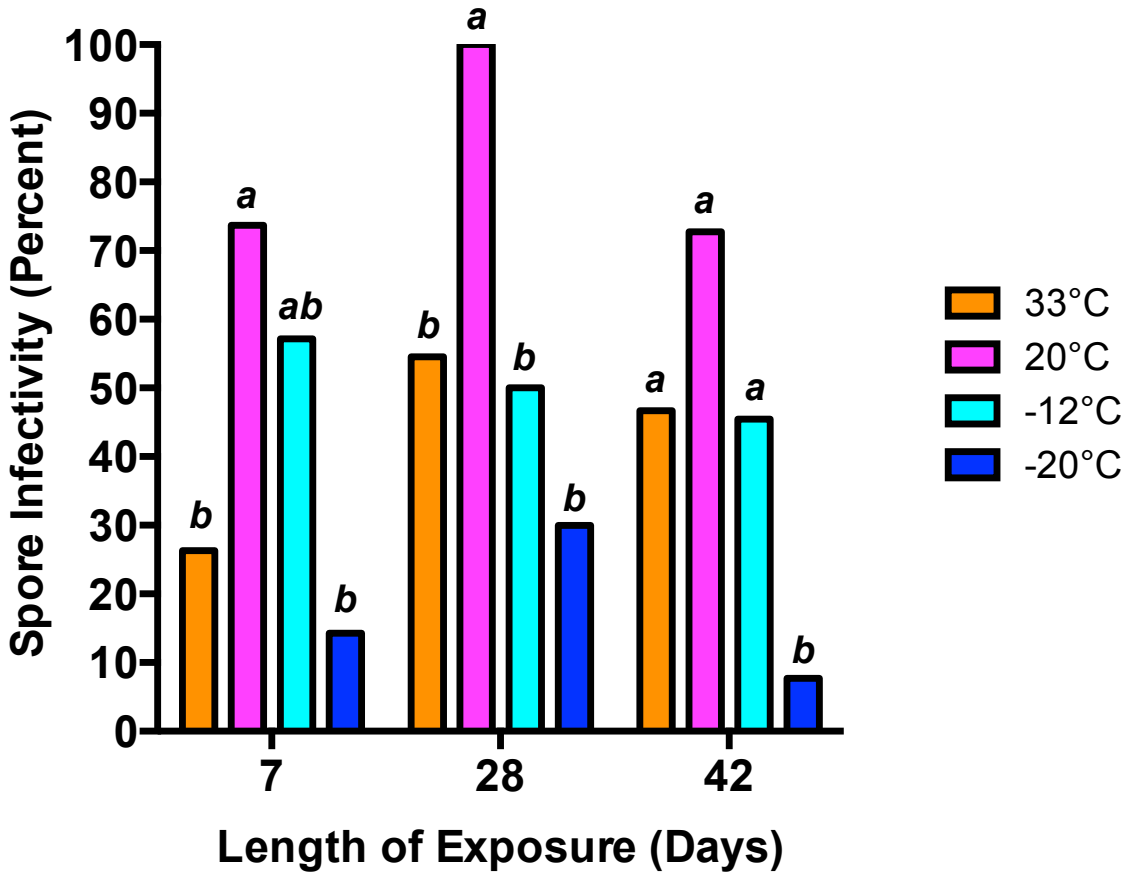

Figure 21. Mean *N. ceranae* spore infectivity for spores suspended in water at 33, 20, -12, and -20°C at three biologically relevant time points. Bars with different letters indicate statistically significant differences ($P<0.05$; Partitioning) among treatments for a given time point. (Day 7 χ^2=12.74 P<0.0052; Day 28 χ^2=17.63 P<0.0005; Day 42 χ^2=10.73 P=0.0133)

Table 8. Summary of χ^2 comparisons of *N. ceranae* spore infectivity for spores maintained in water among all four treatments across three biologically relevant time points. Letters indicate significant differences ($P<0.05$) between treatments based on χ^2 tests for equal proportions followed by partitioning and correction for multiple comparisons.

Treatment	Infectivity (Mean %)	Replicates (n)[g]
Water Day 7	χ^2= 12.74 df=3 *P*=0.0052	
33°C	26 **b**	19
20°C	74 **a**	19
-12°C	57 **ab**	11
-20°C	14 **b**	8
Water Day 28	χ^2= 17.63 df=3 *P*=0.0005	
33°C	55 **b**	17
20°C	100 **a**	15
-12°C	50 **b**	14
-20°C	30 **b**	10
Water Day 42	χ^2= 10.73 df=3 *P*=0.0133	
33°C	47 **a**	15
20°C	73 **a**	11
-12°C	45 **a**	11
-20°C	8 **b**	13

[g] Number of surviving bees inoculated with water-treated spores at the end of a two-week incubation period

2.3.9. Infectivity of N. ceranae spores in 2M Sucrose

As with honey, wax, and water, infectivity for spores in 2M sucrose was analyzed using non-parametric comparisons at three biologically relevant time points (above).

N. ceranae spores suspended in 2M sucrose at 20°C maintained high infectivity over the course of the experiment (Fig. 22), despite displaying moderate viability (Fig.14). Spores maintained at -12, and -20°C experienced a sharp decline in infectivity 48 hrs p.i. (Fig. 22). Infectivity for spores stored at -12°C plateaued after this decrease, and only experienced an ~2% decrease in infectivity over the remainder of the study period. Spores at -20°C experienced a sharper decrease at 48 hrs p.i., and lost an additional ~10% in viability over the remaining 40 days. Infectivity for spores maintained at 33°C underwent a steady decline over the course of the experiment, resulting in only 25% of bees becoming infected at the last experimental time point (Fig. 22). Infectivity on day 42 p.i. was significantly higher for spores stored at 20°C than spores maintained at 33, and -20°C (χ^2=19.60 df=3 *P*=0.0002) (Fig 23, Table 9).

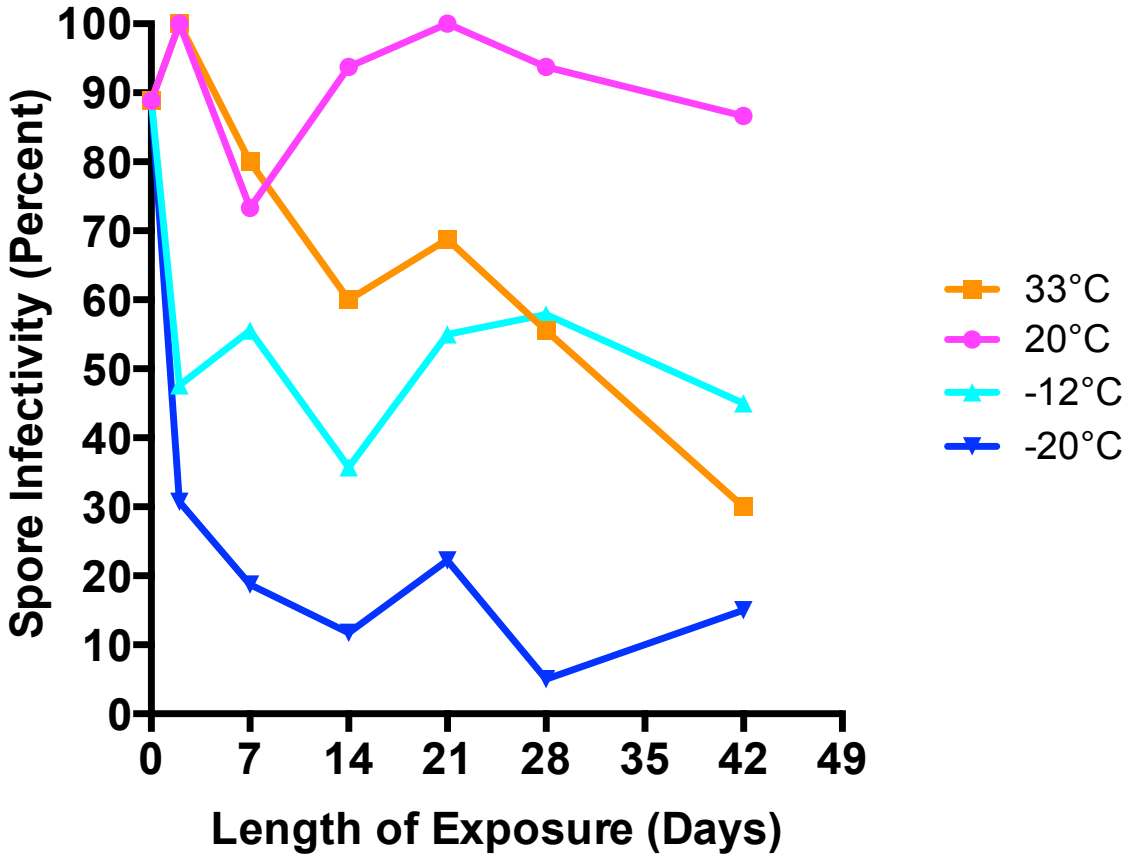

Figure 22. Mean *N. ceranae* infectivity for spores stored in 2M sucrose at 33, 20, -12, and -20°C for up to one year. Infectivity reflects the percentage of surviving, infected bees at the end of a 14-day incubation.

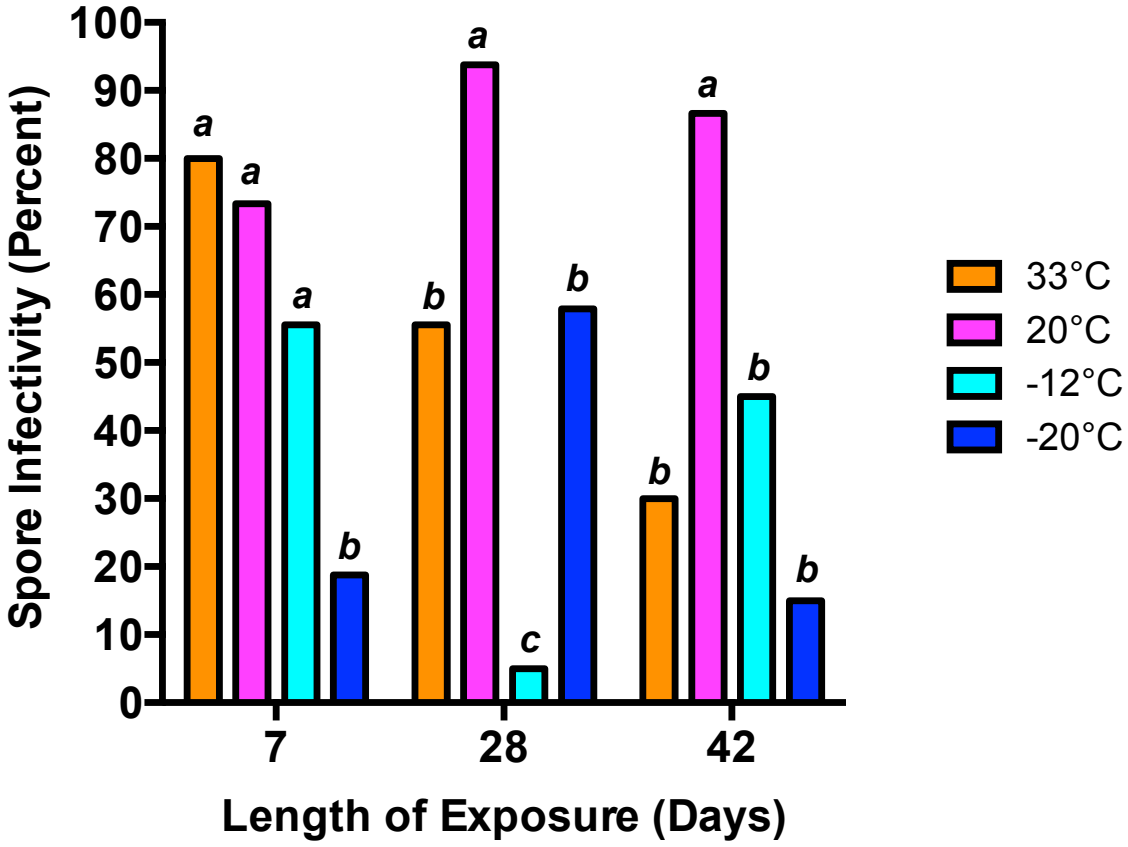

Figure 23. Mean *N. ceranae* spore infectivity for spores maintained in 2M sucrose at 33, 20, -12, and -20°C at three biologically relevant time points. Bars with different letters indicate statistically significant differences (*P<0.05*; Partitioning) among treatments for a given time point. (Day 7 χ^2=14.36 *P*=0.0025; Day 28 χ^2=29.14 P<0.0001; Day 42 χ^2=19.60 *P*=0.0002)

Table 9. Summary of χ^2 comparisons of *N. ceranae* spore infectivity for spores stored in 2M sucrose among all four treatments across three biologically relevant time points. Letters indicate significant differences ($P<0.05$) between treatments based on χ^2 analysis tests for equal proportions followed by partitioning and correction for multiple comparisons.

Treatment	Infectivity (Mean %)	Replicates (n)[h]
2M Sucrose Day 7	χ^2= 14.36 df=3 *P*=0.0025	
33°C	80 **a**	15
20°C	73 **a**	15
-12°C	56 **a**	18
-20°C	19 **b**	16
2M Sucrose Day 28	χ^2= 29.14 df=3 *P*<0.0001	
33°C	56 **b**	18
20°C	94 **a**	16
-12°C	5 **c**	20
-20°C	58 **b**	19
2M Sucrose Day 42	χ^2= 19.60 df=3 *P*=0.0002	
33°C	30 **b**	20
20°C	87 **a**	15
-12°C	45 **b**	20
-20°C	15 **b**	20

[h] Number of surviving bees inoculated with 2M sucrose-treated spores at the end of a two-week incubation period

2.3.10 Summary of half-lives for all Nosema ceranae viability experiments

Half-lives for all *N. ceranae* spore viability experiments are summarized in Table 10. Spores maintained in honey at low temperatures (-12, and -20C) have the highest estimable half-lives (Table 10), while spores maintained on wax have some of the lowest estimable half-lives. This information will be useful when offering new management recommendations to beekeepers.

Table 10. Half-lives of *Nosema ceranae* spores after exposure to different treatment regimes.

Treatment	Half-Life (Days)	95% CI	
		LCL[a]	UCL[b]
Honey			
33°C	31 [c]	22.9	49.8
20°C	149 [c]	82.9	748.4
-12°C	>365 [d]		
-20°C	>365 [d]		
Wax			
33°C	2 [c]	1.9	3.1
20°C	<90 [c]		
-12°C	2 [c]	0.8	6.2
-20°C	<1 [c]		
Water			
33°C	86 [e]		
20°C	222 [e]		
-12°C	222 [e]		
-20°C	6 [e]	3.8	11.7
2M Sucrose			
33°C	156 [e]		
20°C	197 [e]		
-12°C	1,963 [e]		
-20°C	7,938 [e]		

[a] LCL = Lower Confidence Limit
[b] UCL = Upper Confidence Limit
[c] Estimated using a one-phase decay equation
[d] Inestimable due to low decay
[e] Estimated using a linear regression equation

2.4 Discussion

This is the first study to examine the viability and *in vivo* infectivity of *N. ceranae* spores maintained in honey bee-associated matrices under realistic environmental conditions for northern temperate climates. Evaluation of *N. ceranae* spore survival and infectivity under these conditions allows for the enhancement of current management recommendations, offering beekeepers economical, chemical-free strategies to reduce *N. ceranae* spore loads within their operations, improving honey bee health.

Long-term storage of *N. ceranae* spores in honey at low temperatures (-12, and -20°C) proved effective at maintaining spore viability and infectivity, while storage at room and hive temperature (20 and 33°C respectively) had a negative impact on both *N. ceranae* spore survival and infectivity. With the exception of cryopreservation studies (McGowan 2012, McGowan et al. 2016), this contrasts with our current understanding of *N. ceranae* spore biology. Much of the literature describes *N. ceranae* as a cold intolerant and thermotolerant organism, capable of surviving for extended periods of time at high (33-60°C) temperatures (Fenoy et al. 2009, Fries and Forsgren 2009, Martín-Hernández et al. 2009, Higes et al. 2010b, Sánchez Collado et al. 2014). Nevertheless, the bulk of this previous work has examined *N. ceranae* spore viability in suspensions, under laboratory conditions, rather than field-simulated conditions, which undoubtedly contributes to differences in spore viability and infectivity.

In many host-microsporidia systems, cold tolerance is the rule rather than the exception (Weiser 1956, Thomson 1958, Revell 1960, Oshima 1964, Kramer 1970). For example, *Perezia fumiferanae* Thomson, a microsoporidian of *Choristoneura fumierferana* Clemens, can survive 4-6 months at -5°C, frozen in water (Weiser 1956).

E. cuniculi, a parasite of mammalian hosts, also remains infective after exposure to low temperatures (4, -12, and -24°C) in distilled water but not high temperatures (60 and 70°C) (Koudela et al. 1999).

Given the high sugar and low moisture content of honey, the long term survival of *N. ceranae* spores in honey at -12, and -20°C is not particularly surprising. The high sugar:water ratio likely inhibits the formation of ice crystals, acting as a cryoprotectant (similar to glycerol) preventing damage to the spores. This finding is in accordance with McGowan et al. (2016) who found that *N. ceranae* spores exhibited high viability and infectivity after storage at -70°C in 10% glycerol.

Spores maintained at higher temperatures (20 and 33°C) in honey exhibited much lower viability and infectivity over the time course of the experiment than spores stored at -12 and -20°C. These effects may be explained by several hypotheses regarding spore germination, an event governed by combinations of stimuli (including: pH shift, ion concentration, enzyme activity, membrane hydration state, osmolarity, and redox potential) that appear to vary with species (Ishihara 1967, Weidner and Byrd 1982, Pleshinger and Weidner 1985, Undeen et al. 1987, Undeen 1990, Undeen and Frixione 1990, De Graaf et al. 1993, Frixione et al. 1997, Undeen and Vander Meer 1999).

In several species of microsporidia infecting aquatic hosts (namely *N. algerae* Vávra and Undeen), the catabolism of trehalose, the main carbohydrate storage of many microsporidian species (Wood et al. 1970, Undeen and Vander Meer 1999, Keeling and Fast 2002), into subunits of glucose by the enzyme trehalase is considered an important step in the germination process (Undeen et al. 1987, Undeen 1990, Undeen and Vander Meer 1999).

It is not documented whether *N. ceranae* possesses a trehalose store. If this species requires trehalose degradation for germination, it is possible that some of the warmer storage conditions used in these experiments may present some but not all the stimuli (suboptimal stimuli) required for germination, leading to ineffective germination and spore death. Possibly the brood nest temperature and acidic pH of honey may trigger a gradual breakdown of trehalose by trehalase, resulting in lower pressure buildup within the posterior vacuole than is necessary to effectively fire the polar filament during the germination process, and thereby incomplete germination. This mechanism of slow trehalose catabolism has been suggested previously for spores of *Edhazardia aedis* Kudo (Undeen et al. 1993), and it is through this mechanism that spores of *N. algerae* lose viability after exposure to irradiation (Undeen et al. 1984, Undeen and Vander Meer 1990). Although less is known about this mechanism for terrestrial spores, it would explain the variation between percent infectivity and percent viability; spores that had not germinated but were inefficiently breaking down trehalose would appear as viable, but may be incapable of infecting a host. *N. apis* possesses trehalose, and it is interesting to note that the trehalase of *N. apis* is active between 35-45°C (Vandermeer and Gochnauer 1971), and most active at pH 6.0-7.5 (Vandermeer and Gochnauer 1971, De Graaf et al. 1993), which is the pH maintained by the honey bee ventriculus (Hoskins and Harrison 1934). Although the catabolism of trehalose does not appear to play a major role in the germination of *N. apis* (De Graaf, et al.1993, Undeen and Vander Meer 1999), it is conceivable that it may play a role in *N. ceranae* germination.

If *N. ceranae* is similar to *N. apis*, and a large degradation of trehalose does not precede germination, a mechanism involving the breakdown of another storage

carbohydrate, or change in osmolarity, may be influencing the viability and infectivity of the spores. For spores of *N. apis*, De Graaf et al. (1993) suggest that germination is stimulated by monovalent anions. As high viability and infectivity at low temperatures, and lower viability at high temperatures was also observed in 2M sucrose, stimulation of even ineffective germination by the presence of ions seems unlikely because of the absence of monovalent ions in the 2M sucrose. There is also the possibility that the reduction in viability and infectivity observed at high temperatures in honey is not due to ineffective germination, but rather damage to the membrane by some other mechanism. Malone et al (2001) indicate that caged bees inoculated with 10^2 or 10^3 spores maintained in honey had lower spore levels than bees inoculated with the same dosage of spores stored in sugar syrup. They suggest that the observed difference in infectivity may be attributed to the high pH or peroxide activity of honey, but do not propose a mechanism for how these properties impact spore viability. It may be possible that high pH and peroxide activity contribute to the thinning of the apical portion of the spore membrane, resulting in spore death, and thereby lower infectivity, or that they damage transmembrane pathways, preventing or reducing the amount of water required for effective germination from entering the spore.

The results of this experiment suggest that honey may act as an important route of transmission for *N. ceranae*. *N. apis*, a cold-tolerant parasite of the honey bee, is frequently referred to as a wet Nosema, as dysentery is a common symptom, and transmission is primarily via a fecal-oral route (Fries 1993). The phenology of a *N. apis* infection is characterized by low spore levels over the summer when high host population turnover occurs and bees can defecate outside of the colony, followed by an increase in

spore number in the fall, and a peak in the winter when population turnover is low, and the hosts are confined to, and defecate inside, the colony (Fries 1993). In an effort to remove the fecal deposits from within the colony over the winter, the bees ingest *N. apis* spores and perpetuate the infection. Although *N. ceranae* is often referred to as a dry Nosema (dysentery is not a common symptom, therefore a fecal-oral route of transmission seems unlikely), and is traditionally thought of as a heat-tolerant and cold intolerant parasite, the potential for infection of bees with spores from honey may be similar for both parasites. Honey bee populations are at their largest during the summer. For *N. ceranae* spores, displaying moderate viability and infectivity for over 42 days in honey at brood nest temperature is likely sufficient to ensure the parasite population is sustained due to the high number of available hosts during this time. With fewer hosts available (and low turnover) over the winter months, and increased *N. ceranae* spore survival and infectivity in honey at low temperatures, the parasite's chance of reproduction and proliferation within the colony increases, with no presumed fecal-oral route of transmission. The phenology of infection resulting from this mechanism of transmission is most likely increased levels of *N. ceranae* in the spring followed by reduced levels over summer and early fall, with a peak in the winter. This pattern is consistent with infections already observed in temperate climates (Williams et al. 2008b, Gisder et al. 2010, Williams et al. 2011, Copley and Jabaji 2012, Ibrahim et al. 2012, McGowan 2012, Mulholland et al. 2012, Traver et al. 2012), and is similar to the phenology of *N. apis* (see Fries 1993). In addition, *N. ceranae*-infected honey bees have also been shown to self-medicate with honey (Gherman et al. 2014). From the

perspective of the parasite, this is beneficial, as transmission is further enhanced. This mechanism would also support the aforementioned phenology.

The results from the experiment to examine the effect of temperature on the viability and infectivity of *N. ceranae* spores dried onto beeswax indicate that desiccation has a substantial negative impact on both *N. ceranae* spore viability and infectivity. This is consistent with much of the literature, which suggests that spores dried onto a surface are much more susceptible to desiccation than those suspended in liquids or dead hosts (White 1919, Allen 1954, Weiser 1956, Malone et al. 2001).

Fenoy et al. (2009) examined the viability of *N. ceranae* spores dried onto glass slides using DAPI and Sytox Green as fluorescent dyes, however they did not observe a dramatic decrease in spore viability after 1 week of exposure at room temperature. An alternative explanation for these results could be that Fenoy et al. (2009) may have experienced significant autofluorescence of *N. ceranae* spores under the green filter (excitation 470-490 nm) that is used for viewing Sytox Green, contributing to increased apparent viability. This phenomenon was recognized by McGowan (2012) when viewing fresh, unstained spores under the same wavelengths,.

For the current experiment, *N. ceranae* spores maintained on beeswax were wetted to prepare them for viability and infectivity assays. Kramer (1970) found that dried feces-bound spores of *Octosporea muscadomesticae* Flu re-wetted with water before being delivered to susceptible hosts for infection assays resulted in fewer infections than spores given to hosts dry, and suggests that the environmental moisture provided by the water may trigger a futile germination, resulting in reduced infectivity. For the current (beeswax) experiment, it is possible that futile germination was triggered

by preparing samples for assays, leading subsequently to poor viability and infectivity. No extruded polar filaments were observed during the viability assays, however, suggesting that futile germination was not a confounding factor. The sharp decrease in infectivity for spores at 20°C on days 2 and 9 p.i may have been the result of improper inoculation technique; the inoculum may not have been adequately homogenized, resulting in the harnessed bees receiving a low dose or no dose of *N. ceranae* spores, which would result in poor spore production within the bees, and a score of zero for infectivity.

N. ceranae spores maintained in water at -20°C displayed significantly lower viability and lower infectivity than those maintained at -12, 20 and 33°C. Reduced viability and infectivity at -20°C is consistent with previous work which indicates that spores of *N. ceranae* experience increased mortality at low temperatures when stored in high water content liquids (Fenoy et al. 2009, Sánchez Collado et al. 2014).

Contaminated water (e.g., puddles, water feeders) has previously been reported to act as a source of infective *N. apis* spores for foraging honey bees (Bailey, 1955). This may also be true for *N. ceranae*, especially for contaminated ephemeral water sources available to foraging honey bees during the spring and summer months in temperate climates. In this study, *N. ceranae* spores maintained in water at 20°C maintained high viability and infectivity over the course of a six-week exposure. High viability and infectivity over six weeks suggests that the spores are capable of surviving in ephemeral water sources over the spring and summer months in temperate regions. Because of this high survival, it is likely that *N. ceranae* spores maintained in ephemeral water sources at

20°C are capable of infecting foraging honey bees, and their nestmates once the foragers return to the hive and pass the contaminated water along to their nestmates.

For spores of *N. ceranae* stored in 2M sucrose, viability was slightly higher for spores stored at -12 and -20°C than those at 20 and 33°C. For spores maintained in honey, viability was also higher at -12 and -20°C than at 20 and 33°C. Infectivity was higher for spores maintained at higher temperatures at the majority of the time points examined in 2M sucrose. This suggests that in 2M sucrose membrane integrity is preserved (accounting for high viability), while something, perhaps the generation of ice crystals within the spores occurs at lower temperatures, preventing them from germinating effectively. The reduction in infectivity experienced by spores maintained in 2M sucrose at low temperatures compared to those maintained in honey at low temperatures, may be a result of the increased water content, 31.54% in 2M sucrose compared with 14.70-18.30% in honey.

Different *N. ceranae* spore suspensions were used to conduct the viability/infectivity experiments in honey and 2M sucrose. Nevertheless, the reduction in viability and infectivity experienced by spores maintained in honey at 33°C at 42 d.p.i. is still substantial compared to spores maintained at 33°C in 2M sucrose at 42 d.p.i. Malone et al. (2001) observed a similar trend while assessing the viability of *N. apis* spores in sugar syrup and two types of honey at 33°C. In general, Malone et al. (2001) found that *N. apis* spores stored in sugar syrup maintained higher viability, and resulted in a higher number of spores/infected bee than spores maintained in honey. They suggest that this "honey effect" is caused by the pH or peroxide activity of honey.

Given that a "honey effect" has been observed to negatively impact both *N. apis* and *N. ceranae* spores at 33°C, an investigation into what causes this reduction in viability and infectivity in honey at 33°C should be conducted. Identifying the mechanism responsible for *N. ceranae* spore death at 33°C in honey could result in a novel treatment method that would actually eliminate infections by killing the infectious (spore) stage of the parasite, rather than acting on the reproductive stages, which only suppresses infections.

This study has contributed much knowledge to our understanding of basic *N. ceranae* spore biology. It has illustrated that *N. ceranae* spore viability and infectivity are dependent upon storage media and temperature, and it has provided evidence for cold tolerance, which makes *N. ceranae*'s prevalence and persistence in temperate climates much less mysterious. In addition, the high viability and infectivity of *N. ceranae* spores in honey at low temperatures suggest that honey may act as a main route of transmission for the parasite.

Based on the results of these experiments, economical, chemical-free methods for reducing *N. ceranae* spore loads within beekeeping operations that are non-toxic to honey bees are possible. When spore viability is <50%, spores can be considered noninfectious (Undeen et al. 1993). To ensure >50% mortality for *N. ceranae*-contaminated honeycomb, beekeepers should maintain comb at -12°C or colder for 7 days to reduce the viability of any spores present on wax. In addition comb exposed to 33°C for 50 days will reduce the viability of spores in honey to <50%.

Viability for spores maintained in 2M sucrose did not reach a half-life, making management recommendations for fall feed decontamination more difficult. Infectivity

for spores maintained at -20°C in 2M sucrose was reduced by half at ~0.92 d.p.i., and infectivity was reduced to >20% at the last experimental time point. However, because this experiment was terminated at 42 d.p.i, and viability was high across all four treatments at this time point, we recommend beekeepers not barrel feed or re-use feed applied to colonies.

Due to the low presence of water in/on hive equipment, it seems unlikely that contaminated water would act as an important route of transmission for *N. ceranae* within the hive. However, this does not mean that ephemeral water sources in the spring and summer cannot act as sources of spores for *N. ceranae*, as both viability and infectivity were high 79.5% and 73% respectively for spores maintained at 20°C at 42 d.p.i. Beekeepers concerned about *N. ceranae* transmission via water could provide their colonies with water feeders, which is done in some arid regions, and change out the water on a regular basis. Each colony should, of course, have access to its own feeder and not a communal water feeder to reduce the spread of the parasite between colonies.

Future experiments should examine the viability and infectivity of *N. ceranae* spores in bee bread and on wood and plastic; three matrices present within honey bee colonies that could act as potential reservoirs for spores. Despite the efforts in this study *N. ceranae* spores were not adequately separated from bee bread. Future work should include the generation of a chemical-free method of effectively separating *N. ceranae* spores from bee bread to reliably evaluate the viability and infectivity of spores after exposure to various environmental conditions.

2.4.1 General Discussion

The possible transmission of *N. ceranae* via contaminated food sources suggests that the parasite could move rapidly both within and among colonies. This movement could occur through the consumption of honey or sugar syrup, the robbing of weak hives or, given the lack of symptoms associated with the parasite, movement of contaminated honeycomb by beekeepers. It also suggests that *N. ceranae* may be transferred more readily among hives than *N. apis*, which relies heavily on a fecal-oral route of transmission, often through the cleaning of contaminated comb within a colony. Given that *N. apis* is often associated with dysentery and sick/crawling bees (visible symptoms), beekeepers are also less likely to reuse comb from *N.apis*-contaminated colonies without first decontaminating it, thereby reducing the spread of the parasite among colonies.

Given the way in which honey bee colonies are managed in temperate climates (i.e., overwintered), and the excellent survival of *N. ceranae* spores in feed at low temperatures, the phenology of *N. ceranae* will likely continue to mimic that of *N. apis*. The lack of symptoms associated with *N. ceranae*, and the fact that contaminated feed is an effective route of transmission for a parasite, also suggests that *N. ceranae* will continue to displace *N. apis* in areas where the parasite is present.

Possible triggers of germination specific to *N. ceranae* remain to be investigated. Understanding what stimulates parasite reproduction is the first step towards generating effective management techniques. As there was a substantial reduction in viability and infectivity for spores maintained in honey at hive (33°C) temperature, the mechanism responsible for this reduction should be investigated. It could provide insight into some

of the stimuli required for germination, or it may simply reveal a novel treatment method for *N. ceranae*.

2.4.2 Conclusions

This study has contributed substantial knowledge to our understanding of *N. ceranae* spore biology, and demonstrates the importance of conducting biologically relevant experiments. It has provided evidence for cold tolerance, supporting the parasite's persistence in temperate climates, and has illustrated a possible route of transmission for the parasite. The results of this study have also allowed for the development of some economical, chemical-free methods to reduce the spread of *N. ceranae* spores through beekeeping equipment.

Literature Cited

Alaux C., Brunet, J.L., Dussaubat, C., Mondet, F., Tchamitchian, S., et al. (2009) Interactions between *Nosema* microspores and a neonicotinoid weaken honey bees (*Apis mellifera*). Env Microbiol **12**, 774-782.

Alaux C., Folschweiller, M., McDonnell, C., Beslay, D., Cousin, M., Dussaubat, C., Brunet, J.L., Le Conte, Y. (2011) Pathological effects of the microsporidium *Nosema ceranae* on honey bee queen physiology (*Apis mellifera*). J Invertebr Pathol **106**, 380-385.

Allen H., W. (1954) *Nosema* disease of *Gnorimoschema operculella* (Zeller) and *Macrocentrus ancylivorus* Rohwer. Ann Entomol Soc Am **47**, 407-424.

Andreadis T.G., Hall, D.W. (1979) Development, ultrastructure, and mode of transmission of *Amblyospora* sp. (Microspora) in the mosquito. J Protozool **26**, 444-452.

Antúnez K., Martín-Hernández, R., Prieto, L., Meana, A., Zunino, P., Higes, M. (2009) Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (Microsporidia). Environ Microbiol **11**, 2284-2290.

Aufauvre J., Biron, D.G., Vidau, C., Fontbonne, R., Roudel, M., et al. (2012) Parasite-insecticide interactions: a case study of *Nosema ceranae* and fipronil synergy on honeybee. Sci Rep **2**, 326.

Bailey L. (1953) Effect of Fumagillin upon *Nosema apis* (Zander). Nature **171**, 212-213.

Bailey L. (1955) The epidemiology and control of Nosema disease of the honey-bee. Ann Appl Biol **43**, 379-389.

Bailey L. (1967) *Nosema apis* and dysentery of the honey bee. J Apic Res **6**, 121-125.

Bailey L. (1981) Honey bee pathology, 1st Edition, Academic Press, London, UK.

Baldauf S.L., Roger, A.J., Wenk-Siefert, I., Doolittle, W.F. (2000) A kingdom-level phylogeny of eukaryotes based on combined protein data. Science **290**, 972-977.

Baneux P.J.R., Pognan, F. (2003) *In utero* transmission of *Encephalitozoon cuniculi* strain type I in rabbits. Lab Anim **37**, 132-138.

Becnel J.J., Andreadis, T.G. (1999) Microsporidia in insects, in: Wittner M. (Ed.), The microsporidia and microsporidiosis, ASM Press, Washington DC, pp. 447-501.

Becnel J.J., Andreadis, T.G. (2014) Microsporidia in insects, in: Wittner M. and Weiss L.M. (Eds.), Microsporidia: Pathogens of opportunity, John Wiley & Sons Inc., pp. 521-570.

Becnel J.J., Takvorian, P.M., Cali, A. (2014) Checklist of available generic names for microsporidia with type species and type hosts, in: Weiss L.M. and Becnel J.J. (Eds.), Microsporidia: Pathogens of opportunity, John Wiley & Sons Inc., pp. 671-686.

Botías C., Anderson, D.L., Meana, A., Garrido-Bailón, E., Martín-Hernández, R., Higes, M. (2012) Further evidence of an oriental origin for *Nosema ceranae* (Microsporidia: Nosematidae). J Invertebr Pathol **110**, 108-113.

Botías C., Martín-Hernández, R., Barrios, L., Meana, A., Higes, M. (2013) *Nosema* spp. infection and its negative effects on honey bees (*Apis mellifera iberiensis*) at the colony level. Vet Res **44,** 1-14.

Calderone N.W. (2012) Insect pollinated crops, insect pollinators and US agriculture: trend analysis of aggregate data for the period 1992-2009. PLoS ONE **7**, e37235.

Cali A., Owen, R.L. (1988) Micorsporidiosis, in: Balows JR A., Hausler W.J., Ohashi M., and Turano A. (Eds.), Laboratory diagnosis of infectious diseases principles and practice, Springer-Verlag, New York, pp. 929-950.

Cali A., Takvorian, P.M. (2014) Developmental morphology and life cycles of the microsporidia, in: Wittner M. and Weiss L.M. (Eds.), Microsporidia: Pathogens of opportunity, John Wiley & Sons Inc., pp. 71-133.

Canning E.U. (1953) A new microsporidian *Nosema locustae* n. sp., from the fat body of the African migratory locust, *Locusta migratoria migratoroides* R. & F. Parasitol **43**, 287-290.

Cantwell G.E., Shimaunki, H. (1969) Heat treatment as a means of eliminating nosema and increasing production. Am Bee J **109**, 52-54.

Chaimanee V., Warrit, N., Chantawannakul, P. (2010) Infections of *Nosema ceranae* in four different honeybee species. J Invertebr Pathol **105**, 207-210.

Chaimanee V., Chen, Y., Pettis, J.S., Scott Cornman, R., Chantawannakul, P. (2011) Phylogenetic analysis of *Nosema ceranae* isolated from European and Asian honeybees in Northern Thailand. J Invertebr Pathol **107**, 229-233.

Charbonneau L.R., Hillier, N.K., Rogers, R.E., Williams, G.R., Shutler, D. (2016) Effects of *Nosema apis*, *N. ceranae*, and coinfections on honey bee (*Apis mellifera*) learning and memory. Sci Rep **6**, 22626.

Chauzat M.-P., Higes, M., Martín-Hernández, R., Meana, A., Cougoule, N., Faucon, J.-P. (2007) Presence of *Nosema ceranae* in French honey bee colonies. J Apic Res **46**, 127-128.

Chen Y., Evans, J.D., Smith, I.B., Pettis, J.S. (2008) *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J Invertebr Pathol **97**, 186-188.

Chen Y.P., Evans, J.D., Murphy, C., Gutell, R., Zuker, M., Gundensen-Rindal, D., Pettis, J.S. (2009) Morphological, molecular, and phylogenetic characterization of *Nosema ceranae*, a microsporidian parasite isolated from the European honey bee, *Apis mellifera*. J Eukaryot Microbiol **56**, 142-147.

Copley T.R., Jabaji, S.H. (2012) Honeybee glands as possible infection reservoirs of *Nosema ceranae* and *Nosema apis* in naturally infected forager bees. J Appl Microbiol **112**, 15-24.

Cornman R.S., Chen, Y.P., Schatz, M.C., Street, C., Zhao, Y., et al. (2009) Genomic analyses of the microsporidian *Nosema ceranae*, an emergent pathogen of honey bees. PLoS Pathog **5**, e1000466.

Costa C., Lodesani, M., Maistrello, L. (2010) Effect of thymol and resveratrol administered with candy or syrup on the development of *Nosema ceranae* and on the longevity of honeybees (*Apis mellifera* L.) in laboratory conditions. Apidologie **41**, 141-150.

Cox-Foster D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., et al. (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. Science **318**, 283-287.

Currie R.W., Pernal, S.F., Guzmán-Novoa, E. (2010) Honey bee colony losses in Canada. J Apic Res **49**, 104-106.

Darrach M., Page, S. (2016) Statistical overview of the Canadian honey bee industry and the economic contribution of honey bee pollination 2013-2014, AAFC Horticulture and Cross Sectoral Division, Report No. 12447E.

De Graaf D.C., Masschelein, G., Vandergeynst, F., De Brabander, H.F., Jacobs, F.J. (1993) In vitro germination of *Nosema apis* (Mirospora: Nosematidae) spores and its effect on their alpha-alpha-trehalose/D-glucose ratio. J Invertebr Pathol **62**, 220-225.

De Graaf D.C., Raes, H., Sabbe, G., De Rycke, P.H., Jacobs, F.J. (1994a) Early development of *Nosema apis* (Microspora: Nosematidae) in the midgut epithelium of the honeybee (*Apis mellifera*). J Invertebr Pathol **63**, 74-81.

De Graaf D.C., Raes, H., Jacobs, F.J. (1994b) Spore dimorphism in *Nosema apis* (Microsporida, Nosematidae) developmental cycle. J Invertebr Pathol **63**, 92-94.

Didier E.S., Weiss, L.M. (2012) Microsporidiosis: not just in AIDS patients. Curr Opin Infect Dis **24**, 490-495.

Dönhoff E., Leuckart, R. (1857) Über den Fadenpilz im Darm der Biene. Bienenztg **13**, 66-67, 72.

Dunn A.M., Smith, J.E. (2001) Microsporidian life cycles and diversity: the relationship between virulence and transmission. Microb and Infect **3**, 381-388.

Dussaubat C., Brunet, J.L., Higes, M., Colbourne, J.K., Lopez, J., et al. (2012) Gut pathology and responses to the microsporidium *Nosema ceranae* in the honey bee *Apis mellifera*. PLoS ONE **7**, e37017.

Dussaubat C., Maisonnasse, A., Crauser, D., Beslay, D., Costagliola, G., Soubeyrand, S., Kretzchmar, A., Le Conte, Y. (2013) Flight behavior and pheromone changes associated to *Nosema ceranae* infection of honey bee workers (*Apis mellifera*) in field conditions. J Invertebr Pathol **113**, 42-51.

Ebert D., Herre, E.A. (1996) The evolution of parasitic disease. Parasitol Today **12**, 96-101.

Emsen B., Guzmán-Novoa, E., Hamiduzzaman, M.M., Eccles, L., Lacey, B., Ruiz-Pérez, R.A., Nasr, M. (2016) Higher prevalence and levels of *Nosema ceranae* than *Nosema apis* infections in Canadian honey bee colonies. Parasitol Res **115**, 175-181.

Farrar C.L. (1942) Nosema disease contributes to winter losses and queen supersedure. Glean Bee Cult **70**, 660-661.

Farrar C.L. (1947) Nosema losses in package bees as related to queen supersedure and honey yields. J Econ Entomol **40**, 333-338.

Fast N.M., Logsdon, J.M., Jr., Doolittle, W.F. (1999) Phylogenetic analysis of the TATA box binding protein (TBP) gene from *Nosema locustae*: Evidence for a microsporidia-fungi relationship and spliceosomal intron loss. Mol Biol Evol **16**, 1415-1419.

Faucon J.P. (2005) La Nosémose. La santé de l'Abeille **209**, 343-367.

Fenoy S., Rueda, C., Higes, M., Martín-Hernández, R., del Aguila, C. (2009) High-level resistance of *Nosema ceranae*, a parasite of the honeybee, to temperature and desiccation. Appl Environ Microbiol **75**, 6886-6889.

Fine P.E.M. (1975) Vectors and vertical transmission: an epidemiologic perspective. Ann N. Y. Acad Sci **266**, 173-194.

Forsgren E., Fries, I. (2010) Comparative virulence of *Nosema ceranae* and *Nosema apis* in individual European honey bees. Vet Parasitol **170**, 212-217.

Fries I. (1993) *Nosema apis* - a parasite in the honey bee colony. Bee World **74**, 5-19.

Fries I. (1997) Protozoa, in: Morse R.A. and Flottum K. (Eds.), Honey Bee Pests, Predators, and Diseases, A.I. Root Company, Medina, Ohio, U.S.A., pp. 59-76.

Fries I. (2010) *Nosema ceranae* in european honey bees (*Apis mellifera*). J Invertebr Pathol **103 Suppl 1**, S73-79.

Fries I., Forsgren, E. (2009) *Nosema ceranae* fungerar inte som *Nosema apis*. *Nosema ceranae* does not function as *Nosema apis*. Bitidningen **107**, 20-21.

Fries I., Ekbohm, G., Villumstad, E. (1984) *Nosema apis*, sampling techniques and honey yield. J Apic Res **23**, 102-105.

Fries I., Feng, F., Da Silva, A., Slemenda, S.B., Pieniazek, N.J. (1996) *Nosema ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee *Apis cerana* (Hymenoptera, Apidae). Eur J Protistol **32**, 356-365.

Fries I., Martín, R., Meana, A., García-Palencia, P., Higes, M. (2006) Natural infections of *Nosema ceranae* in European honey bees. J Apic Res **45**, 230-233.

Frixione E., Ruiz, L., Cerbon, J., Undeen, A.H. (1997) Germination of *Nosema algerae* (Microspora) spores: Conditional inhibition by D-2O, ethanol and Hg-2+ suggests dependence of water influx upon membrane hydration and specific transmembrane pathways. J Eukaryot Microbiol **44**, 109-116.

Fürst M.A., McMahon, D.P., Osborne, J.L., Paxton, R.J., Brown, M.J.F. (2014) Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature (London) **506**, 364-366.

Genersch E., von der Ohe, W., Kaatz, H., Schroeder, A., Otten, C., et al. (2010) The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie **41**, 332-352.

Germot A., Philippe, H., Le Guyader, H. (1997) Evidence for loss of mitochondria in Microsporidia from a mitochondrial-type HSP70 in *Nosema locustae*. Mol Biochem Parasitol **87**, 159-168.

Gherman B.I., Denner, A., Bobiş, O., Dezmirean, D.S., Mărghitaş, L.A., Schlüns, H., Moritz, R.F.A., Erler, S. (2014) Pathogen-associated self-medication behavior in the honeybee *Apis mellifera*. Behav Ecol and Sociobiol **68**, 1777-1784.

Giersch T., Berg, T., Galea, F., Hornitzky, M. (2009) *Nosema ceranae* infects honey bees (*Apis mellifera*) and contaminates honey in Australia. Apidologie **40**, 117-123.

Gisder S., Hedtke, K., Möckel, N., Frielitz, M.C., Linde, A., Genersch, E. (2010) Five-year cohort study of *Nosema* spp. in Germany: does climate shape virulence and assertiveness of *Nosema ceranae*? Appl Environ Microbiol **76**, 3032-3038.

Gisder S., Möckel, N., Linde, A., Genersch, E. (2011) A cell culture model for *Nosema ceranae* and *Nosema apis* allows new insights into the life cycle of these important honey bee-pathogenic microsporidia. Env Microbiol **13**, 404-413.

Goblirsch M., Huang, Z.Y., Spivak, M. (2013) Physiological and behavioral changes in honey bees (*Apis mellifera*) induced by *Nosema ceranae* infection. PLoS ONE **8**, 1-11.

GraphPad curve fitting guide, GraphPad Software Inc., http://cdn.graphpad.com/docs/prism/6/Prism-6-Curve-Fitting-Guide.pdf.

Gray F.H., Cali, A., Briggs, J.D. (1969) Intracellular stages in the life cycle of the microsporidian *Nosema apis*. J Invertebr Pathol **14**, 391-394.

Guzmán-Novoa E., Hamiduzzaman, M.M., Arechavaleta-Velasco, M.E., Koleoglu, G., Valizadeh, P., Correa-Benítez, A. (2011) *Nosema ceranae* has parasitized Africanized honey bees in Mexico since at least 2004. J Apic Res **50**, 167-169.

Hanson F.R., Eble, T.E. (1949) An antiphage agent isolated from *Aspergillus* sp. J Bacteriol **58**, 527-529.

Higes M., Martín, R., Meana, A. (2006) *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. J Invertebr Pathol **92**, 93-95.

Higes M., García-Palencia, P., Martín-Hernández, R., Meana, A. (2007) Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (Microsporidia). J Invertebr Pathol **94**, 211-217.

Higes M., Martín-Hernández, R., Garrido-Bailón, E., García-Palencia, P., Meana, A. (2008a) Detection of infective *Nosema ceranae* (Microsporidia) spores in corbicular pollen of forager honeybees. J Invertebr Pathol **97**, 76-78.

Higes M., Martín-Hernández, R., Botías, C., Bailón, E.G., González-Porto, A.V., et al. (2008b) How natural infection by *Nosema ceranae* causes honeybee colony collapse. Environ Microbiol **10**, 2659-2669.

Higes M., Martín-Hernández, R., Garrido-Bailón, E., Gonzalez-Porto, A.V., García-Palencia, P., Meana, A., Del Nozal, M.J., Mayo, R., Bernal, J.L. (2009) Honeybee colony collapse due to *Nosema ceranae* in professional apiaries. Environ Microbiol Rep **1**, 110-113.

Higes M., Martín-Hernández, R., Meana, A. (2010a) *Nosema ceranae* in Europe: an emergent type C nosemosis. Apidologie **41**, 375-392.

Higes M., García-Palencia, P., Botías, C., Meana, A., Martín-Hernández, R. (2010b) The differential development of microsporidia infecting worker honey bee (*Apis mellifera*) at increasing incubation temperature. Environ Microbiol Rep **2**, 745-748.

Higes M., Nozal, M.J., Alvaro, A., Barrios, L., Meana, A., Martín-Hernández, R., Bernal, J.L., Bernal, J. (2011) The stability and effectiveness of fumagillin in controlling *Nosema ceranae* (Microsporidia) infection in honey bees (*Apis mellifera*) under laboratory and field conditions. Apidologie **42**, 364-377.

Higes M., Meana, A., Bartolomé, C., Botías, C., Martín-Hernández, R. (2013a) *Nosema ceranae* (Microsporidia), a controversial 21st century honey bee pathogen. Environ Microbiol Rep **5**, 17-29.

Higes M., Juarranz, A., Dias-Almeida, J., Lucena, S., Botías, C., Meana, A., García-Palencia, P., Martín-Hernández, R. (2013b) Apoptosis in the pathogenesis of *Nosema ceranae* (Microsporidia: Nosematidae) in honey bees (*Apis mellifera*). Environ Microbiol Rep **5**, 530-536.

Hirt R.P., Healy, B., Vossbrinck, C.R., Canning, E.U., Embley, T.M. (1997) A mitochondrial Hsp70 orthologue in *Vairimorpha necatrix*: Molecular evidence that microsporidia once contained mitochondria. Curr Biol **7**, 995-998.

Hirt R.P., Logsdon, J.M., Jr., Healy, B., Dorey, M.W., Doolittle, W.F., Embley, T.M. (1999) Microsporidia are related to Fungi: Evidence from the largest subunit of RNA polymerase II and other proteins. Proc Natl Acad Sci **96**, 580-585.

Hoskins W.M., and, Harrison, A.S. (1934) The buffering power of the contents of the ventriculus of the honeybee and its effect upon the toxicity of arsenic. J Econ Entomol **28**, 924-942.

Huang W.-F., Solter, L.F. (2013) Comparative development and tissue tropism of *Nosema apis* and *Nosema ceranae*. J Invertebr Pathol **113**, 35-41.

Huang W.-F., Jiang, J.-H., Chen, Y.-W., Wang, C.-H. (2007) A *Nosema ceranae* isolate from the honeybee *Apis mellifera*. Apidologie **38**, 30-37.

Huang W.F., Solter, L.F., Yau, P.M., Imai, B.S. (2013) *Nosema ceranae* escapes fumagillin control in honey bees. PLoS Pathog **9**, e1003185.

Huang W.F., Solter, L., Aronstein, K., Huang, Z. (2015) Infectivity and virulence of *Nosema ceranae* and *Nosema apis* in commercially available North American honey bees. J Invertebr Pathol **124**, 107-113.

Ibrahim A., Melathopoulos, A.P., Pernal, S.F. (2012) Integrated Management of *Nosema* Detection of Antibiotic Residues Hivelights **25**, 11-17.

Invernizzi C., Abud, C., Tomasco, I.H., Harriet, J., Ramallo, G., Campá, J., Katz, H., Gardiol, G., Mendoza, Y. (2009) Presence of *Nosema ceranae* in honeybees (*Apis mellifera*) in Uruguay. J Invertebr Pathol **101**, 150-153.

Ishihara R. (1967) Stimuli causing extrusion of polar filaments of *Glugea fumiferanae* spores. Can J Microbiol **13**, 1321-1332.

James T.Y., Kauff, F., Schoch, C.L., Matheny, P.B., Hofstetter, V., et al. (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature (London) **443**, 818-822.

James T.Y., Pelin, A., Bonen, L., Ahrendt, S., Sain, D., Corradi, N., Stajich, J.E. (2013) Shared signatures of parasitism and phylogenomics unite cryptomycota and microsporidia. Curr Biol **23**, 1548-1553.

Katinka M.D., Duprat, S., Cornillot, E., Méténier, G., Thomarat, F., et al. (2001) Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi*. Nature **414**, 450-453.

Katznelson H., Jamieson, C.A. (1952) Control of nosema disease of honeybees with fumagillin. Science **115**, 70-71.

Keeling P.J., Fast, N.M. (2002) Microsporidia: Biology and evolution of highly reduced intracellular parasites. Annu Rev Microbiol **56**, 93-116.

Keeling P.J., Luker, M.A., Palmer, J.D. (2000) Evidence from β-tubulin phylogeny that microsporidia evolved from within the fungi. Mol Biol Evol **17**, 23-31.

Kellen W.R., Chapman, H.C., Clark, T.B., Lindegren, J.E. (1966) Transovarian transmission of some *Thelohania* (Nosematidae: Microsporidia) in mosquitoes of California and Louisiana. J Invertebr Pathol **8**, 355-359.

Klee J., Besana, A.M., Genersch, E., Gisder, S., Nanetti, A., et al. (2007) Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. J Invertebr Pathol **96**, 1-10.

Klein A.-M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Tscharntke, T. (2007) Importance of pollinators in changing landscapes for world crops. Proc R Soc B **274**, 303-313.

Koudela B., Kučerová, S., Hudcovic, T. (1999) Effect of low and high temperatures on infectivity of *Encephalitozoon cuniculi* spores suspended in water. Folia Parasitol **46**, 171-174.

Kramer J.P. (1970) Longevity of microsporidian spores with special reference to *Octosporea muscaedomesticae* Flu*. Acta Protozool **8**, 217-224.

Kurze C., Le Conte, Y., Dussaubat, C., Erler, S., Kryger, P., Lewkowski, O., Müeller, T., Widder, M., Moritz, R.F.A. (2015) *Nosema* tolerant honeybees (*Apis mellifera*) escape parasitic manipulation of apoptosis. PLoS ONE **10**, e0140174.

Larsson R. (1986) Ultrastructure Function and Classification of Microsporidia, in: Corliss J.O. and Patterson D.J. (Eds.), Progress in Protistology Biopress Ltd., Bristol, England, UK, pp. 325-390.

Leboeuf A., Nasr, M., Ferland, J., Wislon, G., Jordan, C., et al. (2016) Canadian Association of Professional Apiculturists Statement on Honey Bee Wintering Losses in Canada 2016, http://www.capabees.com/capa-statement-on-honey-bees/.

Letcher P.M., Lopez, S., Schmieder, R., Lee, P.A., Behnke, C., Powell, M.J., McBride, R.C. (2013) Characterization of *Amoeboaphelidium protococcarum*, an algal parasite new to the Cryptomycota isolated from an outdoor algal pond used for the production of biofuel. PLoS ONE **8**, e56232.

Lewis L.C. (1978) Migration of larvae of *Ostrinia nubilalis* (Lepidoptera: Pyralidae) infected with *Nosema pyrausta* (Microsporida: Nosematidae) and subsequent dissemination of this microsporidium. Can Entomol **110**, 897-900.

Lewis L.C., Lynch, R.E. (1978) Foliar application of *Nosema pyrausta* for suppression of populations of European corn borer. Entomophaga **23**, 83-88.

Maistrello L., Lodesani, M., Costa, C., Leonardi, F., Marani, G., Caldon, M., Mutinelli, F., Granato, A. (2008) Screening of natural compounds for the control of nosema disease in honeybees (*Apis mellifera*). Apidologie **39**, 436-445.

Malone L.A., Gatehouse, H.S., Tregidga, E.L. (2001) Effects of time, temperature, and honey on *Nosema apis* (Microsporidia: Nosematidae), a parasite of the honeybee, *Apis mellifera* (Hymenoptera: Apidae). J Invertebr Pathol **77**, 258-268.

Martín-Hernández R., Meana, A., Prieto, L., Salvador, A.M., Garrido-Bailón, E., Higes, M. (2007) Outcome of colonization of *Apis mellifera* by *Nosema ceranae*. Appl Environ Microbiol **73**, 6331-6338.

Martín-Hernández R., Meana, A., García-Palencia, P., Marín, P., Botías, C., Garrido-Bailón, E., Barrios, L., Higes, M. (2009) Effect of Temperature on the biotic potential of honeybee microsporidia. Appl Environ Microbiol **75**, 2554-2557.

Martín-Hernández R., Botías, C., Barrios, L., Martínez-Salvador, A., Meana, A., Mayack, C., Higes, M. (2011) Comparison of the energetic stress associated with experimental *Nosema ceranae* and *Nosema apis* infection of honeybees (*Apis mellifera*). Parasitol Res **109**, 605-612.

Martín-Hernández R., Botías, C., Bailón, E.G., Martinez-Salvador, A., Prieto, L., Meana, A., Higes, M. (2012) Microsporidia infecting *Apis mellifera*: coexistence or competition. Is *Nosema ceranae* replacing *Nosema apis*? Environ Microbiol **14**, 2127-2138.

Mayack C., Naug, D. (2009) Energetic stress in the honeybee *Apis mellifera* from *Nosema ceranae* infection. J Invertebr Pathol **100**, 185-188.

McGowan J. (2012) Viability Assessment and Cryopreservation of the Honey Bee (*Apis mellifera*) Parasite, *Nosema ceranae*, (MSc Dissertation), University of Guelph, Guelph, Ontario, Canada. (https://atrium.lib.uoguelph.ca/xmlui/bitstream/handle/10214/3801/Janine McGowan Thesis Defense Copy July 2012.pdf?sequence=3).

McGowan J., De la Mora, A., Goodwin, P.H., Habash, M., Hamiduzzaman, M.M., Kelly, G.P., Guzmán-Novoa, E. (2016) Viability and infectivity of fresh and cryopreserved *Nosema ceranae* spores. J Microbiol Methods **131**, 16-22.

Milbrath M.O., Xie, X., Huang, Z.Y. (2013) *Nosema ceranae* induced mortality in honey bees (*Apis mellifera*) depends on infection methods. J Invertebr Pathol **114**, 42-44.

Milbrath M.O., van Tran, T., Huang, W.F., Solter, L.F., Tarpy, D.R., Lawrence, F., Huang, Z.Y. (2015) Comparative virulence and competition between *Nosema apis* and *Nosema ceranae* in honey bees (*Apis mellifera*). J Invertebr Pathol **125**, 9-15.

Mulholland G.E., Traver, B.E., Johnson, N.G., Fell, R.D. (2012) Individual Variability of *Nosema ceranae* Infections in *Apis mellifera* Colonies. Insects **3**(4), 1143-1155.

Nägeli K. (1857) Über die neue Krankheit der Seidenraupe und verwandte Organismen. Bot Ztg **15**, 760-761.

Natsopoulou M.E., Doublet, V., Paxton, R.J. (2016) European isolates of the Microsporidia *Nosema apis* and *Nosema ceranae* have similar virulence in laboratory tests on European worker honey bees. Apidologie **47**, 57-65.

Naug D., Gibbs, A. (2009) Behavioral changes mediated by hunger in honeybees infected with *Nosema ceranae*. Apidologie **40**, 595-599.

Nguyen B.K., Mignon, J., Laget, D., de Graaf, D.C., Jacobs, F.J., vanEngelsdorp, D., Brostaux, Y., Saegerman, C., Haubruge, E. (2010) Honey bee colony losses in Belgium during the 2008-9 winter. J Apic Res **49**, 333-339.

O'Mahony E.M., Tay, W.T., Paxton, R.J. (2007) Multiple rRNA variants in a single spore of the microsporidian *Nosema bombi*. J Eukaryot Microbiol **54**, 103-109.

Oshima K. (1964) Method of gathering and purifying active spores of *Nosema bombycis* and preserving them in good condition. Annot Zool Jpn **37**, 94-101.

Paxton R.J. (2010) Does infection by *Nosema ceranae* cause "Colony Collapse Disorder" in honey bees (*Apis mellifera*)? J Apic Res **49**, 80-84.

Paxton R.J., Klee, J., Korpela, S., Fries, I. (2007) *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. Apidologie **38**, 558-565.

Pernal S.F., Clay, H., (eds). (2013) Honey bee diseases and pests, 3rd Edition, Canadian Assoication of Professional Apiculturists (CAPA), Beaverlodge, AB, Canada..

Pettis J.S., Lichtenberg, E.M., Andree, M., Stitzinger, J., Rose, R., Vanengelsdorp, D. (2013) Crop pollination exposes honey bees to pesticides which alters their susceptibility to the gut pathogen *Nosema ceranae*. PLoS One **8**, e70182.

Peyretaillade E., Broussolle, V., Peyret, P., Méténier, G., Gouy, M., Vivarès, C.P. (1998) Microsporidia, amitochondrial protists, possess a 70-kDa heat shock protein gene of mitochondrial evolutionary origin. Mol Biol Evol **15**, 683-689.

Pleshinger J., Weidner, E. (1985) The microsporidian spore invasion tube. IV. Discharge activation beings with pH-triggered Ca2+ influx. J Cell Biol **100**, 1834-1838.

Plischuk S., Martín-Hernández, R., Prieto, L., Lucía, M., Botías, C., Meana, A., Abrahamovich, A.H., Lange, C., Higes, M. (2009) South American native bumblebees (Hymenoptera: Apidae) infected by *Nosema ceranae* (Microsporidia), an emerging pathogen of honeybees (*Apis mellifera*). Environ Microbiol Rep **1**, 131-135.

Porrini M.P., Audisio, M.C., Sabate, D.C., Ibarguren, C., Medici, S.K., Sarlo, E.G., Garrido, P.M., Eguaras, M.J. (2010) Effect of bacterial metabolites on microsporidian *Nosema ceranae* and on its host *Apis mellifera*. Parasitol Res **107**, 381-388.

Porrini M.P., Fernández, N., Garrido, P., Gende, L., Medici, S., Eguaras, M. (2011) *In vivo* evaluation of antiparasitic activity of plant extracts on *Nosema ceranae* (Microsporidia). Apidologie **42**, 700-707.

Retschnig G., Neumann, P., Williams, G.R. (2014) Thiacloprid-*Nosema ceranae* interactions in honey bees: Host survivorship but not parasite reproduction is dependent on pesticide dose. J Invertebr Pathol **118**, 18-19.

Revell I.L. (1960) Longevity of Refrigerated nosema spores - *Nosema apis,* a parasite of honey bees. J Econ Entomol **53**, 1132-1133.

Rinderer T.E. (1976) Honey Bees: Individual Feeding of Large Numbers of Adult Workers. J Econ Entomol **69**, 489-491.

Rutrecht S.T., Brown, M.J.F. (2008) Within colony dynamics of *Nosema bombi* infections: disease establishment, epidemiology and potential vertical transmission. Apidologie **39**, 504-514.

Sagastume S., del Águila, C., Martín-Hernández, R., Higes, M., Henriques-Gil, N. (2011) Polymorphism and recombination for rDNA in the putatively asexual microsporidian *Nosema ceranae*, a pathogen of honeybees. Environ Microbiol **13**, 84-95.

Sagastume S., Martín-Hernández, R., Higes, M., Henriques-Gil, N. (2014) Ribosomal gene polymorphism in small genomes: analysis of different 16S rRNA sequences expressed in the honeybee parasite *Nosema ceranae* (Microsporidia). J Eukaryot Microbiol **61**, 42-50.

Sánchez Collado J.G., Higes, M., Barrio, L., Martín-Hernández, R. (2014) Flow cytometry analysis of *Nosema* species to assess spore viability and longevity. Parasitol Res **113**, 1695-1701.

Shafer A.B.A., Williams, G.R., Shutler, D., Rogers, R.E.L., Stewart, D.T. (2009) Cophylogeny of *Nosema* (Microsporidia: Nosematidae) and bees (Hymenoptera: Apidae) suggests both cospeciation and a host-switch. J Parasitol **95**, 198-203.

Sharpe, D. (2015) Your Chi-Square Test is Statistically Significant: Now What? Pract Assess Res Eval **20**, 1-10.

Shaw R.W., Kent, M.L. (1999) Fish Microsporidia, in: Wittner M. (Ed.), Microsporidia and Microsporidiosis, ADM Press, Washington DC, pp. 418-446.

Sprague V. (1978) Characterization and composition on the genus *Nosema.* Misc Pub Entomol Soc Am **11**, 5-16.

Stentiford G.D., Dunn, A.M. (2014) Microsporidia in Aquatic Invertebrates, in: Wittner M. and Weiss L.M. (Eds.), Microsporidia: Pathogens of Opportunity, John Wiley & Sons Inc., pp. 579-604.

Stevanovic J., Simeunovic, P., Gajic, B., Lakic, N., Radovic, D., Fries, I., Stanimirovic, Z. (2013) Characteristics of *Nosema ceranae* infection in Serbian honey bee colonies. Apidologie **44**, 522-536.

Stevanovic J., Stanimirovic, Z., Genersch, E., Kovacevic, S.R., Ljubenkovic, J., Radakovic, M., Aleksic, N. (2011) Dominance of *Nosema ceranae* in honey bees in the Balkan countries in the absence of symptoms of colony collapse disorder. Apidologie **42**, 49-58.

Tapaszti Z., Forgách, P., Kövágó, C., Békési, L., Bakonyi, T., Rusvai, M. (2009) First detection and dominance of *Nosema ceranae* in Hungarian honeybee colonies. Acta Vet Hung **57**, 383-388.

Tay W.T., O'Mahony, E.M., Paxton, R.J. (2005) Complete rRNA gene sequences reveal that the microsporidium *Nosema bombi* infects diverse bumblebee (*Bombus* spp.) hosts and contains multiple polymorphic sites. J Eukaryot Microbiol **52**, 505-513.

Terry R.S., Smith, J.E., Bouchon, D., Rigaud, T., Duncanson, P., Sharpe, R.G., Dunn, A.M. (1999) Ultrastructural characterisation and molecular taxonomic identification of *Nosema granulosis* n. sp., a transovarially transmitted feminising (TTF) microsporidium. J Eukaryot Microbiol **46**, 492-499.

Thomson H.M. (1958) Some aspects of the epidemiology of amicrosporidian parasite of the spruce budworm, *Choristoneura fumiferana* (Clem.). Can J Zool **36**, 309-316.

Traver B.E., Fell, R.D. (2011a) *Nosema ceranae* in drone honey bees (*Apis mellifera*). J Invertebr Pathol **107**, 234-236.

Traver B.E., Fell, R.D. (2011b) Prevalence and infection intensity of *Nosema* in honey bee (*Apis mellifera* L.) colonies in Virginia. J Invertebr Pathol **107**, 43-49.

Traver B.E., Fell, R.D. (2015) A scientific note: Survey for *Nosema* spp. in preserved *Apis* spp. Apidologie **46**, 194-196.

Traver B.E., Williams, M.R., Fell, R.D. (2012) Comparison of within hive sampling and seasonal activity of *Nosema ceranae* in honey bee colonies. J Invertebr Pathol **109**, 187-193.

Undeen A.H. (1990) A Proposed Mechanism for the Germination of Microsporidian (Protozoa: Microspora) Spores. J Theor Biol **142**, 223-236.

Undeen A.H., Vander Meer, R.K. (1990) The effect of ultra-violet radiation on the germination of *Nosema algerae* Vávra and Undeen (Microsporidia: Nosematidae) Spores. J Protozool **37**, 194-199.

Undeen A.H., Frixione, E. (1990) The role of osmotic pressure in the germination of *Nosema algerae* spores. J Protozool **37**, 561-567.

Undeen A.H., Vander Meer, R.K. (1999) Microsporidian intrasporal sugars and their role in germination. J Invertebr Pathol **73**, 294-302.

Undeen A.H., Vander Meer, R.K., Smittle, B.J., Avery, S.W. (1984) The effect of gamma-irradiation on *Nosema algerae* (Microspora: Nosematidae) Spore Viability, Germination, and Carbohydrates. J Protozool **31**, 479-482.

Undeen A.H., Elgazzar, L.M., Vander Meer, R.K., Narang, S. (1987) Trehalose levels and trehalase activity in germinated and ungerminated spores of *Nosema algerae* (Microspora: Nosematidae). J Invertebr Pathol **50**, 230-237.

Undeen A.H., Johnson, M.A., Becnel, J.J. (1993) The effects of temperature on the survival of *Edhazardia aedis* (Microspora: Amblyosporidae), a pathogen of *Aedes aegypti*. J Invertebr Pathol **61**, 303-307.

Van de Peer Y., Ben Ali, A., Meyer, A. (2000) Microsporidia: Accumulating molecular evidence that a group of amitochondriate and suspectedly primitive eukaryotes are just curious fungi. Gene **246**, 1-8.

van den Heever J.P., Thompson, T.S., Otto, S.J.G., Curtis, J.M., Ibrahim, A., Pernal, S.F. (2015a) Evaluation of Fumagilin-B® and other potential alternative chemotherapies against *Nosema ceranae* -infected honeybees (*Apis mellifera*) in cage trial assays. Apidologie **47**, 617-630.

van den Heever J.P., Thompson, T.S., Otto, S.J.G., Curtis, J.M., Ibrahim, A., Pernal, S.F. (2015b) The effect of dicyclohexylamine and fumagillin on *Nosema ceranae* -infected honey bees (*Apis mellifera*) mortality in cage trial assays. Apidologie **47**, 663-670.

van der Zee R., Pisa, L., Andonov, S., Brodschneider, R., Charrière, J.-D., et al. (2012) Managed honey bee colony losses in Canada, China, Europe, Israel and Turkey, for the winters of 2008-9 and 2009-10. J Apic Res **51**, 91-114.

van der Zee R., Gómez-Moracho, T., Pisa, L., Sagastume, S., García-Palencia, P., Maside, X., Bartolomé, C., Martín-Hernández, R., Higes, M. (2014) Virulence and polar tube protein genetic diversity of *Nosema ceranae* (Microsporidia) field isolates from Northern and Southern Europe in honeybees (*Apis mellifera iberiensis*). Environ Microbiol Rep **6**, 401-413.

Vandermeer J.W., Gochnauer, T.A. (1971) Trehalase Activity Associated with Spores of *Nosema apis*. J Invertebr Pathol **17**, 38-41.

vanEngelsdorp D., Underwood, R., Caron, D., Hayes, J.J. (2007) An estimate of managed colony losses in the winter of 2006-2007. Am Bee J **147**, 599-603.

vanEngelsdorp D., Hayes, J., Jr., Underwood, R.M., Pettis, J. (2008) A survey of honey bee colony losses in the US, fall 2007 to spring 2008. PLoS ONE **3**, e4071.

vanEngelsdorp D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E., et al. (2009) Colony collapse disorder: a descriptive study. PLoS ONE **4**, e6481.

vanEngelsdorp D., Hayes, J., Jr., Underwood, R.M., Caron, D., Pettis, J. (2011) A survey of managed honey bee colony losses in the USA, fall 2009 to winter 2010. J Apic Res **50**, 1-10.

Vávra J., Larsson, J.I.R. (2014) Structure of Microsporidia, in: Wittner M. and Weiss L.M. (Eds.), Microsporidia: Pathogens of Opportunity, John Wiley & Sons Inc., pp. 1-70.

Vidau C., Diogon, M., Aufauvre, J., Fontbonne, R., Viguès, B., et al. (2011) Exposure to Sublethal Doses of Fipronil and Thiacloprid Highly Increases Mortality of Honeybees Previously Infected by *Nosema ceranae*. PLoS ONE **6**, e21550.

Vossbrinck C.R., Debrunner-Vossbrinck, B.A. (2005) Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. Folia Parasitol **52**, 131-142.

Vossbrinck C.R., Debrunner-Vossbrinck, B.A., Weiss, L.M. (2014) Phylogeny of the Microsporidia, in: Wittner M. and Weiss L.M. (Eds.), Microsporidia: Pathogens of Opportunity, 1st Edition, John Wiley & Sons Inc., pp. 203-220.

Weidner E., Byrd, W. (1982) The Microsporidian Spore Invasion Tube 2. Role of Calcium in the Activation of Invasion Tube Discharge. J Cell Biol **93**, 970-975.

Weiser J. (1956) Protozzoare Infectionen im Kampfe gegen Insekten. Z. PflKrankh PflPath PflSchutz **63**, 625-638.

White G.F. (1919) *Nosema* - Disease. U.S. Department of Agriculture Bulletin 780, pp. 59.

Williams G.R., Shafer, A.B., Rogers, R.E., Shutler, D., Stewart, D.T. (2008a) First detection of *Nosema ceranae*, a microsporidian parasite of European honey bees (*Apis mellifera*), in Canada and central USA. J Invertebr Pathol **97**(2), 189-192.

Williams G.R., Sampson, M.A., Shutler, D., Rogers, R.E. (2008b) Does fumagillin control the recently detected invasive parasite *Nosema ceranae* in western honey bees (*Apis mellifera*)? J Invertebr Pathol **99**, 342-344.

Williams G.R., Shutler, D., Little, C.M., Burgher-MacLellan, K.L., Rogers, R.E.L. (2011) The microsporidian *Nosema ceranae*, the antibiotic Fumagilin-B ®, and western honey bee (*Apis mellifera*) colony strength. Apidologie **42**, 15-22.

Williams G.R., Shutler, D., Burgher-MacLellan, K.L., Rogers, R.E. (2014) Infra-population and -community dynamics of the parasites *Nosema apis* and *Nosema ceranae*, and consequences for honey bee (*Apis mellifera*) hosts. PLoS ONE **9**, e99465.

Wood P.J., Siddiqui, J.R., Vandermeer, J.W., Gochnauer, T.A. (1970) Carbohydrates in *Nosema apis* spores. Carbohydr Res **15**, 154-158.

Zander E. (1909) Tierische Parasite als Krankenheitserreger bei der Biene. Bienenztg **31**, 196.

Zar, J. H. (2010) Biostatistical analysis, 5th edition, Pearson Education Inc., Upper Saddle River, NJ, pp. 189-244.