The Effects of *Nosema ceranae* on Honey Bee Health

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Acknowledgements

I took an invertebrate zoology course many years ago from Dr. Malcolm Butler at North Dakota State University in which one section of the course was devoted entirely to insects. ZOO 450 showcased the significance of a world whose inhabitants have six legs. From that day forward, I made a promise to myself that I would one day pursue graduate studies in entomology. When I was accepted into the Ph.D. program at the University of Minnesota, all the years I had spent prior to that point working uncomfortable jobs, often involving long hours alone in the cold, rain, and dark for not much pay made completing the degree a personal goal. I thought I could finish my Ph.D. solely on my contributions, but this way of thinking was very wrong.

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Dedication

This dissertation is dedicated to all Goblirschs

And Rupert, Trooper, Jack, and Bitey, who were always “four-feet trotting behind.”

– Rudyard Kipling

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CHAPTER 1 - INTRODUCTION

Nosema Disease of the Honey Bee (*Apis mellifera*)

Michael Goblirsch

ABSTRACT

Honey bees are arguably the world's most iconic pollinator. The presence of honey bees in our landscapes has long invoked images of vitality, diligence, and cooperation. Unfortunately, the current state of honey bee health and productivity paints a rather different picture for this beneficial insect. The survival of honey bees, as well as the livelihoods of those who benefit from their labor (e.g., beekeepers, growers, and ranchers) is under threat from the culmination of a number of detractors to bee health. Exposure to pesticides, poor forage, mite parasites, and pathogens has resulted in heavy annual death of honey bee colonies in the U.S., Europe, and other parts of the world. Among the suspects thought to contribute to bee decline, the fungal pathogen, *Nosema ceranae*, is found at high prevalence in both healthy and declining/dead colonies. Since *N. ceranae* is thought to be a novel pathogen of the European honey bee, *Apis mellifera*, much remains unknown about its pathology at the individual and colony levels, as well as how infection may interact and synergize with other factors thought to be responsible for declining honey bee populations. Here, I review the breadth of research conducted on *Nosema* infection of honey bees in general. I give particular attention to observations on the detection of infection, cytopathology, viability and infectivity of spores, and incidence and geographic distribution. I rely on what is known about a similar and long-recognized pathogen of *A. mellifera*, *N. apis*, for context on the host-parasite relationship

of *Nosema* infection of honey bees. As one part of my dissertation research, I applied background information about the effects of infection on the different castes to show how this may disrupt the social structure of a honey bee colony and contribute to its decline. The level of a honey bee colony or even a whole bee are complicated systems to study intracellular microbes such as *Nosema* that threaten honey bee health and productivity. Continuous cell lines have been lacking to further our understanding of the pathogenicity of *Nosema* infection in a simplified environment removed from the confounding effects of other factors such as poor nutrition or pesticides. For the second part of my dissertation research, I addressed this deficiency in honey bee research by developing an *in vitro* system derived from host cells that could be utilized for observing infection in a simplified environment. Whenever possible, I provide suggestions for future research that could broaden our understanding of this pathogen and ultimately improve honey bee health.

KEYWORDS: colony collapse disorder, fungal pathogen, Microsporidia, social behavior

1.1 Honey Bees: The Hands that Feed, Enlighten, and Amuse Us

1.1.1 Honey Bees Occupy the 'Human Niche'

Honey bees have been part of the human experience for thousands of years. During the course of our history with bees, we have progressed from simply harvesting honey from wild colonies to practicing sophisticated management schemes that involve rearing colonies in artificial nests and transporting them significant distances to pollinate commercial crops. Our curiosity and fascination for honey bees, channeled through science, has transformed them from a stinging mob to a model organism, advancing the knowledge base in diverse areas such as pollination ecology, development and aging, toxicology, genetics, animal behavior, and most notably, the evolution of sociality.

As beekeepers, we have applied the experiences and knowledge garnered from our ongoing relationship with honey bees towards management practices that inch these beneficial insects closer to status as a domesticated livestock, requiring our intervention to sustain its populations. Similar to other livestock, we use selective breeding, supplemental nutrition, and therapeutics to minimize the negative effects of disease stemming from impoverished forage, xenobiotics (e.g., agrochemicals and beekeeper applied miticides), mite parasites, and pathogens. Unfortunately, annual levels of colony mortality in the U.S., parts of Europe, and several other countries run well-above normal attrition (Stokstad, 2007; van Engelsdorp et al., 2009; van der Zee et al., 2012; OPERA, 2013; Spleen et al., 2013), forcing many to question whether the inputs of time and money into the management of this "livestock" are in vain.

I began research for my doctoral dissertation at approximately the same time when beekeepers started experiencing a new wave of large scale colony death in the U.S.

(2006-2007). Also at this time, I began keeping bees as a hobby and soon found myself in a similar predicament as many other beekeepers – frustrated by the tribulations of trying to maintain healthy colonies. Although for me beekeeping is a hobby, for others it can be a source of income and a way of life that continues to be tested. After all, it has been only two decades since beekeepers in the U.S. were confronted with the devastating impact from the introduction and rapid dispersal of the ectoparasite, *Varroa destructor* (Wenner and Bushing, 1996). It did not take long after the introduction of the *Varroa* mite, that the beekeeping industry recognized the correlation between mite levels and colony decline and death. However, the same does not hold true for our current bee health crisis, known as Colony Collapse Disorder (CCD).

1.1.2 Colony Collapse Disorder

Colony Collapse Disorder has become part of the lexicon of the beekeeping community, and is defined as a rapid loss of adult bees from the hive, leaving behind the queen, a small population of young workers, and ample amounts of sealed brood and food stores (i.e., pollen and honey). Colony Collapse Disorder was put forth as one explanation for the mysterious manner in which colonies, that looked healthy based on prior inspections, seemed to "disappear" suddenly for unknown reasons. To date, no cause has been identified that can sufficiently account for the magnitude of colony death experienced annually in the U.S. and other parts of the world. However, there are a number of identifiable factors, including infestation by *Varroa* mites, exposure to pesticides, limited access to diverse and abundant forage, and numerous pathogens that

are prevalent at any given time in most honey bee colonies that may interact and contribute to decline and death.

One pattern that is evident from reports from beekeepers and researchers is the high prevalence and burden of pathogens such as viruses and Microsporidia within dead and dying colonies (Cox-Foster et al., 2007; Cornman et al., 2012). Prior to the occurrence of CCD, *Nosema apis* was considered to be the only species of Microsporidia known to infect the European honey bee, *Apis mellifera*. Symptoms of colonies infected with *N. apis* include increased frequency of queen supersedure (Farrar, 1947; Furgala, 1962; Jay, 1967), and reduced honey yield (Farrar, 1947; Fries et al., 1984), pollen collection (Anderson and Giacon, 1992), and overwintering survival (Farrar, 1942; Farrar, 1947). Infections with *N. apis* used to be a common occurrence for beekeepers in the U.S., and for the most part, only caused significant decline of the colony when the rate of worker death from infection surpassed the egg-laying rate of the queen plus the task performance of the remaining workers to satisfy the demands of the colony.

1.1.3 Impetus for Dissertation Research

In the aftermath of CCD, intense surveillance efforts routinely report the presence of a "new *Nosema*" traditionally associated with the Asian honey bee, *A. cerana*. The widespread presence of *N. ceranae* in beekeeping operations has caused reason for concern since *A. mellifera* is thought to be a novel host and much is unknown about how infection with this new *Nosema* plays out on the physiology, behavior, and longevity of honey bees. There is a need for greater understanding of how these obligate intracellular pathogens enter, reproduce in, and disperse from host cells. Although research methods

are available to study the host response of honey bees to *Nosema* infection at the colony, individual, and molecular levels, one level is overtly lacking – the level of the cell. It is the cell of the honey bee where *Nosema* gains access to and initiates the exploitation of host resources for the benefit of their own propagation. *In vitro* systems derived from host cells to study *Nosema* and other microbes such as viruses that have a negative impact on bee health have been a longtime coming. Therefore, I set out to achieve two goals for my dissertation. First, I wanted to examine the effects of *N. ceranae* infection on the physiology, behavior, and longevity of worker honey bees under laboratory and field relevant conditions and determine if there are parallels or contradictions with what has been observed with *N. apis*. Second, I wanted to understand the cytopathology of *Nosema* infection in a simplified, controlled host environment composed of honey bee cells. Since no continuous honey bee cell lines were available to observe the effects of infection at the intracellular level at the time I started my dissertation research, I set out to develop an *in vitro* culture method that could be applied towards establishing young and continuous honey bee cell lines isolated from embryonic tissues.

1.2 Characterization of *Nosema*

1.2.1 Significance of Microsporidia to Human and Animal Welfare

Nosema belongs to the phylum Microsporidia, a group of highly evolved, single-celled fungi (Weiss et al., 1999; Thomarat et al., 2004) that infect and cause disease in a wide array of animal hosts. Many Microsporidia have implications for human health (e.g., *Encephalitozoon* and *Enterocytozoon* in immunocompromised patients) (Didier and Weiss, 2011) or cause economic injury to non-insect hosts (e.g., gill disease caused by

Loma salmonae in salmonids) (Constantine, 1999), as well as in insects of economic significance other than honey bees (e.g., Pébrine disease caused mainly by *N. bombyc*is in the silkworm moth) (Pasteur, 1870; Ma et al., 2013). The derived condition of Microsporidia has led to unique adaptations in genomic organization, ultrastructure, and infection an apparatus conducive for life within a host cell. For example, Microsporidia have some of the smallest genomes for eukaryotes making them amenable to high-throughput genomic sequencing. Genomic sequencing of Microsporidia is an intense area of research and will facilitate the identification and functional analysis of proteins and other factors involved with such poorly understood processes as spore germination. The genomes of both *N. apis* and *N. ceranae* have been sequenced (Cornman et al., 2009; Chen et al., 2013). Coupled with *in vitro* systems, knowledge of the transcriptome or proteome could be applied towards the development of cell-based therapies that specifically target *Nosema* while posing minimal harm to the host or other non-target organisms. For example, the genome-sequencing effort for *N. ceranae* revealed the presence of parasite-mediated RNA interference (RNAi) capability. The subsequent application of double-stranded RNA homologous to ADP/ATP transporter genes specific to *N. ceranae* resulted in the silencing of transcripts encoding these proteins (Paldi et al., 2010).

1.2.2 Detection, Identification, and Quantification of *Nosema* in Honey Bees

Nosema disease is not a novel phenomenon to honey bees, as Zander discovered (1909) and was the first to describe the morphology and life cycle of *N. apis* (1911). For nearly 100 years, *N. apis* was the only microsporidian thought to infect and cause disease

in *A. mellifera*; therefore, it has provided a basis for current studies with *N. ceranae* to compare the histopathology in host tissues affected directly or indirectly by infection. Diagnosis of infection can be performed using traditional techniques such as viewing and counting spores with a haemocytometer under magnification (≥ 200X) with bright-field or phase contrast microscopy (Cantwell, 1970). Since the identification of *Nosema* spores is relatively straightforward, this method allows for an accurate assessment of the presence/absence of infection. Information on the presence/absence of spores is useful for quantifying the overall proportion of infected individuals in a colony, as well as the proportion of infected bees based on a specific parameter (e.g., proportion of infected foragers). However, because *N. apis* and *N. ceranae* are only discernible definitively by their ultrastructural features, the specificity of Cantwell's (1970) method is confined to the resolving power of the objective lens used.

Alternatively, PCR amplification of conserved genes such as the small subunit ribosomal RNA gene from DNA extracted from spores provides both a highly specific and sensitive method for detection and quantification (Chen et al., 2009; Carletto et al., 2013; although see: Ptaszyńska et al. 2014, on loop-mediated isothermal amplification, which enables a 10^3-fold increase in sensitivity compared to traditional PCR). One study compared the specificity and sensitivity of microscopy to real-time PCR for the detection of *Nosema* infection and showed that the former failed to identify the presence of *N. ceranae* spores in 50% of the samples that tested positive by PCR (Traver and Fell, 2011a). This finding may demonstrate a disparity between species in the production of mature spores, where *N. ceranae* may not be as prolific as *N. apis* (Martín-Hernández et al., 2009; Mulholland et al., 2012). Furthermore, ascribing the degree of illness based on

the number of spores in a sample may be misleading as apparently healthy or asymptomatic bees can have high spore loads and vice versa (Meana et al., 2009).

Vegetative forms and immature spores of *N. apis* and *N. ceranae* have only been observed in the epithelial cells that line the midgut of the adult honey bee. However, PCR signals specific for DNA isolated from *N. apis* and *N. ceranae* have been detected in the hypopharyngeal glands, salivary glands, fat body, and Malpighian tubules, but not brain or muscle (Chen et al., 2009; Gisder et al., 2010; Copley and Jabaji, 2012; Huang and Solter, 2013). Outside of the alimentary tract, environmental spores of *N. apis* and *N. ceranae* have been observed on the mouthparts of workers, suggesting a possible mechanism of horizontal transmission via trophallaxis (Smith, 2012; Huang and Solter, 2013). Late larval and pupal mortality purportedly due to infection by *N. apis* was observed in the Cape honey bee, *A. m. capensis* (Buys, 1972). Additional evidence is scant as to whether the egg, larval, or pupal stages are susceptible to infection. Contrary to some other species of *Nosema* (Raina et al., 1995; Terry et al., 1997), there is no evidence of transovarial transmission from an infected queen to her eggs. The lack of vertical transmission is likely a result of infection being restricted to the midgut epithelial cells.

Spores of *N. apis* have been observed in the hemolymph of honey bee larvae (Gilliam and Shimanuki, 1967), and drone pupae have tested positive by PCR for *N. ceranae* (Traver and Fell, 2011b). *Nosema ceranae* has also been detected by PCR in royal jelly (Cox-Foster et al., 2007). Positive detection of either *Nosema* spores or DNA in royal jelly supports the likelihood that the immature stages are exposed through the food supplied to them by nurse bees, but demonstration of infection in these stages has

not been forthcoming. One study was unsuccessful at germinating spores and infecting the larva stage with *N. apis* (Hassanein, 1953). Spores require specific stimuli that they encounter in the chemical milieu of the digestive tract to initiate germination. It is possible that these conditions exist only in the adult.

1.2.3 Life Cycle of *Nosema* in Honey Bees

The midgut of the honey bee provides an interface between the external environment and the internal milieu of tissues and hemolymph. It is the site where nutrients are digested and assimilated by the body and is one port of entry for pathogens to gain access to the host. Unlike the foregut and hindgut, the midgut is not lined with cuticle but is comprised of a single layer of epithelium anchored to the basement membrane that supports neuronal and muscle attachment. The midgut epithelium is heterotypic for cell type, composed mainly of columnar cells that secrete digestive enzymes and the peritrophic matrix. The peritrophic matrix can be thought of as an acellular "sleeve" that protects the epithelium from mechanical and chemical damage and forms a barrier to invasion by pathogens (Lehane, 1997). Additionally, goblet, exocrine, and regenerative cells are interspersed among the columnar cells and have specific functions such as regulating the pH of the gut lumen, producing and secreting protein precursors, and replacing damaged and dying cells.

Unlike *N. apis* and *N. ceranae*, most other species of *Nosema* are systemic pathogens that are not constrained to a particular tissue that they can infect and utilize for reproduction (Huang et al., 2013). Even the closely related bumblebee pathogen, *N. bombi*, has been shown to infect multiple tissues (Fries et al., 2001). Although there may

be differences in the timing of specific infection events, the fine morphology and trajectory of development has been described in detail and is similar between *N. apis* and *N. ceranae* (Figure 1.1) (Gray et al., 1969; Youssef and Hammond, 1971; Liu, 1984; Fries, 1988; Fries, 1989a; Fries, 1989b; Liu, 1989; de Graaf et al., 1994; Fries et al., 1996; Higes et al., 2007; Chen et al., 2009). True to their genus, *N. apis* and *N. ceranae* are diplokaryotic throughout their life cycle. Spores gain access to the host cell and initiate proliferation by rapidly extruding a long tube that is attached anteriorly to the spore by an anchoring disc (Xu and Weiss, 2005). The extruded polar filament of a germinated spore penetrates a host cell, providing a conduit for the transfer of the binucleate sporoplasm. Presporal development, or merogony, begins within hours after inoculation with the sporoplasm. Merogony proceeds through a variable number of nuclear and/or cytoplasmic divisions. Binary fission leads to the production of clusters or chains of meronts, which are often quadrinucleate (Gray et al., 1969; Fries et al., 1996; Higes et al., 2007). Meronts of *N. apis* and *N. ceranae* have a single, thin plasma membrane that remains in direct contact with the host cytoplasm and is similar to the early developmental stages of a related genus, *Vairimorpha* (Cali et al., 2011). There is no evidence for the formation of a parasitophorous vacuole as displayed by members of the genus *Encephalitozoon* (Youssef and Hammond, 1971; Scanlon et al., 2004). Not all Microsporidia utilize a parasitophorous vacuole as part of their development. The function of this organelle is purported to be an exploitation strategy of the pathogen in that the meront repositions host mitochondria along the interface of the vacuole to increase the surface area for the import of host ATP (Hacker et al., 2014). The absence of the parasitophorous vacuole in *Nosema* leads to the possibility that these species use

other means of obtaining a continuous supply of ATP from the host, such as nucleotide transporters embedded in the plasma membrane of the parasite (Tsaousis et al., 2008; Paldi et al., 2010).

Meronts of *N. apis* and *N. ceranae* transform into either the primary spore or mature, environmental spore between 48 and 96 hours after infection (Fries, 1988; de Graaf et al., 1994; Higes et al., 2007). The primary spore is produced through binary fission of the sporont to form two sporoblasts, each having a thin spore wall and short polar filament. The primary spore is capable of transmitting infection to adjacent cells through autoinfection (i.e., spontaneous germination) (Becnel and Andreadis, 1999). The major differences in development and morphology between the primary spore and the mature spore are the thick spore wall and longer polar filament of the later. Ultimately, an infected cell becomes tightly packed with parasites (Figure 1.2), leading to the rupture and release of spores into the gut lumen. Mature spores can pass through the rectum in the feces, serving as an inoculum for other bees, or they can remain in the midgut to infect other cells.

It is important to note that most observations on the life cycle of *Nosema* in honey bees were conducted under conditions where bees were placed in cages held at a constant temperature, given *ad libitum* access to sucrose solution, and restricted from alleviating mature spores from the alimentary tract through cleansing flights. In the natural hive environment, temperatures fluctuate, the diet of the bee is not restricted to carbohydrates, and bees are able to leave the hive freely for defecation, which could lead to deviations in the progression of the development of the parasite from that observed under controlled conditions in the laboratory (Fries, 1993; Higes et al., 2010).

1.2.4 Cytopathic Effects of *Nosema* in Honey Bees

Compared to uninfected cells, cells infected with *Nosema* show extensive lysis or degeneration (Liu, 1984; Higes et al., 2007; Dussaubat et al., 2012). Other indicators of cell injury and/or death have been observed by microscopy and include a loss of glycogen particles (i.e., carbohydrate-based energy reserves) and detachment of ribosomes from the rough endoplasmic reticulum (RER) (i.e., protein synthesis apparatus) (Liu, 1984). Loss of glycogen and loose aggregation of ribosomes may be a consequence of damage to the mitochondria, the site of ATP-producing oxidative phosphorylation in the host cell. Utilization of glycogen reserves indicates that an infected cell has switched from a more efficient means of energy production to the less efficient anaerobic glycolysis, possibly to compensate for competition and/or depletion of ATP by the amitochondriate *Nosema*. As ATP is depleted, it is unavailable to drive the efflux/influx of sodium and potassium across the plasma membrane via sodium pumps. Pump failure retains sodium within the cell and causes swelling of the RER and detachment of ribosomes, resulting in necrotic cell death (Kumar et al., 2012). Cells likewise can proceed through programmed cell death, or apoptosis. The ultimate outcome for a cell infected with *Nosema* is likely death, but understanding the mechanism of death is important for determining the degree to which it is mediated by the host to limit the spread of infection to other cells or whether the pathogen has some control over host cell metabolic processes so it can persist and exploit resources for the production of progeny.

Immunohistochemistry techniques and assays that measure the activity of specific enzymes or levels of substrates involved with the cascades of cell death have been used to explore whether cells infected with *Nosema* proceed through apoptosis or necrosis.

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and immunohistochemistry for caspase-3 have been used to examine apoptosis in midgut epithelial cells infected with *N. ceranae*. In a healthy cell, caspase-3 exists as an inactive proenzyme; however, early in the process of apoptosis it is cleaved, and the residues dimerize to form an active enzyme. Fragmented or damaged DNA late in the process of apoptosis provides the substrate for terminal deoxynucleotidyl transferase to catalyze the addition of dUTP, which is coupled to a detection molecule. Using both of these methods, Higes et al. (2013) concluded that *N. ceranae* inhibits apoptosis as caspase-3 and dUTP-labeled molecules were widely distributed throughout uninfected tissues, but had negligible presence in infected tissues. This finding confirms what may be the norm for Microsporidia (Scanlon et al., 1999) and alludes to a conserved strategy utilized by intracellular microorganisms for development (Nash et al., 1998).

Cells that are necrotic release lactate dehydrogenase (LDH) as their plasma membranes become permeable. Lactate dehydrogenase is a necrosis-specific marker that can be detected by colorimetric absorbance with a microplate reader (Chan et al., 2013). It would interesting to see if the infected tissues from Higes et al. (2013) had elevated levels of LDH, as this would corroborate changes seen in the ultrastructure of organelles of cells infected with *N. apis* (Liu, 1984).

1.2.5 Viability and Infectivity of *Nosema* in Bees Under Natural Conditions

The environmental spore is the vehicle of infection for all Microsporidia; it is resistant to desiccation, abrasion, and temperature extremes because of the mechanical protection offered by the chitinous spore wall. Honey bees become infected with *Nosema*

by ingesting spores excreted in the feces of an infected bee. Sources of spores in the environment include comb and water that has been contaminated with feces (L'Arrivee, 1965; Bailey 1981). In addition to dispersal through defecation by an infected bee, Valera et al. (2010) found that the feces of the European bee-eater, *Merops apiaster*, an insectivorous bird that preys primarily on bees, wasps, and hornets, contained viable spores of *N. ceranae*. These authors' pose that bee-eaters that have consumed infected bees may contribute to the dissemination of *Nosema* over a broad geographic range as the they migrate from wintering sites in tropical Africa and India to breeding grounds in northern Africa and southern Europe (Valera et al., 2010). A similar mechanism of dissemination has not been identified in the Americas where *N. ceranae* is widely distributed. However, the transportation of colonies long distances for the pollination of commercial crops such as almonds in California or placement in large apiaries may serve as mechanisms that promote the dispersal of *Nosema* and other pathogens.

Spores of *N. ceranae* have been found in the corbicular pollen of returning foragers, and these spores were capable of causing infection when fed to *Nosema*-free bees (Higes et al., 2008). This finding suggests that floral resources can become contaminated with the feces of an infected bee or that spores adherent to the body of a worker become mixed into the pollen during grooming behavior. Workers performing comb cleaning are more likely to become infected than queens and drones that do not perform these behaviors (Fries, 1993). As bees emerge from pupation, they may become infected with spores as they chew their way through the wax covering of the cell (Bailey, 1981; Malone and Gatehouse, 1998). The potential for exposure of uninfected bees to spores increases when conditions are not conducive for cleansing flights (e.g., long

periods of rain or temperatures below 40°C), which allow infected bees to eliminate spores outside of the hive environment in the bolus of the feces (Moeller, 1972).

1.2.6 Viability and Infectivity of *Nosema* in Bees Under Experimental Conditions

Exposure requires that a sufficient number of spores be ingested to cause infection. Based on studies of infected bees in cages, the number of spores needed to cause infection in 50 and 100% of adult workers (i.e., ID_{50} and ID_{100}) has been found to be comparable for both *N. apis* and *N. ceranae* and are in the range of 10^2 and 10^4 spores, respectively (Forsgren and Fries, 2010). To obtain large numbers of spores that can be subsequently used as inocula to study pathological effects on uninfected bees, infectious material must be isolated and purified from the alimentary tracts of infected bees. Purification is frequently done by segregating spores from other microbes and debris by passing homogenized tissues through a Percoll gradient with high-speed centrifugation.

Reliable methods for determining the viability of purified spores typically employ differential stains followed by observation using phase contrast and fluorescence microscopy or other technologies such as flow cytometry. The conventional approach for assessing spore viability is by a dye exclusion technique where "live" spores with intact membranes exclude a dye such as trypan blue or similar differential stain and nonviable spores with compromised membranes take up the dye (Stoddart, 2011). This approach has been applied to identify factors that minimize the effects of storage conditions such as temperature, time, and storage buffer as an attempt to preserve the viability of purified spores and would eliminate the time and materials needed for additional purifications.

The viability of *N. ceranae* spores treated with a brief exposure (1, 2, 4, or 6 hours) to temperatures of 35°C or 60°C were assessed using the permeable stain, SYTOX Green (Fenoy et al., 2009). Spores inspected immediately or 1 month after temperature treatment showed comparable levels of staining to control spores held at 4°C for 1 month in 1X PBS (≥ 92.0%). However, maintaining *N. ceranae* and *N. apis* spores at 4°C in PBS for an extended period was not sufficient to sustain viability as levels declined (i.e., increase in SYTOX Green) by approximately one third and three quarters the viability of freshly isolated spores after 6 and 12 months, respectively (Fenoy et al., 2009).

The use of flow cytometry provides an efficient and reproducible method for accurately assessing the viability of *Nosema* spores compared to fluorescence microscopy (Peng et al., 2014). Recently, flow cytometry was used to assess the viability of *Nosema* spores stained with propidium iodide (PI). Spores that were freshly isolated, stored for 20 days at room temperature, or autoclaved were then segregated by light scattering filters to determine spore size and complexity. From these populations the intensity of PI fluorescence was measured to differentiate the number of viable spores from the number of PI-positive (i.e., dead) spores (Sánchez Collado et al., 2014). Using this method, the percentage of dead spores was found to be less than 15% for both *N. apis* and *N. ceranae* after 10 months at 4°C or room temperature (~25°C). Storage for 10 months at 33°C for both species and -20°C for *N. ceranae* had a significant negative effect, decreasing viability by at least 50% in all cases. Decreased viability under these conditions was correlated to decreased infectivity when the treated spores were fed to uninfected bees (Sánchez Collado et al., 2014).

Even though a spore may appear viable (i.e., excludes dye), it may not be infectious. In other words, it may not be competent to respond to stimuli of the alimentary tract and complete the germination process. One method to ascertain whether seemingly viable spores exposed to different conditions are infectious is to administer them to uninfected bees and measure outcomes such as the proportion infected and/or spore production. Malone et al. (2001) dried spores of *N. apis* on glass slides and kept them at 33, 40, 45, and 49°C for 3 or 5 days. Spores were then suspended in sucrose solution and fed to uninfected bees. Approximately half as many bees became infected when fed spores stored at 40, 45, and 49°C compared to bees given spores stored at 33°C or control spores stored at room temperature for 24 hours, suggesting that temperature is one factor that influences the ability of the spore to germinate (Malone et al., 2001).

Activation and completion of germination remains a poorly understood process. The large diversity of hosts and external environments where Microsporidia are found indicates that this process is adaptable by species. Interestingly, differences in the activation of germination have been demonstrated between *N. apis* and *N. ceranae* spores stored at low temperatures (4°C). Freshly isolated spores air dried on glass slides and kept at 4°C for 4 days showed that 80% of *N. apis* compared to 10% of *N. ceranae* spores germinated in the presence of 0.1 M sucrose in PBS (Gisder et al., 2010). Interestingly, these authors noticed that the extruded polar filament of many of the *N. ceranae* spores were relatively short, suggesting that they may be the primary life stage and not the mature environmental form. It is possible that the primary spore requires different conditions to activate and complete germination compared to the environmental spore.

1.2.7 Comparative Virulence of *Nosema apis* and *Nosema ceranae*

In vivo studies, involving the use of cages to isolate bees experimentally infected with *N. apis*, *N. ceranae*, or both species, provide insight into comparing differences in pathogen growth rate, infectivity, and transmission, as well as for comparing consequences to host physiology such as changes in the immune response or host survivorship. One study showed that experimental infection of adult bees with 10^5 spores of *N. ceranae* led to 100% mortality 8 days after infection (Higes et al., 2007). This finding prompted concern that this "new" *Nosema* may be more virulent than the "traditional" *N. apis*. Subsequently, Forsgren and Fries (2010) infected newly emerged bees with 10, 10^2, 10^3, and 10^4 spores of *N. apis* or *N. ceranae*, which were doses at least 10-fold lower than Higes et al. (2007), and noticed that *N. ceranae* was slower to increase in spore number compared to *N. apis* up to 12 days after infection. However, this difference was temporary as the total spore load and proportion of infected bees were equivalent between the two species 14 days after infection (Forsgren and Fries, 2010). In addition, these authors examined within host competition from mixed infections using inocula of 1:9, 9:1, or equal ratios of both species. Using DNA yield as a proxy for spore number, it was shown that the species that originated as the lower inoculum produced the greater number of spores 14 days after infection (Forsgren and Fries, 2010). The authors hypothesize that this effect may be due to negative-frequency dependent selection. Negative-frequency dependent selection is a mechanism to explain how asexually reproducing organisms like *Nosema* maintain genetic diversity within an essentially clonal population and ultimately lead to the development of unique haplotypes (Weeks and Hoffmann, 2008). Multiple haplotypes have been detected in isolates of *N. ceranae*

spores from individual bees and different geographic regions (Williams et al., 2008; Gómez-Moracho et al., 2014). Variation within the species may explain why *N. ceranae* isolates from Spain are purported to be more virulent than isolates from other regions of the world (Paxton et al., 2007). An alternative explanation for why *N. ceranae* had such a pronounced negative effect on the survival of honey bees from Spain, but not honey bees from Northern Europe, may pertain to the degree of host tolerance to infection of the later. For example, honey bees in the region (i.e., Denmark) have been under selection for tolerance to *Nosema* for decades (Huang et al., 2012; Huang et al., 2014).

1.2.8 Global Distribution and High Prevalence of *Nosema ceranae*

Fries et al. (1996) were the first to describe an infection by *N. ceranae* in the Asian honey bee. Almost a decade later, *N. ceranae* was first reported in a putatively new host, the European honey bee (Fries et al., 2006; Higes et al., 2006). Discovery of *N. ceranae* as a novel pathogen infecting the European honey bee corresponded with the emergence of CCD. Cox-Foster et al. (2007) applied a metagenomic approach to identify potential microbial markers of CCD. Findings from this study showed that *N. ceranae* had a high prevalence in both collapsed (i.e., dead) (100%) and normal colonies (80.9%). Levels of *N. apis* were also high in collapsed colonies (90%) but moderate in healthy colonies (47.6%) (Cox-Foster et al., 2007). High levels of *N. ceranae* in dead and dying colonies prompted the near simultaneous reporting of detection in different regions of the world (Table 1.1). *Nosema ceranae* is now considered to be widespread (Klee et al., 2007), and has been found in every continent where honey bees are present. The first report of incidence by continent includes: Asia – Fries et al., 1996; Europe – Higes et al.,

2006; North America – Cox-Foster et al., 2007; Australia – Giersch, 2009; South America – Invernizzi et al., 2009; and Africa – Higes et al., 2009a.

Nosema ceranae is hypothesized to have originated in Asia with *A. cerana* as its primary host (Fries et al., 1996; Botias et al., 2012). Evidence from archived samples suggests that *N. ceranae* had an expanded geographical range that existed several years, if not decades, before it was first detected as a natural infection in colonies of the European honey bee in Spain. Samples of honey bees dating back to 1995 collected from 12 U.S. states revealed the presence of *N. ceranae*, but not *N. apis*, according to amplification of ribosomal RNA genes (Chen et al., 2008). Archived material collected in Finland between 1986 and 2006 showed that some samples from as early as 1998 were positive for *N. ceranae* based on restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene (Paxton et al., 2007). Likewise, archived material from Italy collected before 2010 showed that some samples from as early as 1993 were positive for *N. ceranae* (Ferroglio et al., 2013).

In the Western Hemisphere, 17 of 19 samples of Africanized honey bees (*A. m. scutellata*) collected in Mexico in 2004 were found to be positive for *N. ceranae* (Guzman-Novoa et al., 2011). Moreover, Africanized drones collected in Brazil in 1979 showed the presence of spores by microscopy and were positive for both *N. ceranae* and *N. apis* by duplex PCR (Teixeira et al., 2013). Interestingly, Clark (1980) described a “second” microsporidian in infected honey bees from the Southeastern U.S.; however, it is unlikely that the spores observed were *N. ceranae* due to the presence of a single nucleus instead of a diplonucleus characteristic for this species.

1.3 Effects of *Nosema* on Colony Demography and the Division of Labor

1.3.1 Caste-specific Pathology from *Nosema* Infection

The reproductive division of labor exemplified by honey bee queens and workers and the haplodiploid condition that exists between fertilized females and unfertilized males (i.e., drones) establishes a unique set of conditions to examine differences in susceptibility to pathogens such as *Nosema* and determine the potential impact on the colony based on the sex, role, and proportion of infected individuals. The ovaries of a honey bee queen represent one-half of the reproductive organs of a colony, the other half coming from the sperm contributed by drones during mating. In temperate climates during the summer months, a fertilized queen can lay several thousand eggs a day. She secretes pheromones, notably queen mandibular pheromone (QMP), which have regulatory effects on worker physiology and behavior, such as stimulating the assembly of the retinue, inhibiting the development of worker ovaries (i.e., as means to control worker reproduction), and modulating worker behavioral maturation (Slessor et al., 2005). The amount of QMP declines as a queen ages or starts to fail in her ability to lay eggs, prompting the workers to rear a new queen (Winston, 1987). Because a queen is long-lived and her fecundity correlates positively with colony growth and metrics of productivity such as honey yield (Nelson and Gary, 1983; Rangel et al., 2013b), it is no surprise that "poor queens" are one factor perceived to be responsible for failing colony health (van Engelsdorp et al., 2013).

1.3.2 Effects of *Nosema* on Queen Health

The association between *Nosema* infection and queen health was originally recognized with *N. apis* in the middle of the twentieth century. Several studies noted that queens infected with *N. apis* were more likely to be replaced through supersedure compared to uninfected queens (Farrar, 1947; Furgala, 1962; Jay, 1967). Alaux et al. (2011) looked at the profile of QMP in queens as a proximate mechanism to explain above normal frequencies of supersedure from *Nosema* infection. Findings from this research showed that levels of the QMP compounds, 9-oxodec-2-enoic acid (9-ODA) and 9-hydroxy-2-enoic acid (9-HDA) were significantly greater, while methyl p-hydroxybenzoate (HOB) was significantly lower, in the heads of queens infected with *N. ceranae* compared to controls (Alaux et al., 2011). Declining levels of HOB may provide a signal to workers about queen failure and prompt them to respond by initiating queen rearing. Further research is needed to test whether the concentrations of HOB produced from queens infected with *N. ceranae* under laboratory conditions are capable of altering worker behavior (e.g., disrupting retinue behavior) when administered in the natural hive setting. Interestingly, HOB (i.e., methylparaben) is an antifungal agent commonly added to artificial diets used to rear insects (Cohen, 2003). Although speculative, suppression of HOB production in queens infected by *N. ceranae*, albeit a highly evolved fungus, may facilitate growth and transmission of the pathogen.

Nosema infection induces changes to other factors of reproductive health that may compromise the egg-laying ability of the queen. Terminal oöcytes of queens infected with *N. apis* show evidence of autolysis, and mitochondria, endoplasmic reticulum, ribosomes, and yolk granules appear unevenly dispersed or in a state of deterioration

(Liu, 1992). While yolk material undergoes degeneration in the ovaries, levels of the yolk precursor protein, vitellogenin (Vg), increase in the hemolymph of infected queens. Eight days after inoculation, queens infected with *N. ceranae* were found to have nearly twice the amount of Vg in hemolymph compared to controls (Alaux et al., 2011). The reason levels of Vg are elevated in the hemolymph of infected queens is unclear. In addition to uptake by developing oöcytes for use in egg production (Engels, 1974), Vg is a potent antioxidant (Seehuus et al., 2006). The upregulation and secretion of Vg into the hemolymph may not be sufficient to prevent an early death for an infected queen but it may attenuate harmful reactive oxygen species produced from elevated respiration rates in infected midgut tissues (Higes et al., 2009b). On the other hand, Vg is continuously synthesized in the fat body and secreted into the hemolymph of mature queens (Fluri et al., 1981), where it is routed to the ovaries for egg maturation (Hagedorn and Kunkel, 1979). Elevated Vg levels in the hemolymph of infected queens may be a temporary condition due to the deterioration of the ovaries and specific receptors that permit binding and uptake.

1.3.3 Effects of *Nosema* on Drone Health

The contribution that drones make in shaping the health of a honey bee colony may be underappreciated and often overlooked when studying the impact that factors such as infectious diseases have on colony productivity and survival. However, the degree of genetic variation among workers from different patrilines, produced from a queen that has mated with up to two-dozen drones, allows variability in the repertoire of responses for a colony confronted with changing environmental conditions or stressors

such as pathogens. Drone pupae and adults are susceptible to natural infection with *N. ceranae* (Traver and Fell, 2011b). While spore loads may not reach as high a levels as in workers, evidence suggests that infection with *N. ceranae* has a greater negative effect on drone body mass and survival (Retschnig, et al., 2014a). The biological significance of this finding is unclear but raises some important questions about drone health. Do infected adult drones weigh less because workers recognize that they are infected and stop feeding them to avoid transmission? Is there an effect to the quality (e.g., viability) or quantity of sperm from infected drones? Infections can decrease male fertility in humans and other animals (Bachir and Jarvi, 2014). Although *N. ceranae* is restricted to tissues outside of the genital tract, infection may contribute to a change in somatic processes that ultimately reduce the fertilization potential and reproductive success of infected drones.

1.3.4 Effects of *Nosema* on Workers and Disruption of the Division of Labor

The social organization of a honey bee colony is maintained by an age-based division of labor. After emergence to the adult stage, young workers tend to remain in the brood nest for the first 2 weeks of life performing nursing duties such as feeding and tending to the larvae and queen. Workers transition to a different set of tasks that include guarding or foraging for nectar and pollen outside of the nest at about 3 weeks of age, and continue to perform these behaviors for the remainder of their lives, which is an additional 3 weeks if they emerged during the summer months (Winston, 1987). The chronological sequence of behaviors that workers display is not necessarily fixed, but can show plasticity if circumstances imposed at the colony level cause a task-niche to be

underserved. For example, nurse bees can transition prematurely to foraging behavior if they perceive a shortage of food stores or a loss of foragers due to predation (and vice versa), or they can maintain nursing duties beyond the normal age for this behavior if older foragers are confined to the nest during inclement weather (Robinson et al., 1992; Huang and Robinson, 1996; Schulz et al., 1998; Amdam and Omholt, 2003). The perception of colony needs at the level of an individual worker operates through response thresholds to environmental and internal stimuli as well as through communication among nestmates to alter physiological processes of the worker that results in the execution of an appropriate task.

Physiological processes provide a framework that regulates the transition from nursing duties exhibited by young workers to foraging behavior exhibited by older workers (Robinson, 1998). Adipose-like tissue called fat body lines a worker's abdomen and is the site of Vg synthesis. Vitellogenin is seemingly a "social protein" that has multiple roles in honey bee health; it is utilized by the queen during egg development, it provides protection against reactive oxygen species, and is a reserve for proteins and lipids (Münch et al., 2008; Havukainen et al., 2013). Nurse bees secrete large amounts of Vg into the hemolymph (Fluri et al., 1982; Pinto et al., 2000). Since workers normally do not produce eggs, their utilization of Vg has been repurposed to fulfill a role that is different from the ancestral maternal condition displayed by the queen – a source of food for the brood. The heads of workers contain glands where Vg becomes localized, suggesting that these glands have receptors that facilitate the transport of Vg from fat body tissues (Seehuus et al., 2007). The hypopharyngeal glands are enlarged in nurse

bees and secrete copious amounts of a protein-rich mixture that gets deposited as food in comb cells containing larvae.

As workers age, the size and activity of the hypopharyngeal glands begin to decrease as another structure in the head, the corpora allata, begins to increase. The corpora allata is part of the brain that synthesizes juvenile hormone (JH), which is then released into the hemolymph to act on responsive tissues. Juvenile hormone is thought to act on regulatory elements in the fat body and inhibit the transcription of Vg (Pinto et al., 2000; Amdam and Omholt, 2003). The increase in JH titer in the hemolymph marks the transition from the nurse phase to the forager phase of a worker's life (Rutz et al., 1976; Fluri et al., 1982; Robinson, 1987; Huang et al.1991; Huang et al., 1994; Jassim et al., 2000). As JH titer increases, there is a corresponding decrease in the synthesis of Vg and regression of the hypopharyngeal glands, making these bees no longer efficient at performing nursing behavior (Robinson, 1992).

The interaction of Vg and JH are thought to repress the expression of each other by positive feedback loops acting through the insulin-IGF-1 nutrient signaling pathway (Amdam and Omholt, 2003; Corona et al., 2007; Ament et al., 2008). When workers are young they are maternal-like and have high levels of Vg, which in turn suppresses the synthesis of JH and makes them competent to perform nursing behaviors. Since nurse bees stay in the brood nest, it ensures that the pleiotropic effects of Vg are kept within the colony. However, as workers age, JH titer is high, which suppresses the synthesis of Vg, making these bees competent to perform behaviors of high risk such as guarding the nest entrance or foraging without the threat of losing Vg to the external environment (Amdam and Omholt, 2003).

The regulatory interaction between Vg and JH provides one explanation for how honey bees express the sequence of complex behaviors homologous to the reproductive care exhibited by their solitary ancestors (Amdam et al., 2006). Importantly, experiments using RNAi or topical application of JH or its analog (e.g., methoprene) demonstrate how factors can interfere with the normal progression of worker ontogeny. For example, when Vg expression is suppressed via RNAi, JH titer spikes in the hemolymph of nurse-aged bees to trigger premature foraging and a shortened lifespan (Guidugli et al., 2005; Amdam et al., 2007; Nelson et al., 2007; Marco Antonio et al., 2008). The application of JH or its analog, methoprene, produced similar effects when applied topically to nurse-aged bees (Robinson 1985; Robinson 1987; Robinson et al., 1989; Sasagawa et al., 1989).

Physiological processes regulating host development are sensitive to the effects of parasites and pathogens (Hurd et al., 2001). It is a reasonable argument that the infection of midgut tissues with *Nosema* can have downstream effects on the expression of Vg, JH, or both as a parasite-mediated mechanism that permits exploitation of host resources. Alteration of Vg and JH levels due to infection would likely manifest as expression of age inappropriate behaviors and other physiological changes. For example, the hypopharyngeal glands of nurse-aged bees infected with *N. apis* are reduced in size and function (Wang and Moeller, 1969; Liu, 1990). Workers infected with *Nosema* have shortened lifespans and spend more time outside of the nest engaged in risky behaviors such as guarding the nest entrance, performing orientation flights, and foraging (Hassanein, 1953; Wang and Moeller, 1970; Lin et al., 2001; Higes et al., 2008; Woyciechowski and Moroń, 2009; Alaux et al., 2010; Vidau et al., 2011; Aufauvre et al., 2012; Goblirsch et al., 2013a; Aufauvre et al., 2014).

Premature foraging may be a consequence of the energetic demands that *N. ceranae* imposes on workers when they become infected at a young age (Mayack and Naug, 2009). Additionally, energetic stress due to infection can affect homing ability (Wolf et al., 2014). The success rate of workers returning to their colony after flying away from it during for a foraging trip is significantly reduced for workers infected with *Nosema* (Kralj and Fuchs, 2010; Wolf et al., 2014). Workers infected with *N. ceranae* have lower levels of trehalose, which can reduce flying capability by one-third compared to uninfected bees (Mayack and Naug, 2010). Interestingly, flying capability can be restored to the same level as controls if infected bees are given free access to sucrose solution (Mayack and Naug, 2010). The hypothesis that infected bees are "hungry" is supported by the finding that sucrose responsiveness is elevated in workers infected with *N. ceranae* and that these bees are less likely to share food with nestmates (Naug and Gibbs, 2009). *Ad libitum* access to food stores such as nectar/honey and pollen (i.e., bee bread) may offset the drain on carbohydrate and protein metabolism from infection (Basualdo et al., 2014).

1.3.5 Dissertation Research on the Effects of *Nosema* on Vitellogenin/Juvenile Hormone Signaling

Evidence from both *N. apis* and *N. ceranae* show that infection with these pathogens can disrupt the age-polyethism of honey bee workers, mainly by advancing the transition from the nursing phase to the foraging phase and ultimately resulting in premature death (Wang and Moeller, 1970; Goblirsch et al., 2013a). As part of my dissertation research, I proposed that the age-advancing effects of infection operate by

disrupting underlying regulatory mechanisms that control the interaction of Vg/JH. To test this hypothesis, I infected bees with *N. ceranae* and put them in cages or small field colonies. I then measured levels of Vg transcript from fat body tissues using quantitative PCR and JH titer in hemolymph using a radioimmunoassay from bees I collected at regular intervals during a 15 day (field colonies) or 16 day (cages) incubation period.

Compared to controls, I observed a trend for nurse-aged bees infected with *N. ceranae* to have elevated levels of JH in cages and field colonies (Goblirsch et al., 2013a). This finding is corroborated by research showing that the profile of JH titer for workers infected with *Nosema* is atypical for their age compared to uninfected workers (Fisher and Sanborn, 1962; Liu, 1989; Lin et al., 2001; Ares et al., 2012). In addition, I found that the expression of Vg was depressed for infected nurse-aged workers in cages but not field colonies. A previous study revealed that Vg expression was downregulated in 7-day old bees (Antúnez et al., 2009), which is comparable to what I observed in 8-day old bees (Goblirsch et al., 2013a). Since nurse bees typically consume large amounts of pollen that becomes digested and used for the synthesis of proteins found in brood food, it is possible that low levels of Vg in infected bees is the result of changes to fundamental nutritional and metabolic pathways (Holt et al., 2013; Vidau et al., 2014). Interestingly, I observed elevated levels of Vg expression for infected workers in cages at 16 days of age. I am uncertain about the relevance of this finding, but it has been observed previously in infected queens (Alaux et al., 2011) and workers from colonies with low levels of *Nosema* infection (Antúnez et al., 2013). The pleiotropic role of Vg in the development of the queen and workers may provide insight into how its expression and synthesis is affected by infection. Furthermore, this knowledge could promote the development of

therapeutics or management schemes that beekeepers could employ to mitigate the effects of *Nosema* on the worker caste to keep the colony's Vg potential within the hive.

The downstream effects to disrupting nutritional and metabolic pathways of the worker may be evident by changes in pheromone production. Modulation of pheromone production has been shown in workers infected with *Nosema.* Ethyl oleate (EO) is synthesized by older workers and has an inhibitory effect on the pace of behavioral development of younger workers. Older workers likely expose younger bees to EO during trophallaxis (Leoncini et al., 2004). Dussaubat et al. (2010) showed a positive correlation between spore load and ethyl oleate (EO) production in infected bees. Because *Nosema* infection has been shown to cause workers to transition prematurely from nursing to foraging behavior, elevated EO production in infected workers may be an additional outcome of changes in the underlying endocrine signaling physiology that regulates pheromone synthesis.

1.4 Interactions and Bee Health

1.4.1 Interactions with *Nosema* that Negatively Affect Bee Health

The interaction of *Nosema* with xenobiotics, viruses, or when it is introduced into other bee species poses additional problems for bee health. Many factors thought to contribute negatively to honey bee health likely do not act alone but are additive or synergistic with one or more stressors that exacerbate morbidity in individual bees and/or accelerate colony mortality. Xenobiotics such as pesticides, fungicides, herbicides, and acaricides are one factor in which extensive research has been initiated to better understand their effects on bee health. Foraging bees are exposed to xenobiotics from the

external environment (e.g., spray drift, systemic expression in plant tissues) during the collection of nectar and pollen, which they bring back to the colony as food for adults and brood. Additionally, the internal hive environment can become contaminated through beekeeper-applied therapeutics such as acaricides that are used to control *Varroa* mites and other pests. Residues of over 100 different pesticides and their metabolites have been detected on bees or in pollen, honey, wax, and hive equipment (Bogdanov, 2006; Chauzat and Faucon, 2007; Frazier et al., 2008; Chauzat et al., 2009; Mullin et al., 2010; Lambert et al., 2013; Pettis et al., 2013). The amount of any individual pesticide frequently detected in the hive may not be sufficient to threaten colony survival by causing acute toxicity of a large number of bees, but combinations of pesticides and/or their metabolites may have cumulative effects that over time create synergisms with other widespread colony stressors such as pathogens or poor nutrition (Sanchez-Bayo and Goka, 2014). For example, honey bees that are exposed to high levels of fungicides have been shown to be more susceptible to infection with *N. ceranae*. The increased risk to infection is hypothesized to result from the inability of bees exposed to the fungicides to mount an effective immune response against *N. ceranae* (Pettis et al., 2013).

1.4.2 Interaction of *Nosema* with Pesticides

Neonicotinoids and phenylpyrazoles are recent classes of insecticides with low toxicity to mammals compared to traditional formulations (e.g., organophosphates and carbamates). For this reason, they are used intensively to control insect and other arthropod pests of crops, landscapes, livestock, and companion animals. Neonicotinoids are agonists of neuronal nicotinic acetylcholine receptors and act by blocking the binding

capability of the natural ligand, acetylcholine. Phenylpyrazoles act by blocking γ-aminobutyric acid (GABA) and glutamate-gated chloride ion channels. Both pesticides result in overstimulation of nervous and/or muscle tissues, leading to death. Sublethal doses of neonicotinoids or other pesticides combined with entomopathogenic fungi such as Microsporidia have become part of an integrated approach that utilizes two modes of action to enhance control of insect pests of agricultural systems (Purwar and Sachan, 2006). The presence of these xenobiotics in the environment could affect non-target species such as honey bees harboring natural infections of *Nosema*. Studies looking at the interaction of chronic exposure to the neonicotinoids, imidacloprid and thiacloprid, with *N. ceranae* infection observed significant increases in honey bee mortality compared to infection alone (Alaux et al., 2010; Retschnig et al., 2014b). These findings are similar to other studies examining the interaction of the phenylpyrazole, fipronil, with *N. ceranae* (Vidau et al., 2011; Aufauvre et al., 2012; but see Aufauvre et al., 2014). Xenobiotics have been shown to induce apoptotic cell death in the midgut epithelial cells of honeybee larvae (Gregorc and Bowen, 2000), therefore, an *in vitro* system comprised of honey bee cells could be used to observe cell death from exposure to combinations of pesticides and *Nosema* infection as a proximate mechanism to explain premature bee death.

In addition to effects on survivorship, the interaction of pesticides and *Nosema* infection may change the dynamics of the host-parasite relationship, such as allowing exploitation of host resources for spore production. Honey bees infected with *Nosema* were found to have elevated spore loads after exposure to either neonicotinoids or phenylpyrazoles (Vidau et al., 2011; Pettis et al., 2012). One plausible explanation for the occurrence of higher spore loads in exposed bees may be that *Nosema* is opportunistic

and takes advantage of the antibiotic potential of pesticides to reduce populations of beneficial fungi and other symbionts that reside in the digestive tract (e.g., *Aspergillus*, *Penicillium*, *Rhizopus*, and *Cladosporium*) and act as a defense against invasion from foreign pathogens (Yoder et al., 2013).

Both *Nosema* infection and pesticide exposure are ubiquitous events for a honey bee colony; therefore, it is difficult to tease apart which event, infection or exposure, occurs first and alters physiological processes allowing the subsequent event to exacerbate the rate of decline in host health. Infection with *N. ceranae* has been shown to upregulate cytochrome P450 monooxygenase and glutathione-S-transferase, and downregulate catalase and glutathione peroxidase in the midgut of the honey bee (Aufauvre et al., 2014). These genes are responsible for metabolizing xenobiotic substrates for detoxification or provide protection against oxidative damage by catalyzing hydrogen peroxide to water and oxygen, respectively. Honey bees have half the number of cytochrome P450 monooxygenase genes as most other insects (Claudianos et al., 2006). These findings provide some clues as to what the outcome will be when pesticides challenge the detoxification system of honey bees already overwhelmed from *Nosema* infection (Johnson et al., 2009). It could be that detoxification processes in midgut cells become overwhelmed from exposure to pesticides, increasing susceptibility to *Nosema* infection.

From an applied perspective, I perceive *Nosema* infection and pesticide exposure as seemingly continuous events in the life of a honey bee colony. One approach to assessing colony-level health would be to treat them as a single factor and focus on other events, such as mite levels, infection(s) with virus or viral titers, and nutrition to see if

their addition or subtraction can offset effects stemming from the pesticide-*Nosema* interaction.

1.4.3 Interaction of *Nosema* with Viruses

Honey bees are host to at least 18 viruses, most of which are nonenveloped, single-stranded, positive sense RNA viruses, or picornaviruses. Picornaviruses are both highly prevalent and detrimental to honey bee health and productivity. Those most frequently detected in beekeeping operations include black queen cell virus (BQCV), deformed wing virus (DWV), Kashmir bee virus (KBV), and sacbrood virus (SBV) (Chen and Siede, 2007; Welch et al., 2009). Bailey et al. (1983) observed co-infections of BQCV and *N. apis* during a 3-year observation period. Their data showed that BQCV and *N. apis* cycled in unison, with levels of both pathogens peaking in May or June. Peak infections of *N. apis* and BQCV at this time of year may provide an explanation for the classic symptom of "spring dwindle," where the adult bees of a heavily-infected colony succumb before they can be replaced by young bees as brood-rearing resumes after its winter dearth (White, 1918). Seasonal cycling of common bee viruses and *Nosema* was demonstrated more recently with comprehensive molecular techniques including microarrays and deep sequencing of the honey bee microbiome using samples taken from a large scale migratory beekeeping operation (Runckel et al., 2011). These findings present the possibility that viruses and *Nosema* may act synergistically to affect honey bee health.

The interaction of *Nosema* and viruses has been reported for bees co-infected with *N. ceranae* and chronic bee paralysis virus (CBPV) or DWV. One study showed that

experimental co-infection of adult bees with *N. ceranae* and CBPV resulted in increased replication of CBPV but not mortality compared to bees infected with CBPV alone (Toplak et al., 2013). Costa et al. (2011) found a significant negative correlation between *N. ceranae* spore load and DWV titer in midgut tissues of adult honey bee workers. Furthermore, a survey of 322 colonies in Hawaii showed that while at least 89% were infected with *N. ceranae*, there was no significant correlation between spore load and DWV titer by PCR (Martin et al., 2013). One possible explanation why there is not a positive correlation between *Nosema* spore load and DWV titer may depend on the specific demographics of bees that are sampled. Honey bees that are symptomatic for DWV reveal replication, based on the detection of negative-strand viral RNA, in tissues throughout the body, which include hemolymph, gut, legs, head, thorax, abdomen, and most notably, the wings (Boncristiani et al., 2009). Food-borne transmission is one mechanism by which DWV can establish infection (Chen et al., 2006a; Chen et al., 2006b). However, asymptomatic bees do not show signs of replication in the gut or abdomen, prompting speculation that a compromise in the integrity of the gut lining is needed for DWV to gain access to gut epithelial cells and/or enter circulation via the hemolymph (Boncristiani et al., 2009). Deformed wing virus can infect the immature stages of the honey bee, whereas current evidence only shows that the adult is the only life stage that can become infected with *Nosema*. Newly emerged bees that are symptomatic for DWV (e.g., wing deformities) die shortly after emergence (Chen et al., 2005; Dainat et al., 2012a). Even if these bees were inoculated with spores upon emergence, the time until death may not be sufficient for *Nosema* to complete its development and produce a large number of spores. On the other hand, asymptomatic

bees appear relatively healthy and have a lifespan that supports the production of a large number of spores. *Nosema apis* and *N. ceranae* need at least 10 days post inoculation to reach fully developed infections (i.e., ~20 x 10^6 spores/species) (Forsgren and Fries, 2010). If a sample consists mainly of asymptomatic bees, it may not be surprising that these bees have high spore loads but low DWV titer and vice versa.

1.4.4 Spillover of *Nosema ceranae* to Other Bee Species

Evidence of infection with *N. ceranae* in other bee species has been examined. One study used cages experiments to infect different honey bee species, including the dwarf honey bee, *A. florea*, and the giant honey bee, *A. dorsata*, from South and Southeast Asia with isolates of *N. ceranae* from Canada and Thailand. Both honey bee species were susceptible to infection with the Thai isolate but the Canadian isolate was only infective to *A. dorsata* (Chaimanee et al., 2013). This finding supports an Asian origin for *N. ceranae*, but may also demonstrate strain virulence in that the isolate from Canada was capable of producing an infection in *A. mellifera* as well.

Infection with *N. ceranae* has also been found in several species of *Bombus* from China (Li et al., 2012), Argentina (Plischuk et al., 2009), and the United Kingdom (Graystock et al., 2013; Fürst et al., 2014), albeit at low to moderate prevalence (6.1%, 10.8%, 21%, and 7%, respectively). *Nosema ceranae* may be associated with the decline in abundance and richness of species of *Bombus*; however, much remains unknown (e.g., infectivity and pathogenicity) about the impact this pathogen is having on populations of these bees and other beneficial insects in their native ranges. One study inoculated workers of *B. terrestris* with 6.5 x 10^3 *N. ceranae* spores and observed infection levels of

nearly 100%, amplification of spore number, and a significant reduction in longevity during a 15-day experiment period under controlled conditions (Graystock et al., 2013).

The use of bees other than honey bees for commercial pollination has led to the development of artificial rearing techniques for some species. Several species of solitary bee from the family Megachilidae, as well as the primitively social bumblebee can be reared in the laboratory (Bosch and Kemp, 2002; Evans et al., 2007) making it possible to study the effects of spillover from infection on metrics of individual bee and social living (e.g., foraging efficiency, survivorship, and reproductive success) in a controlled environment. However, this represents just a small subset of the thousands of other bees where the effects from *Nosema* can only be assessed using anecdotal evidence, such as prevalence of infection. It is a challenge for future research to develop methods leading to an increase in the number of bee species that can either be reared in the laboratory or assessed by minimally invasive techniques to further our understanding of the pathological effects due to the spillover of *N. apis*, *N. ceranae*, and other Microsporidia in wild bee populations (Paxton et al., 1997).

A honey bee colony is a highly complex unit comprised of thousands of individuals. The members that make up this unit come from “all walks of bee life” in the sense that each bee has an identity that is shaped by its date of birth, the patriline it comes from, its task performance history, and its breadth of interactions with its environment (e.g., exposure to pathogens). When isolated from the colony, an individual honey bee cannot perform all the functions necessary to survive and reproduce. Since the root cause(s) responsible for the decline of honey bees is likely multifactorial, it becomes difficult to get a representative picture of colony health from sampling only a few bees.

The interaction of factors and the diverse identities that comprise a honey bee colony point to the need for a more basic system where the experimenter can regulate the input of a single or combination of factors and observe the outcome. A continuous cell line would provide such a system and could serve as a starting point for higher order studies (e.g., individual bee or colony-level).

1.5 Honey Bee *In Vitro* Systems

1.5.1 A Different Approach to Studying *Nosema* and Other Bee Diseases

During the course of my research with *Nosema*, I began collaborating with Professor Tim Kurtti to develop a honey bee cell culture system. Although it was not my original intent to develop a cell line, serendipity led me to realize the potential that an *in vitro* system derived from honey bee cells could have in expanding my understanding of the effects of intracellular pathogens such as *Nosema* and viruses. Cell culture is a basic tool in biology that involves removing a small amount of tissue from a whole organism, introducing this material into a sterile vessel containing a liquid medium that approximates the physiological environment from where the material was excised, and maintaining the viability and proliferation of the cells. Cell culture provides a simplified environment where variables, such as the introduction of pathogens or foreign DNA can be manipulated, and the resulting outcomes used to predict what would happen at the level of the whole organism.

Cell culture has an extensive history in insect biology and disease as it has advanced our understanding of the contribution and interplay of different cell types that make up complex systems and has expanded the knowledge base on the dynamics of the

host-pathogen relationship. Grace (1962) isolated the first continuously replicating insect cell line from the ovarian tissues of the emperor moth, *Antheraea eucalypti*. Since this achievement, over 500 continuous insect lines have been developed, mainly of dipteran or lepidopteran origin (~80%) (Lynn, 2003). One of the original motivations for the development of cell lines from insects was to propagate viruses axenically for the development of biopesticides for the control of pests of economic importance (Arif and Pavlik, 2013). However, the finding that foreign genes can be expressed in insect cells through baculovirus-mediated expression vectors (Smith et al., 1985) has subsequently spurred large scale production of recombinant proteins (e.g., vaccines) (Airenne et al., 2013) that have application in the treatment of human diseases.

1.5.2 Previous Work on Honey Bee Cell Culture

Infection biology and transgenesis have expanded the utility of existing, and the need for additional, cell lines derived from insects. Ironically, the celebrity-like status of the honey bee has not placed it at the forefront for the development of *in vitro* systems from this model organism. Until recently, no cell lines were available from the honey bee (Kitagishi et al., 2011; Goblirsch et al., 2013b). Furthermore, the number of studies reporting the use of honey bee tissues as a source of material for initiating cell cultures is surprisingly few. Primary cultures have been reported using honey bee embryonic cells (Giauffret et al., 1967; Bergem et al., 2006; Chan et al., 2010) and larval and pupal cells (Stanley 1968; Giauffret, 1971; Beisser et al., 1990; Gascuel et al., 1994; Gisselmann et al., 2003). Neurons dissociated from antennal lobes or mushroom bodies of honey bee pupae and adults have proved valuable for studies on the morphology, development, and

biochemical function of the olfactory system (Kriessl and Bicker, 1992; Bicker and Kriessl, 1994; Kloppenburg et al., 1999; Malun et al., 2003; Barbara et al., 2008). However, the heterogeneous cell types and relatively short-term viability characteristic of most primary cultures make reproducibility a challenge.

Continuous cell lines composed of a single type are desirable because of the potential for an unlimited source of biological material. Despite previous efforts, the paucity of continuous cell lines for the honey bee suggests factors such as the developmental stage and/or tissue origin of source material, media composition, or culture environment need to be identified or refined to support adaptation and long-term proliferation to the artificial conditions. Mitotically active cultures have been maintained for 3 months using honey bee pre-gastrula embryos (36 – 40 hours after oviposition) (Bergem et al., 2006). Zhang et al. (2009) maintained midgut epithelial cells isolated from *A. cerana* larvae in Grace's medium supplemented with 20% FBS for approximately 5 months. Mitsuhashi (2001) developed a medium (MGM-464) and tested its suitability on primary cultures established from embryonic tissues of the honey bee, as well as embryos and other tissues from several orders of insect. Honey bee embryonic cells cultured with MGM-464 were short-lived, which may be attributed to toxicity from high levels of the media constituents (Mitsuhashi, 2001). The use of transgene expression systems to introduce "immortalizing" genes has been attempted to circumvent the long adaptation period typical for insect cells transitioning from a primary culture to a continuous cell line (Brooks and Kurtti, 1971; Vaughan, 1985). For example, insertion of the human c-myc oncogene by lipofection resulted in the isolation of a reputed

continuous honey bee cell line; however, there is no evidence that this line is available to the research community (Kitagishi et al., 2011).

1.5.3 A Continuous Cell Line Established from Honey Bee Embryos

I combined traditional techniques used to establish primary cell cultures from honey bee embryos with culture medium specifically developed for the culture of tick cells (Munderloh and Kurtti, 1989). I maintained these primary cultures in sealed vessels at 32°C in a non-humidified incubator. Most primary cultures survived at least 6 months when exposed to these conditions, and several could be passaged to establish young cell lines. After a long period of adaptation, I was able to isolate a honey bee cell line from one of my primary cultures (Goblirsch et al., 2013b). The cell line, named AmE-711, is composed mainly of fibroblast-type cells with a diploid karyotype. The doubling time of AmE-711 cells was estimated to be approximately 4 days, and the line has undergone at least 50 transfers and remains in culture since its isolation in July of 2011. The AmE-711 will be a useful tool to study the mechanisms of infection from the many intracellular microbes known to cause disease in honey bees, such as viruses and *Nosema*.

1.6 Conclusions and Future Directions

Beekeepers and researchers have long dealt with the problem of *Nosema* disease in honey bee colonies. Past observations on how infestations with the "old *Nosema*", *N. apis*, contribute to queen failure, atypical worker ontogeny, and colony decline serve as a reference for understanding the current challenges imposed by the "new *Nosema*", *N. ceranae*. Much like its predecessor, *N. ceranae* produces a similar pathology as seen in

the expression of abnormal worker behavior and physiology. Workers infected with *N. ceranae* have shortened lifespans and exhibit an early onset of foraging behavior. Contributing to the condition of premature foraging is a disruption of underlying regulatory mechanisms, namely the interaction Vg and JH.

My thesis is comprised of two areas of study, which ultimately complement each other, but were not originally set out to do this. Chapter II contains research conducted on the effects of *Nosema ceranae* infection on worker honey bees. This chapter has been published [Goblirsch M, Huang ZY, and Spivak M. 2013a. "Physiological and behavioral changes in honey bees (*Apis mellifera*) induced by *Nosema ceranae* infection." *PLoS ONE*. 8(3):e58165]. For this component of my thesis, I established methods for infecting newly-emerged honey bee workers with spores of *Nosema ceranae* purified from infected bees. I compared the survivorship and age of foraging onset of inoculated worker bees to un-inoculated controls in cages and field relevant conditions, respectively. It was possible that some of my control bees were infected naturally within the colony, as all colonies now have *N. ceranae*. It was also possible that some of my inoculated bees never became infected, so to make sure I really saw an effect on foraging from infection, I compared only *Nosema*-infected bees to uninfected control bees. I have included a table in Appendix 1 showing this comparison. In addition, I analyzed abdominal tissues from workers for relative expression of the Vg gene by quantitative-reverse transcription PCR (q-rtPCR) and hemolymph for juvenile hormone titer by radioimmunoassay collected from cage and field bees of known age and infection status. A handbook containing a chapter for standard methods for conducting research with *Nosema* in *A. mellifera* was published at roughly the same time as my research on *N.*

ceranae (Fries et al., 2013). Based on this valuable reference, I have compiled a summary on how I would improve future research in the area of *Nosema* using bees in cages and colony studies (see: Appendix 2).

The third chapter of my thesis contains an account of my research where I set out to develop a continuous honey bee cell line. Chapter III has been published [Goblirsch MJ, Spivak MS, Kurtti TJ. 2013b. "A cell line resource derived from honey bee (*Apis mellifera*) embryonic tissues." *PLoS ONE*. 8(7):e69831]. For this component of my research, I established methods that promoted the long-term survival and growth of primary cultures and young cell lines isolated from honey bee embryonic tissues. I used a culture medium previously shown to support *in vitro* growth of tick and other insect cell lines (Munderloh and Kurtti, 1989; Sagers et al., 1996; Munderloh et al., 1999). From approximately 100 primary cultures, I was able to establish one line that remains in culture, is routinely passaged, and can be revived from cryopreservation in liquid nitrogen. I have characterized this line, named AmE-711, using conventional PCR and karyology and found that it conforms to the species from where the tissues were obtained to initiate the primary cultures. I incubated AmE-711 cells at different temperatures and with different commercial media to see if this had an effect on overall cell growth. These experiments provided a segue for work performed for Chapter IV where I further characterized the growth of AmE-711 honey bee cells exposed to different permutations of the culture media. Now that a continuous cell line is available for honey bees, others can use it to study *Nosema*, viruses, or a combination of biotic and abiotic factors.

Researchers throughout the U.S. and several other countries have requested the AmE-711 cell line. Upon receiving the cells, most laboratories have encountered

difficulty in maintaining the line. Difficulty in culturing the AmE-711 cell line may be because it is a nascent line. The direction that I will pursue for my future research will be to focus on refining the culture conditions used to maintain the AmE-711 line. One objective of this research will be to simplify the culture medium by trying to adapt AmE-711 cells to a medium that is commercially prepared. Another objective will be to identify and characterize a putative factor that has emerged after I had thawed an ampoule of cells from my frozen stocks. My preliminary studies demonstrate that this factor is capable of killing AmE-711 cells but has no effect when applied to tick cells, and is not filtered out by passing medium containing the factor through a 0.22 μm syringe-driven filter. Characterizing this unknown "killing" factor will set in motion my long term plans that will include studying the effects of intracellular pathogens (e.g., DWV) on bee cell physiology, or testing the interaction of xenobiotics with pathogens to better understand how these factors can form synergies as observed under colony conditions. I am excited about the many opportunities that exist for demonstrating the utility of the AmE-711 cell line in honey bee research.

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1.8 FIGURE LEGENDS

Figure 1.1 Life cycle of *Nosema apis*.

A spore that has been ingested by a bee (1) encounters stimuli in the gut that activate the germination process. Eversion of the polar filament results in the injection of the sporoplasm into a midgut epithelial cell (2-3). The developing parasites undergo several rounds of binary fission (i.e., merogony)(4-10), followed by thickening of the chitinous spore wall and lengthening of the polar filament (i.e., sporogony)(11-12). The accumulation of mature, or environmental, spores causes the host cell to lyse and release the infectious progeny into the lumen of the gut (13). Primary, or vegetative, spores can also transmit infection, or autoinfect, adjacent host cells. Reprinted with permission from Springer Life Sciences © (see: Appendix 3).

Figure 1.2 Infection of AmE-711 cells with *Nosema ceranae.*

Spores of *N. ceranae* were germinated in the presence of AmE-711 cells (N, host nucleus). Sporogonic stages (I, blue arrows) were observed 7 days after inoculation. Mature spores with thickened spore walls (S, yellow arrow) and germinated cases (E, black arrow). Scale bar = 10 µm.

1.9 TABLE LEGENDS

Table 1.1 Geographic distribution and first incidence of *Nosema ceranae*.

Geographic distribution of the first incidence of *Nosema ceranae* infection in species from the family Apidae based on histological examination or PCR products amplified from extracted DNA. Data were gathered from peer-reviewed publications or reporting by government agencies. Date of incidence may differ from date of citation due to the use of archived material or delays in publication.

Table 1.1 Footnote.

* Original description of *N. ceranae* infection in the Asian honey bee, *Apis cerana*.

† First report of infection of *N. ceranae* in the European honey bee, *A. mellifera*.

Figure 1.1

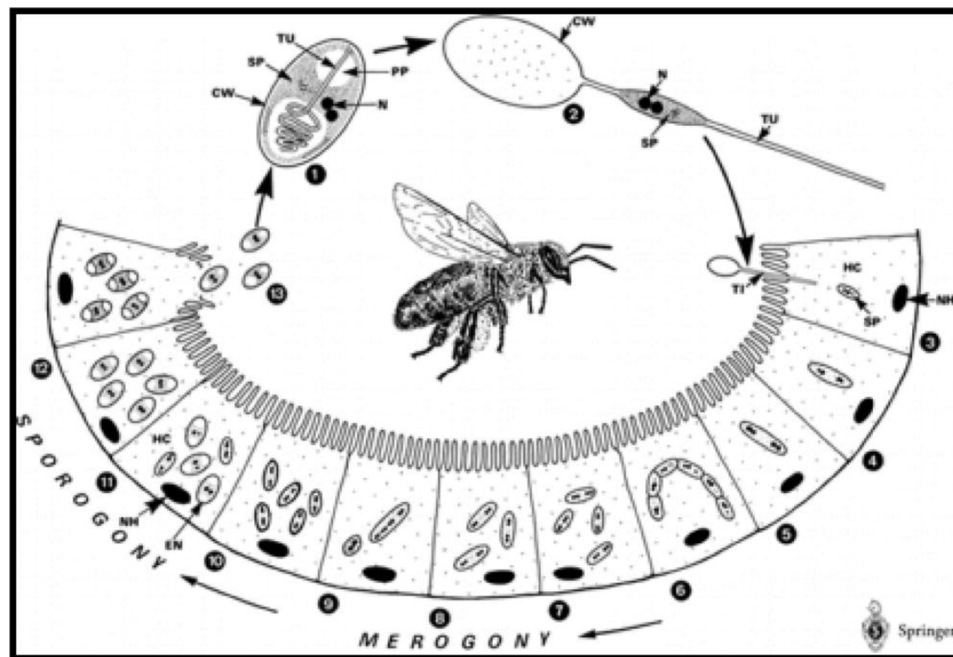

Figure 1.2

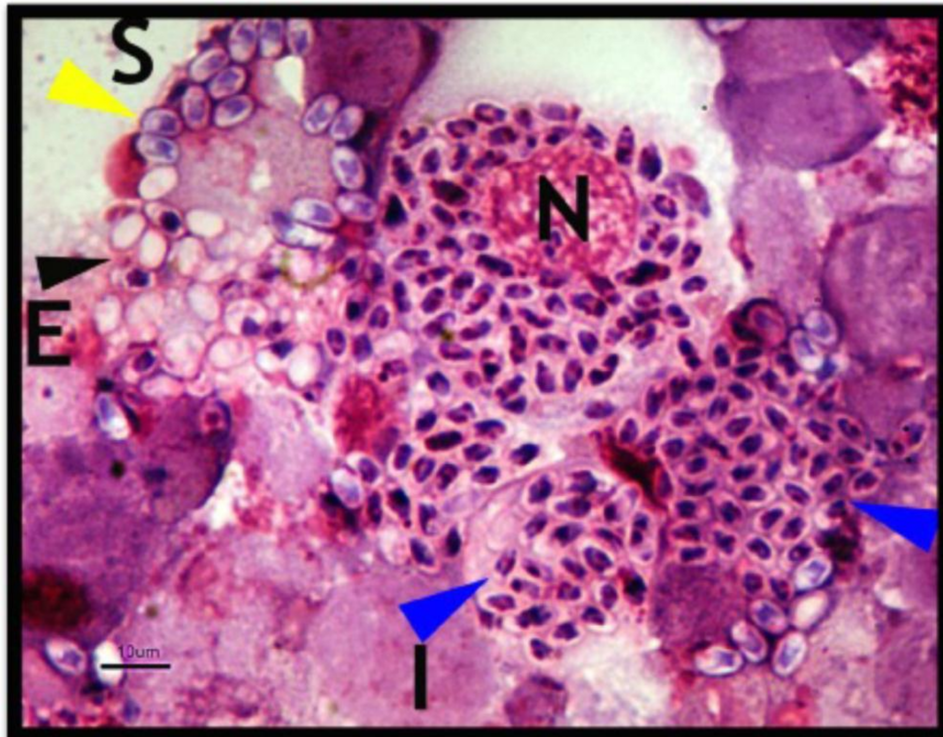

Table 1.1

Continent	Country: Region	Year	Species/Strain Infected	Reference
Africa	Algeria	2009	*A. m. intermissa*	Higes et al. 2009a. *J Apicult Res*. 48(3):217-9.
Asia	China: Beijing	1996*	*A. cerana*	Fries et al. 1996. *Eur J Protistol*. 32(3):356-65.
	Taiwan	2004†	*A. mellifera*	Huang et al. 2007. *Apidologie*. 38(1):30-7.
	Vietnam	2006	*A. mellifera*	Klee et al. 2007. *J Invertebr Pathol*. 96(1):1-10.
	Israel	2010	*A. m. ligustica, A. m. caucasica, A. m. syriaca*	Soroker et al. 2010. *Apidologie.* 42(2):192-9.
	Turkey	2010	*A. mellifera*	Whitaker et al. 2011. *Apidologie*. 42(2):174-80.
	Iran	2011	*A. mellifera*	Nabian et al. 2011. *Iran J Parasitol*. 6(3):89-95.
	Japan	2011	*A. mellifera*	Yoshiyama, Kimura. 2011. *J Invertebr Pathol*. 106:263-7.
	Thailand	2011	*A. cerana, A. florea, A. mellifera*	Suwannapong et al. 2011. *J Invertebr Pathol*. 106:236-41.

	China: Gansu, Qinghai, Sichuan, and Inner Mongolia	2012	*Bombus* spp.	Li et al. 2012. *Int J Parasitol*. 42(1):49-61.
Australia	Queensland, New South Wales, Victoria, and South Australia	2009	*A. mellifera*	Giersch et al. 2009. *Apidologie*. 40(2):117-23.
	New Zealand	2010	*A. mellifera*	New Zealand Ministry for Primary Industries: biosecurity.govt.nz/pests/nosema-ceranae.
Europe	Italy	1993[1], 2005[2]	*A. mellifera*	[1]Ferroglio et al. 2013. *J Apicult Res.* 52(2):60-1. [2]Klee et al. 2007. *J Invertebr Pathol*. 96(1):1-10.
	Finland	1998[1], 2006[2]	*A. mellifera*	[1]Paxton et al. 2007. *Apidologie*. 38(6)558-65. [2]Klee et al. 2007. *J Invertebr Pathol*. 96(1):1-10.
	Denmark	2004	*A. mellifera*	Klee et al. 2007. *J Invertebr Pathol*. 96(1):1-10.
	Spain	2004	*A. mellifera,*	Klee et al. 2007. *J Invertebr Pathol*. 96(1):1-10.
		2006	*A. m. iberiensis*	Higes et al. 2006. *J Invertebr Pathol*. 92(2):93-5.
	Greece	2004[1], 2010[2]	*A. mellifera*	[1]Klee et al. 2007. *J Invertebr Pathol*. 96(1):1-10. [2]Hatjina et al. 2010. *J Apicult Res*. 49(1):116-18.
	Germany	2006	*A. mellifera*	Klee et al. 2007. *J Invertebr Pathol*. 96(1):1-10.
	Serbia	2006	*A. mellifera*	Klee et al. 2007. *J Invertebr Pathol*. 96(1):1-10.

Norway	2006	*A. mellifera*	Paxton et al. 2007. *Apidologie*. 38(6)558-65.
Sweden	2006	*A. mellifera*	Klee et al. 2007. *J Invertebr Pathol*. 96(1):1-10.
France	2007	*A. mellifera*	Chauzat et al. 2007. *J Apicult Res*. 46(2):127-8.
Poland	2007	*A. mellifera*	Topolska, Kasprzak. 2007. *Med Weter*. 63(11):S1504-6.
Hungary	2009	*A. mellifera*	Tapaszti et al. 2009. *Acta Vet Hung*. 57(3):383-8.
Slovakia	2009	*A. mellifera*	Staroň et al. 2012. *Slovak J Anim Sci*. 45(1):36-8.
Bosnia and Herzegovina	2010	*A. mellifera*	Santrac et al. 2010. *J Apicult Res*. 49(1):100-1.
Croatia	2010	*A. mellifera*	Gajger et al. 2010. *J Apicult Res*. 49(3):340-1.
Netherlands	2010	*A. mellifera*	van der Zee. 2010. *Bijenhouden*. 9:3-5.
Scotland	2013	*A. mellifera*	Bollan et al. 2013. *Parasitol Res*. 112(2):751-9.
United Kingdom	2013	*B. terrestris*	Graystock et al. 2013. *J Invertebr Pathol*. 114(2):114-9.
Lithuania	2014	*A. mellifera*	Blažytė-Čereškienė et al. 2014. *J Apicult Res.* 53(3):374-6.

Region	Country/Location	Year	Host	Reference
North America	United States: Washington D.C., California, Florida, Hawaii, Pennsylvania, Minnesota	1995[1], 2004[2], 2007[3], 2008[4]	*A. mellifera*	[1]Chen et al. 2008. *J Invertebr Pathol*. 97(2):186-8. [2]Klee et al. 2007. *J Invertebr Pathol*. 96(1):1-10. [3]Cox-Foster et al. 2007. *Science*. 318(5848):283-7. [4]Williams et al. 2008. *J Invertebr Pathol*. 97(2):189-92.
	Mexico	2004	*A. m. scutellata*	Guzman-Novoa et al. 2011. *J Apicult Res*. 50:167-9.
	Canada: Nova Scotia, Prince Edward Island	2008	*A. mellifera*	Williams et al. *J Invertebr Pathol*. 97(2):189-92.
	Costa Rica	2008	*A. m. scutellata*	Calderon et al. 2008. *J Apicult Res*. 47(4):328-9.
South America	Brazil	1979[1], 2006[2]	*A. m. scutellata*	[1]Teixeira et al. 2013. *J Invertebr Pathol*. 114(3):250-4. [2]Klee et al. 2007. *J Invertebr Pathol*. 96(1):1-10.
	Argentina	2009	*B. atratus, B. morio, B. bellicosus*	Plischuk et al. 2009. *Microbiol Rep*. 1(2):131-5.
		2009	*A. mellifera*	Medici et al. 2012. *Parasitol Res*. 110(2):859-64.
	Uruguay	2009	*A. mellifera*	Invernizzi et al. 2009. *J Invertebr Pathol*. 101(2):150-3.
	Chile	2012	*A. mellifera*	Martínez et al. 2012. *Parasitol Res*. 111(2):601-7.
	Belize	2013	*A. m. scutellata*	Rangel et al. 2013a. *J Apicult Res*. 52(2):62-6.

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CHAPTER 2

Physiological and Behavioral Changes in Honey Bees (*Apis mellifera*) Induced by *Nosema ceranae* Infection

Mike Goblirsch, Zachary Y. Huang, and Marla Spivak

SUMMARY

Persistent exposure to mite pests, poor nutrition, pesticides, and pathogens threaten honey bee survival. In healthy colonies, the interaction of the yolk precursor protein, vitellogenin (Vg), and endocrine factor, juvenile hormone (JH), functions as a pacemaker driving the sequence of behaviors that workers perform throughout their lives. Young bees perform nursing duties within the hive and have high Vg and low JH; as older bees transition to foraging, this trend reverses. Pathogens and parasites can alter this regulatory network. For example, infection with the microsporidian, *Nosema apis*, has been shown to advance behavioral maturation in workers. We investigated the effects of infection with a recent honey bee pathogen on physiological factors underlying the division of labor in workers. Bees infected with *N. ceranae*, were nearly twice as likely to engage in precocious foraging and lived 9 days less, on average, compared to controls. We also show that Vg transcript was low, while JH titer spiked, in infected nurse-aged bees in cages. This pattern of expression is atypical and the reverse of what would be expected for healthy, non-infected bees. Disruption of the basic underpinnings of

temporal polyethism due to infection may be a contributing factor to recent high colony mortality, as workers may lose flexibility in their response to colony demands.

KEYWORDS: double-repressor hypothesis, endocrine factor, eusociality, insect pathology

2.1 INTRODUCTION

The economic and ecological importance of honey bees (*Apis mellifera*) as pollinators of many cultivated and native plants make them an important system for studying the effects of illness at both the individual and colony or social levels. In the U.S. and worldwide, it has become increasingly difficult to keep colonies alive as bees are challenged with numerous factors that threaten their survival. Mite pests, pathogens, pesticides, and nutritional deficiencies create a combination of circumstances that can interact negatively to jeopardize colony health (Spivak et al., 2011; Dainat et al., 2012b). For the U.S. specifically, the outcome of this health crisis has been losses of nearly one-third of colonies annually since 2006 (van Engelsdorp et al., 2012). This recurring level of death may be unsustainable for the beekeeping industry, and could debase the value of crops and other products requiring pollination (Calderone, 2012).

Many pathogens and parasites are common and widespread in non-symptomatic, or apparently healthy, colonies (Runckel et al., 2011). This burden emphasizes the buffering capacity of honey bee societies, but makes it difficult to state with confidence the impact a specific factor has on colony health. Fortunately, a wealth of research is available describing physiological factors that influence the age-based division of labor of workers within healthy colonies (reviewed by: Robinson, 1992; Amdam et al., 2011). We relied on this research background to explore mechanisms that could interfere with worker behavior following exposure to a single specific pathogen, *Nosema ceranae*, considered to be a factor in colony decline (Spivak et al., 2011).

The honey bee worker caste displays an age-based division of labor. In northern temperate climates, workers that emerge as adults during the summer live 6 wks on average. Upon emergence, workers less than 3 wks of age remain in the hive as nurses, feeding the larvae and queen, or performing nest maintenance. At about 3 wks, workers transition from nursing duties to foraging for nectar and pollen outside the nest. Once workers transition to foraging, they typically live an additional 2–3 wks, regardless of the age at which the shift occurs (Winston, 1987). To maintain colony cohesion, workers demonstrate a level of flexibility by advancing, delaying, or reverting behavioral development in response to the needs of the colony such as a loss of foragers from predation or confinement of foragers during inclement weather (Huang and Robinson, 1996; Schulz et al., 1998; Amdam and Omholt, 2003).

The yolk precursor protein, vitellogenin (Vg), and endocrine factor, juvenile hormone (JH), are thought to be physiological regulators underlying behavioral development in honey bee workers (Robinson and Vargo, 1997; Amdam et al., 2003; Nelson et al., 2007). Vitellogenin has multiple roles in honey bee health; it is essential for egg development, has immune response and antioxidant properties, and serves as a nutrient reserve and lipid carrier (Münch et al., 2008). Nurse bees have high Vg in fat body tissues and low JH in hemolymph, whereas at the onset of foraging, older bees have low Vg and high JH (Huang et al., 1994; Amdam and Omholt, 2003). The regulatory interaction between Vg and JH has been demonstrated experimentally in nurse-aged bees using RNA interference, where knockdown of Vg expression resulted in increased JH titer, rapid foraging onset, and a shortened lifespan (Guidugli et al., 2005). Moreover,

topical application of JH has been shown to induce precocious foraging (Robinson et al., 1989).

Nosema ceranae is a spore-forming fungus that infects and reproduces inside epithelial cells of the midgut. During the course of infection, millions of spores are produced and released into the environment when a bee defecates. These spores are a source of infection for other bees. For a colony containing thousands of workers, infection and untimely death of one bee may be an insignificant loss. However, as infection spreads to a large number of bees a cascade effect could ensue to challenge colony survival: as the infected population dies, the queen may be unable to lay enough eggs to replace the loss, and remaining workers may lose flexibility in their behavioral development to respond to task demand.

Studies detailing the pathological effects of *N. ceranae* on individual workers have focused mainly on consequences to mortality (Higes et al., 2007; Forsgren and Fries, 2010). Some studies have examined the effects of infection on expression of immune-related peptides (Antúnez et al., 2009; Chaimanee et al., 2012) or looked at changes in nutritional or energetic states (Mayack and Naug, 2009; Mayack and Naug, 2010). However, our understanding of underlying physiological effects contributing to the disease process is incomplete. Moreover, behavioral studies linking changes seen in the physiology of infected bees are lacking and would help ascertain effects to host and/or colony fitness. We observed bees infected with *N. ceranae* in field colonies to establish the age of foraging onset. We then measured physiological responses, specifically Vg and JH, of infected bees in cages and field colonies. We used qRT-PCR

and a radioimmunoassay to measure relative Vg transcript in tissues and JH titer in hemolymph, respectively. Our objective was to determine whether infection with *N. ceranae* disrupts fundamental physiological processes underlying ancestral reproductive traits that honey bees co-opted as a mechanism to control social behavior (Amdam and Omholt, 2003; Amdam et al., 2003).

2.2 MATERIALS AND METHODS

2.2.1 Ethics Statement

No specific permits were required for the described field studies. Observations were conducted at the University of Minnesota apiary; therefore, no specific permissions were required for this location. The apiary is the property of the University of Minnesota and not privately-owned or protected in any way. Field studies involved observing the European honey bee (*Apis mellifera*), which is neither an endangered or protected species.

2.2.2 Spore Purification

Nosema ceranae spores were procured for infection studies by Percoll purification. Briefly, bees were collected and anesthetized on ice from a colony known to contain individuals infected with *Nosema* sp. as determined by microscopy. The alimentary tract was removed from a sufficient number of bees to ensure a large source of inoculum. The alimentary tracts were pooled in a sterile 50mL conical tube containing 15mL of sterile water and homogenized using a Polytron tissue homogenizer (Brinkmann

Instruments, Westbury, NY). The homogenate was passed through a gauze filter to remove debris and then brought to a volume of 40mL with sterile water. The suspension was centrifuged at 1500g for 10min at 4°C. The supernatant was discarded and the pellet was resuspended by vortexing in 40mL of sterile water, followed by centrifugation at 1500g for 10min at 4°C. This step was repeated 2 times. The supernatant was discarded and the pellet was resuspended by vortexing in 20mL of 100% Percoll (Sigma-Aldrich, St. Louis, MO) adjusted to a pH of 7.0 with 1N HCl. The suspension was centrifuged at 2500g for 30min at 4°C. The Percoll column was carefully removed and discarded, and the pellet was washed once with 5mL of sterile water. The final pellet was resuspended in sterile water and stored at 4°C until use. Confirmation to species was performed by conventional PCR using previously described primers (Chen et al., 2008).

2.2.3 Inoculation of Bees

Frames of capped brood were removed from colonies with no known, apparent symptoms of disease, and incubated overnight at 32°C and 70% relative humidity (RH). The following day, bees that had emerged were collected from frames within 24h after eclosion. Each bee that was to be inoculated was restrained between the thumb and forefinger by laboratory staff and orally administered 10^4 *N. ceranae* spores in 5μL of sucrose solution (50% w:v) or an equal volume of sucrose solution without spores using micropipettes. Bees were held individually in 20mL vials after inoculation for ≥ 20min to ensure the inoculum was ingested. Bees that did not consume the inoculum were discarded.

2.2.4 Mortality Assessment

Bees were inoculated within 24h after eclosion as above and put in 11 x 11 x 11cm cages according to experimental group along with 30 paint-marked un-inoculated bees to provide social interaction and serve as controls that received minimal handling. Cages were placed in a darkened environmental chamber maintained at 28°C and 70% RH. Bees were given *ad libitum* access to 50% sucrose solution and water supplied in gravity feeders, and 5g MegaBee protein patty (Dadant & Sons, Inc., Hamilton, IL) placed in a weigh boat. Cages were rotated daily to minimize position effects, and sucrose, water, and protein patty were replaced weekly. Mortality in each cage was recorded and dead bees were removed daily. Three replicate cages of 30 bees, except cage 3 from the infected group that contained 33 bees, were tested for both infected and control conditions. Mortality results are presented as the daily mean percent survival.

2.2.5 Behavioral Observations

To determine whether infection leads to precocious foraging, bees were inoculated within 24h after eclosion with 10^4 *N. ceranae* spores or sucrose solution as above, except trial 2 where *Nosema*-infected bees received 10^5 spores per bee. Bees were paint-marked to identify treatment and introduced into field colonies containing an egg-laying queen and 5 frames equalized for brood, food, and adults. One hundred bees per treatment were introduced into each of 8 field colonies for trial 1 and 2 and each of 10 field colonies for trial 3. Typically, bees transition from in-hive tasks to foraging behavior at 3 wks of age; therefore, foraging behavior was observed daily from 7 until 21

days of age, which was considered as the precocious window for this behavior. Foraging was observed during a 30-min observation session by blocking colony entrances with 8 mesh hardware cloth and recording all paint-marked bees that returned. Only paint-marked bees with pollen loads in their corbiculae were considered as foragers. Marked foragers were removed upon appearance; however, other marked bees not showing evidence of foraging were not collected and may have been counted on subsequent days. The foraging assay was conducted over 3 trials, twice in 2010 (July and August) and once in 2011 (July).

2.2.6 Sample Collection for Physiological Measures

Bees received either 10^4 *N. ceranae* spores or sucrose solution within 24h after eclosion as above and were placed in cages containing paint-marked bees that were not inoculated to provide social interaction. Un-inoculated bees were members of the same cohort as inoculated bees, but received minimal handling. Eight replicate cages containing 120 inoculated and 80 un-inoculated bees per cage were established per treatment. Dead bees were removed regularly and cages were maintained as above.

At 4, 8, 12, and 16 days of age, 8–10 bees were randomly selected and removed from each cage. Selected bees were grouped by cage into 15mL sterile conical tubes on ice to induce anesthetization. Anesthetized bees were immobilized and $\geq 1\mu L$ of hemolymph was obtained from each bee by piercing the intersegmental membrane between the second and third abdominal tergites and drawing hemolymph up by capillary action using a Drummond Wiretrol (Drummond Scientific Company, Broomall, PA).

Hemolymph was discharged into a 12 x 125mm glass tube with Teflon-lined cap containing 500µL of chilled acetonitrile and stored at −20°C until JH analysis. The sting apparatus and as much of the attached alimentary tract as possible was removed by grasping it with forceps and pulling away from the terminal abdominal segments. The sting apparatus and attached alimentary tissues were placed in 1mL of sterile water in a microcentrifuge tube and kept at 4°C until inspection for spores by microscopy. Next, the head was severed and discarded, and the remaining carcass was put into a microcentrifuge tube and flash frozen in liquid nitrogen and stored at −80°C until Vg analysis.

To compare levels of Vg and JH in infected versus control bees under natural conditions, an additional 80 bees per treatment were introduced into each of 10 field colonies containing a queen and equal amounts of brood, food, and adults. Field colonies were the same as those used in trial 3 for foraging observations. Bees were inoculated with either 10^4 *N. ceranae* spores or sucrose within 24h after eclosion as above and given paint marks to allow identification by treatment. At 7 and 15 days of age, 4 bees per treatment were collected on ice from each colony. Bees were processed for infection status, hemolymph collection, and tissue processing as above. Only those bees from the *Nosema*-infected group that showed presence of spores in gut homogenates by microscopy were used for Vg and JH analysis (Table 2.1). Absence of spores in gut homogenates of sucrose controls was also confirmed by microscopy before further analysis for Vg and JH.

2.2.7 Radioimmunoassay to Quantify Juvenile Hormone

A chiral-specific radioimmunoassay was used to quantify JH titer in hemolymph collected from individual bees (Huang et al., 1994). One mL of hexane and 0.5mL of 0.9% NaCl were added to samples and centrifuged at 2000g for 10min. The JH-containing hexane phase was transferred to a new tube. This process was repeated to increase extraction efficiency. Hexane was evaporated using a Savant drying system (Thermo Fischer Scientific, Inc., Pittsburgh, PA). Dried samples were chilled on ice and washed with 100µL of methanol by vortexing. A 10µL aliquot was transferred to a new tube and the methanol was evaporated. Next, 200µL of premixed JH antiserum (1:28,000) and 10,000 DPM [10-3H(N)]-JH (Perkin Elmer, Inc., Waltham, MA) were added to the tubes. The tubes were incubated for 2h, when radiolabeled JH and JH in the hemolymph competed for binding to the antibody. The reaction was slowed by chilling the tubes in ice water for 10min. To absorb unbound JH, samples were incubated in 500µL of dextran-coated charcoal for 2.5min. The tubes were centrifuged at 2000g for 3min to pellet the charcoal. The supernatant containing bound JH was decanted into 20mL scintillation vials and 5mL of Ultima Gold scintillation cocktail (Perkin Elmer, Inc., Waltham, MA) was added. Radioactivity was quantified and values were evaluated against standards fitted by non-linear regression. Samples were run in duplicate. All glassware was baked at 500°C for 3.5h prior to use to minimize JH adsorption, and all reagents were HPLC grade. The JH antiserum was provided by David Borst (Central Florida University).

2.2.8 Quantitative-Reverse Transcription Polymerase Chain Reaction for Relative Vitellogenin Gene Expression

Total RNA was extracted using an RNAqueous kit (Ambion, Austin, TX) for cage studies or TRIzol method for field studies. DNA contamination was degraded with 10U DNAse I (Ambion, Austin, TX) at 37°C for 1h followed by 75°C for 10min. cDNA was synthesized by incubating 8μL total RNA with mastermix containing 50U Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), 20U RNaseOUT ribonuclease inhibitor (Invitrogen, Carlsbad, CA), 2μM dNTPs, 2nM poly(dT)$_{18}$, and 0.1nM poly(dT)$_{12–18}$ at 42°C for 50min followed by 15min at 70°C as described previously (Evans, 2006).

Transcript was assayed by qPCR. Two μL of cDNA served as template in a reaction containing 1U Taq polymerase, 1X buffer, 1mM dNTPs, 2mM $MgCl_2$, 0.2μM of forward (5'-agttccgaccgacgacga-3') and reverse (5'-ttccctcccacggagtcc-3') primers (Genbank Entry: NP_001011578) specific for amplification of the honey bee Vg precursor gene, and 1X SYBR-Green (Applied Biosystems, Foster City, CA) for a total volume of 25μL. Reactions were run using a thermal profile consisting of 95°C for 30sec, followed by 40 cycles of a 4-step protocol consisting of 95°C for 20sec, 60°C for 30sec, 72°C for 1min, and 78°C for 20sec. Fluorescence measurements were taken repeatedly during the 78°C step. A 3-step melt curve was performed to ensure proper amplification of the target gene. The reference gene, β-actin, was amplified with forward (5'-ttgtatgccaacactgtccttt-3') and reverse (5'-tggcgcgatgatcttaattt-3') primers (Genbank Entry: NC_007076) to normalize data according to RNA amount in each sample. All

primers were published previously and validated experimentally (Evans, 2006; Corona et al., 2007).

2.2.9 Statistical Analyses

Data were analyzed using R version 2.13.0 (2011-04-13) statistical software with car package. Mortality data were analyzed by Cox proportional-hazards regression model with splines package. Differences in the number of returning marked pollen foragers were analyzed by Chi-squared test. Differences in Vg transcript and JH titer were analyzed by mixed model ANOVA with nlme package. Infection status and age of bees were modeled as fixed effects and cage or colony of origin were modeled as random effects. JH data were natural logarithm transformed to meet assumptions of a normal distribution for residuals and to stabilize variances. All statistical tests used $\alpha=0.05$ to establish significance. Data are reported as means ± SE.

2.3 RESULTS

2.3.1 Infected Bees Have a Shorter Lifespan Compared to Controls

Nosema ceranae had a negative effect on lifespan ($z=3.97$, n=363, $p<0.0001$; Figure 2.1). Infection increased the daily mortality by a factor of 1.99. Survivorship was above 75% and indistinguishable between infected and control bees from 1 through 14 days of age. However, the number of surviving infected bees dropped below 50% by 16 days of age, whereas controls did not reach this level until 25 days of age. Therefore, most control bees lived, on average, 9 days longer than infected bees.

To account for mortality associated with handling during inoculation, an additional 30 bees that received minimal handling were put into each cage (i.e., un-inoculated cagemates). Daily mortality was significantly greater for bees that received handling during inoculation compared to un-inoculated cagemates (z=4.46, n=363, p<0.0001). Handling increased the daily mortality by a factor of 2.93. Un-inoculated cagemates for both infected and control conditions had survivorship levels ≥ 60% throughout the experiment. There was no difference in the daily mortality rate between un-inoculated cagemates in *Nosema*-infected or control cages (z=1.59, n=363, p=0.11).

2.3.2 Infection with *Nosema ceranae* Results in Premature Foraging

A greater number of infected marked bees compared to non-infected marked bees were recorded at entrances to field colonies starting at 7 through 16 days of age (Figure 2.2A). There were ≥ 30 marked bees recorded on 13 of 15 days for the infected group compared to only 2 of 15 days for controls. In all, there were 206, 79, and 261 marked bees (total=546) recorded for the infected group compared to 130, 64, and 151 marked bees (total=345) for sucrose controls for trials 1, 2, and 3, respectively (Table 2.2).

It is unknown whether all returning marked bees were engaged in foraging; therefore, only bees carrying loads of pollen were recorded as foragers for the following analysis. The distribution shows that there were ≥ 10 foragers recorded on 8 of 15 days for the infected group compared to only 1 of 15 days for controls (Figure 2.2B). Foragers from the infected group outnumbered controls at nearly 2 to 1 for each trial and the 3 trials combined. In all, there were 73, 29, and 51 foragers (total=153) for the infected

group compared to 38, 15, and 23 foragers (total=76) for sucrose controls for trials 1, 2, and 3, respectively. Chi-squared analysis showed significantly more foragers were collected from the infected group for trial 1 (χ^2=11.19, p=0.0008), trial 2 (χ^2=3.95, p=0.047), trial 3 (χ^2=10.23, p=0.0014), and the 3 trials combined (χ^2=26.38, $p<0.0001$) compared to controls (Table 2.2). For trial 2, all colonies were inspected on days 4, 12, and 21 after the introduction of paint-marked bees and it was noted that there were relatively equal numbers of bees from both treatment groups present. Therefore, we assume that uninfected control bees did not die at a faster rate than *Nosema*-infected bees during the field trials, which is in agreement with our mortality assessment.

2.3.3 Infection Has a Negative Effect on Vitellogenin Transcript Over Time for Bees in Cages, but Not Field Conditions

There was a significant interaction between infection status and age of bees on relative Vg transcript in caged bees ($F_{3,191}$=3.04; p=0.030; Figure 2.3A). Control bees exhibited a normal Vg profile for adult worker ontogeny (Amdam and Omholt, 2003): relative levels peaked at 8 days of age and then decreased linearly through 16 days of age. *Nosema*-infected bees showed the opposite trend: relative levels were low at 4 and 8 days of age with a 1.9-fold and 3.5-fold decrease in Vg, respectively, compared to control bees. After day 8, Vg levels increased linearly for infected bees. At 16 days of age, infected bees had a 3.3-fold increase in relative Vg compared to age-matched controls. The main effects of infection status or age of bees on relative Vg transcript were not significant ($p>0.05$).

Levels of relative Vg for infected bees from field colonies were not different from controls ($F_{1,80}$=0.22; p=0.64; Figure 2.4A). However, there was an effect of age of bees on Vg transcript ($F_{1,80}$=4.91; p=0.030). As would be expected, levels of Vg decreased with the age of the worker. Transcript abundance was comparable between controls (-1.32 Vg units) and infected bees (-1.09 Vg units) at 7 days of age but decreased by 1.4-fold and 2.2-fold by 15 days of age, respectively. There was no interaction between infection status and age of bees on relative Vg transcript ($F_{1,80}$=0.80; p=0.37).

2.3.4 Change in Juvenile Hormone Titer in Hemolymph of Bees in Cages and Field Conditions

Infection did not show an effect on JH titer of bees in cages ($F_{1,14}$=0.47, p=0.51). However, infected bees had a 1.4-fold increase in JH titer, on average, compared to controls throughout the time course of the study (Figure 2.3B). The trend for bees from the infected group revealed a spike in JH titer in 8-day-old bees (1580.18ng/mL), which was an increase of 2.1-fold compared to the average level for 8-day-old control bees (750.09ng/mL). Juvenile hormone titer showed a linear decline from 8 days of age through 16 days of age for the infected group. In contrast, JH titer remained static throughout the time course for control bees. Bees older than 16 days of age were not collected; therefore, it is unknown whether JH titer for controls would have been elevated as predicted for workers $\geq$ 3 wks old (Amdam and Omholt, 2003). There was a significant effect of age on JH titer ($F_{3,212}$=8.22, p<0.0001). Eight-day-old bees in cages had at least a 2-fold increase in average JH titer compared to bees at the other time points.

There was no interaction between infection status and age of bees in cages on JH titer ($F_{3,212}$=2.00, p=0.11).

Infected bees in field colonies had nearly a 3-fold increase in average JH titer compared to controls (Figure 2.4B); this difference was marginally significant ($F_{1,119}$=3.81; p=0.053). Juvenile hormone was low for both infected and control bees at 7 days of age, but infected bees had a 2.2-fold increase in JH titer in hemolymph (34.10ng/mL) compared to age-matched controls (15.67ng/mL). Juvenile hormone titer for infected bees rose sharply at 15 days of age and was 2.9-fold greater than age-matched controls. There was a significant difference in JH titer among bees of different ages in field colonies ($F_{1,119}$=100.21, p<0.0001). As would be expected for aging workers, 15-day-old bees averaged a 12.7-fold increase in JH titer compared to 7-day-old bees. The interaction between infection status and age of bees on JH titer was not significant ($F_{1,119}$=0.95; p=0.33).

2.4 DISCUSSION

Nosema ceranae is a common and widespread pathogen of honey bees in the U.S. and worldwide (Botías et al., 2012; Medici et al., 2012). In this report, we demonstrate that *N. ceranae* can trigger premature foraging and shorten the lifespan of infected workers. We also show that Vg transcript was low, while JH titer spiked, in infected nurse-aged bees. This pattern is atypical and the reverse of what would be expected for healthy, non-infected bees. In honey bees, the transition from nursing inside the brood nest to foraging outside the hive is thought to be under regulatory control through the

interaction of the yolk precursor protein, Vg, and endocrine factor, JH (Huang and Robinson, 1996). This interaction serves as an underlying physiological mechanism that is essential for allowing plasticity in behavioral responses by workers to dynamic colony and ecological environments (Amdam et al., 2011). In *Nosema*-infected bees, this regulatory framework is apparently disrupted.

In our experiment examining the effects of infection on lifespan, we found that controls lived, on average, 9 days longer than infected bees. During summer months, a typical worker bee lives 3–6 wks (Sakagami and Fukuda, 1968), with the final 2–3 wks devoted to foraging (Winston, 1987). Assuming there is a similar mortality rate due to infection in field colonies, decreasing worker lifespan by 9 days, especially during the foraging phase, can be significant. Any nectar and/or pollen that would have been collected by the infected bee during these 9 days of life would be lost. Moreover, if the infected bee was part of the colony's foraging force that performs scouting behavior for novel floral patches (Seeley, 1983), the loss could be compounded as information detailing patch location would not be communicated.

Parasites can accelerate or delay host development as a strategy that exploits host resources and maximizes transmission (Hurd et al., 2001). Lengthening the immature development of insect hosts has been demonstrated with other *Nosema* spp. (Solter et al., 1990; Blaser and Schmid-Hempel, 2005). In contrast, our findings suggest that *N. ceranae* accelerates honey bee behavioral development. We observed a greater number of infected bees outside the nest and engaged in foraging compared to controls. Evidence from other studies has shown anatomical or behavioral changes that are congruent with

accelerated development resulting from infection with a similar pathogen, *N. apis*. Bees infected with *N. apis* were found to have reduced size and function of hypopharyngeal glands, which secrete a protein-rich medium that nurses feed to larvae (Wang and Moeller, 1969; Liu, 1990). Furthermore, bees infected with *N. apis* have been shown to have a reduced lifespan, and were more likely to be found at the nest entrance engaged in guarding, or outside the nest performing orientation flights or foraging behavior (Hassanein, 1953; Wang and Moeller, 1970; Lin et al., 2001; Woyciechowski and Moroń, 2009).

In addition to changes in the rate of foraging onset, our data show that infection can disrupt the underlying physiology that regulates the age-specific expression of behaviors in workers. The trend in levels of Vg and JH showed an inverse relationship: when relative Vg transcript was at its lowest, JH titer was at its highest and vice versa for infected bees in cages. This finding is in agreement with research showing how Vg and JH act through positive feedback loops to negatively affect the synthesis of each other to regulate the age-specific expression of nursing or foraging behaviors (Amdam et al., 2006). In our cage studies, infected nurse-aged bees had an elevated JH titer that peaked at 8 days of age. This finding is corroborated by a recent study that showed higher levels of JH in bees infected with *N. ceranae* compared to bees infected with *N. apis* or uninfected controls (Ares et al., 2012). We also saw an interaction between infection and age of bees on relative Vg transcript; however, the main effect of *N. ceranae* infection on Vg levels was not significant for bees in cages or field colonies. This finding is similar to a recent report that showed Vg transcript to be unchanged following infection with *N.*

ceranae (Antúnez et al., 2009). Our finding may suggest the strict inhibitory relationship that exists between Vg and JH may be an example of how a pathogen can decouple normally correlated behavioral syndromes by disrupting underlying physiology (Barber and Dingemanse, 2010).

We saw considerable variability in the levels of relative Vg transcript and JH titer in our cage experiments. In fact, if cage of origin was modeled as a fixed effect in the ANOVA, a significant effect of cage could be observed. Modeling cages as a fixed effect is not statistically valid, but is mentioned here to illustrate a shortcoming of cage studies. The variability in bees among cages may be due to an absence of a queen, brood, or other environmental cues that workers typically encounter in the natural hive setting. It has been shown previously that contact pheromone produced by the mandibular glands of the queen suppresses JH biosynthesis in workers, resulting in low JH titer and a delay in foraging onset (Kaatz et al., 1992; Pankiw et al., 1998). Moreover, in the absence of primer pheromone produced by larvae, nurse-aged workers have lower Vg in fat body stores (Smedal, et al., 2009). Interaction among workers may also affect behavioral development. In groups of bees lacking older individuals, 5−10% will display accelerated development (Page et al., 1992). If bees expressing a precocious phenotype, independent of treatment, were present in our cages they may have acted on other bees to suppress JH synthesis and behavioral maturation (Huang and Robinson, 1992). Placing bees in cages without the modulatory effects of queen and brood pheromones may not be the best way to study basic factors regulating behavioral development. Data from the field colonies, containing a queen and brood, better represent the effects of *N. ceranae* infection on Vg

and JH.

The positive trend in relative Vg transcript seen in infected bees in cages over time may be due to variability among cages or it could be a host response induced by parasitism. Vitellogenin has life-extending properties, protecting workers from oxidative stress (Seehuus et al., 2006). *Nosema ceranae*, like all microsporidia, lack mitochondria or have relict mitochondria-like structures with limited ATP-producing capability; therefore, it is characteristic of these fungi to aggregate near host mitochondria so as to exploit endogenous ATP (Tsaousis et al., 2008). Infection can result in the production of millions of spores in as little as 1−2 wks (Fries, 2010). An infected bee may respond to this high rate of fungal proliferation by increasing levels of Vg to counter toxic reactive oxygen species produced from the elevated respiration rates in infected host midgut cells (Dussaubat et al., 2012).

High annual mortality of honey bee colonies in the U.S. and worldwide due to persistent exposure to mite pests, poor nutrition, pesticides, and pathogens has created a need for alternative approaches to evaluate bee health. Diagnostic assays that establish the presence/absence of disease may provide limited information about the overall health of a colony (Aronstein et al., 2011; Traver et al., 2012). Currently, the standard method to diagnosis *Nosema* spp. infection is to estimate the average number of spores per bee by microscopy in a composite sample of 50–100 bees. Based on research presented here, it may be more informative to estimate the proportion of infected bees in a colony. If few bees are infected, the queen may be able to lay enough eggs to replace the loss, and uninfected bees may retain flexibility to revert to nurse bee physiology and behaviors as

needed. On the other hand, if a high proportion of bees are infected, this flexibility in behavioral development may deteriorate, leading to colony decline. As it was not time-efficient to determine the proportion of infected bees, it may prove beneficial in future research to gauge colony health and productivity by examining genetic, endocrine, or other biochemical markers. Specifically, sampling for Vg or JH may provide a "medical record" of a colony's ability to respond to infection by pathogens or other stressors. For example, poor nutrition could correlate with overall low levels of Vg in nurse-aged bees, as well as provide an indication of the antioxidant capacity of workers (Seehuus et al., 2006; Nelson et al., 2007).

In a honey bee colony, impairment of worker health through infection, as we have shown here, can result in alteration of behavioral performance and premature death. How the progression of an infectious disease impairs task performance and productivity of not only infected, but healthy workers, should be a goal of research exploring further the challenges to honey bee colony health.

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2.6 FIGURE LEGENDS

Figure 2.1 ***Nosema ceranae*** **shortens honey bee lifespan.**

Mean survival (%) of caged bees fed 10^4 *N. ceranae* spores (*Nosema*-infected) or sucrose solution (Sucrose control) at day 0 (i.e., within 24h after adult emergence). Age-matched cagemates that were not inoculated received minimal handling and were placed into infected (*Nosema* cagemate) or control (Sucrose cagemate) cages to simulate social interaction. n=30 bees per treatment/cage (except cage 3 from the infected group that had 33 bees), 3 replicate cages per treatment. Infection had a significant negative effect on lifespan (z=3.97, n=363, p<0.0001).

Figure 2.2 Honey bees infected with *Nosema ceranae* forage prematurely.

A. Distribution of returning marked bees during a daily 30-min observation period from 7 through 21 days of age after eclosion. Data are summarized over 3 independent trials.

B. Distribution of returning marked pollen foragers collected daily during a 30-minute observation period from 7 through 21 days of age after eclosion. Data are summarized over 3 independent trials. Significantly more marked pollen foragers were collected from the *Nosema*-infected group compared to controls for the 3 trials combined (χ^2=26.38, p<0.0001).

Figure 2.3 ***Nosema ceranae*** **disrupts honey bee behavioral physiology.**

A. Vitellogenin transcript normalized to the reference gene, β-actin, (mean ± SE) for caged bees fed *N.* ceranae spores (*Nosema*-infected) or sucrose solution (Sucrose control) at day 0 (i.e., within 24h after adult emergence). n ≥ 17 per treatment per day. There was a significant interaction between infection status and age of bees on relative Vg transcript ($F_{3,191}$=3.04; p=0.030).

B. JH titer (mean ± SE) for caged bees fed *N.* ceranae spores (*Nosema*-infected) or sucrose solution (Sucrose control) at day 0 (i.e., within 24h after adult emergence). n ≥ 20 per treatment per day. The interaction effect of infection status on JH titer over time was not significant ($F_{3,212}$=2.00, p=0.11).

Figure 2.4 Honey bees infected with ***Nosema ceranae*** **have elevated JH titer under field conditions.**

A. Vitellogenin transcript normalized to the reference gene, β-actin, (mean ± SE) for bees fed *N.* ceranae spores (*Nosema*-infected) or sucrose solution (Sucrose control) at day 0 (i.e., within 24h after adult emergence) and placed into field colonies. n ≥ 22 per treatment per day. There was no significant interaction effect of *N. ceranae* infection on relative Vg transcript over time ($F_{1,80}$=0.80; p=0.37).

B. JH titer (mean ± SE) for bees fed *N. ceranae* spores (*Nosema*-infected) or sucrose solution (Sucrose control) at day 0 (i.e., within 24h after adult emergence) and placed into field colonies. n ≥ 31 per treatment per day. The effect of infection status on JH titer was marginally significant ($F_{1,119}$=3.81, p=0.053).

2.7 TABLE LEGENDS

Table 2.1 *Nosema ceranae* spore loads.

Mean number of spores per bee ($x10^6$) at different ages after eclosion for bees used for Vg and JH analysis from cage and field studies.

Table 2.2 Experimental bees observed at entrances to field colonies.

The number of returning marked bees, and of those, the number of returning pollen foragers observed at entrances to field colonies during daily 30-min observations for 3 independent trials.

Table 2.2 Footnote. Bees were introduced into field colonies after receiving either *N. ceranae* spores (Infected) or sucrose solution (Control) within 24h after eclosion. Marked bees were counted once during the daily observation period. Marked foragers were collected without replacement. Counts between rows within category followed by different letters are significant for the marked pollen forager data (Chi-squared test, $p<0.05$).

Table 2.1

Source of infected bees	Mean spores per bee ± SE (x 10^6)			
Cage studies (days of age from eclosion)	4	8	12	16
	0.1 ± 0.0	6.1 ± 0.8	29.7 ± 2.3	27.4 ± 4.3
Field studies (days of age from eclosion)	7	15		
	0.5 ± 0.1	17.4 ± 3.4		

Table 2.2

	Marked returning bees		Marked pollen foragers	
Trial	Control	Inoculated	Control	Inoculated
1. July, 2010	130	206	38[a]	73[b]
2. August, 2010	64	79	15[a]	29[b]
3. July, 2011	151	261	23[a]	51[b]
Total	345	546	76[a]	153[b]

Figure 2.1

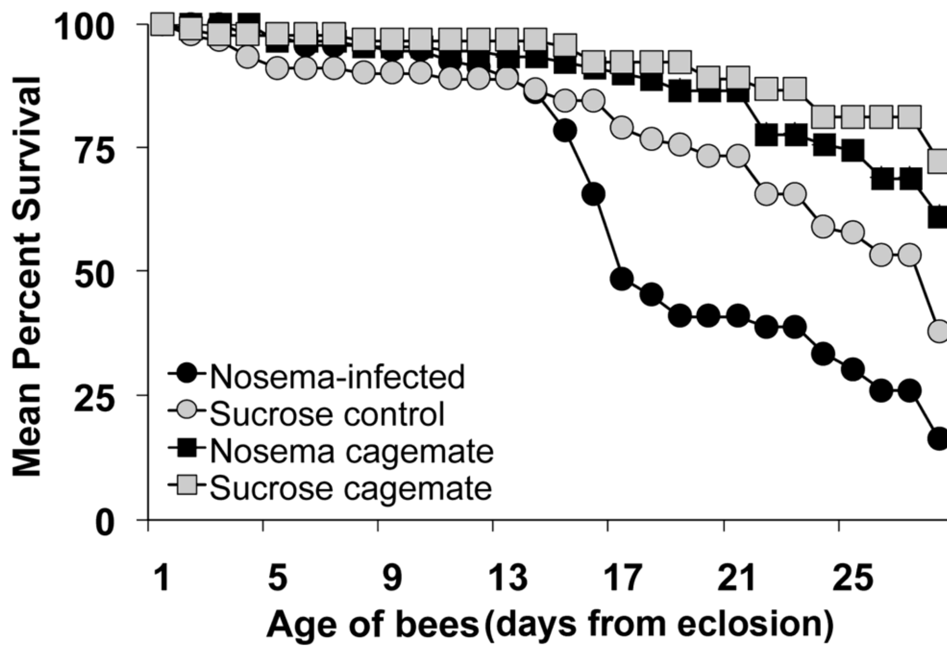


Figure 2.2

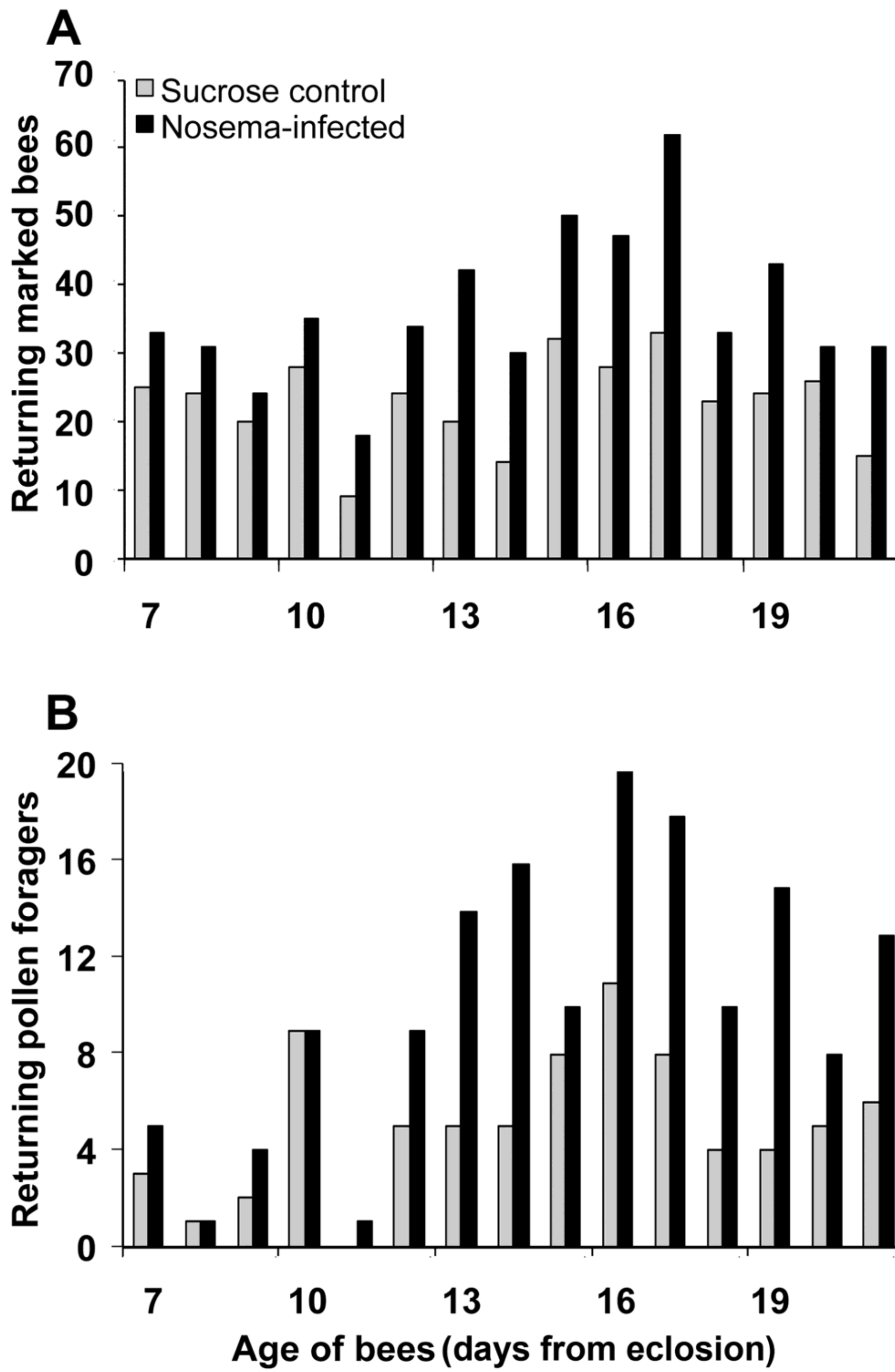


Figure 2.3

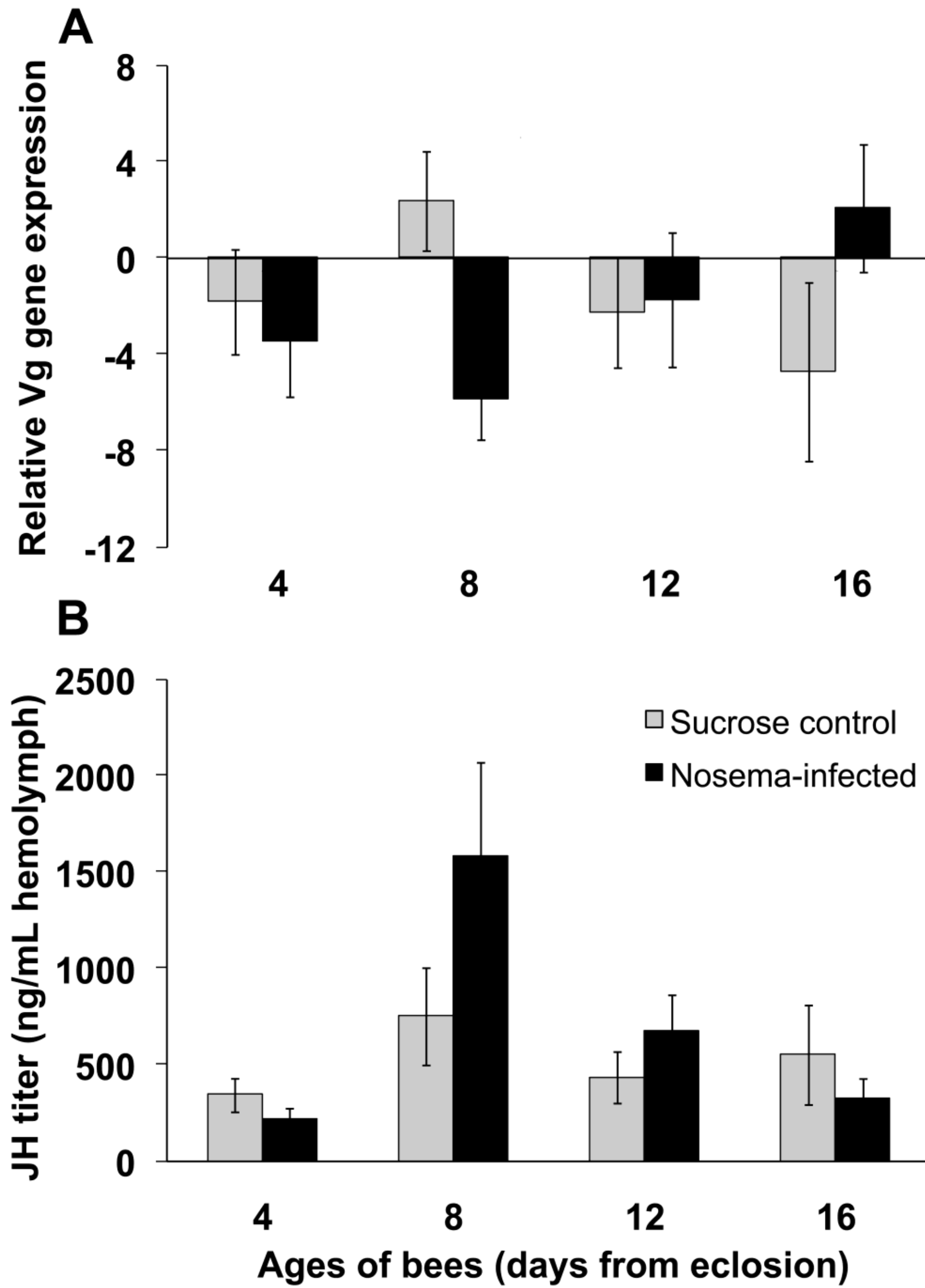
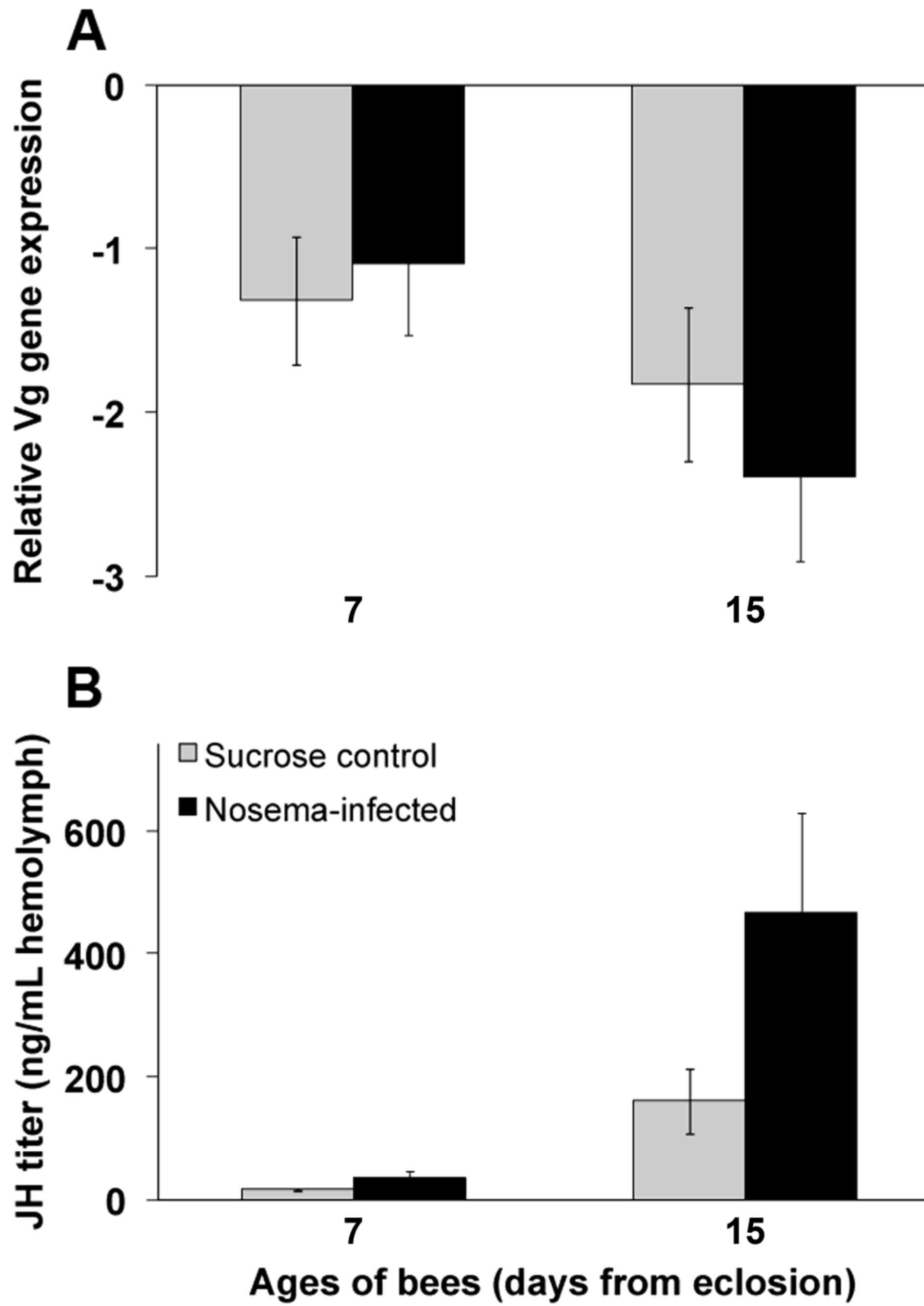


Figure 2.4



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CHAPTER 3

A Cell Line Resource Derived from Honey Bee (*Apis mellifera*) Embryonic Tissues

Michael J. Goblirsch, Marla S. Spivak, and Timothy J. Kurtti

SUMMARY

A major hindrance to the study of honey bee pathogens or the effects of pesticides and nutritional deficiencies is the lack of controlled *in vitro* culture systems comprised of honey bee cells. Such systems are important to determine the impact of these stress factors on the developmental and cell biology of honey bees. We have developed a method incorporating established insect cell culture techniques that supports sustained growth of honey bee cells *in vitro*. We used honey bee eggs mid to late in their embryogenesis to establish primary cultures, as these eggs contain cells that are progressively dividing. Primary cultures were initiated in modified Leibovitz's L15 medium and incubated at 32ºC. Serial transfer of material from several primary cultures was maintained and has led to the isolation of young cell lines. A cell line (AmE-711) has been established that is composed mainly of fibroblast-type cells that form an adherent monolayer. Most cells in the line are diploid (2n = 32) and have the *Apis mellifera* karyotype as revealed by Giemsa stain. The partial sequence for the mitochondrial-encoded cytochrome c oxidase subunit I (*Cox 1*) gene in the cell line is

identical to those from honey bee tissues and a consensus sequence for *A. mellifera*. The population doubling time is approximately 4 days. Importantly, the cell line is continuously subcultured every 10 – 14 days when split at a 1:3 ratio and is cryopreserved in liquid nitrogen. The cell culture system we have developed has potential application for studies aimed at honey bee development, genetics, pathogenesis, transgenesis, and toxicology.

KEYWORDS: bioassay, honey bee cell line, Hymenoptera, *in vitro*, intracellular pathogens

3.1 INTRODUCTION

Established insect cell lines and primary culture methods are numerous and frequently used for diverse research interests such as understanding the transmission and pathogenesis of disease causing microbes. For example, one motivation for the establishment of the first continuous cell line from an insect, which was isolated from ovarian tissues of the emperor moth, *Antheraea eucalypti* (Grace, 1962), was to propagate viruses axenically for the purpose of developing control measures for agricultural and forest pests (Arif and Pavlik, 2013). In the fifty years since this line was established, there have been over 500 continuous (i.e., immortalized) insect lines that have been developed, the vast majority (~80%) of which are dipteran or lepidopteran (Lynn, 2003). Moreover, advances in baculovirus expression systems used for recombinant protein production has made insect cell lines effective substrates for commercial and research applications (Sokolenko et al., 2012). Underrepresented, however, in the catalogue of insect lines are those derived from the order Hymenoptera (i.e., bees, wasps, and ants). Continuous cell lines from the hymenopteran lineage have been reported from only 6 species, including the pine sawfly *Neodiprion lecontei* (Sohi and Ennis, 1981) and the parasitoid wasps *Trichogramma pretiosum* (Lynn and Hung, 1986), *T. confusum*, *T. exiguum* (Lynn and Hung, 1991), *Mormoniella vitripennis* (Wahrman and Zhu, 1993), and *Hyposoter didymator* (Rocher et al., 2004).

Despite the economic and ecological importance of honey bees as pollinators of many cultivated and native plants, there is a surprising lack in availability of controlled *in vitro* systems, especially given that several threats to honey bee health are obligate

intracellular pathogens that are abundant and widespread across colonies (Runckel et al., 2011). A limited number of studies have documented attempts at culturing honey bee embryonic cells (Giauffret et al., 1967; Bergem et al., 2006; Chan et al., 2010) and larval and pupal cells (Stanley 1968; Giauffret, 1971; Kreissl and Bicker 1992; Gascuel et al., 1994; Gisselmann et al., 2003; Hunter, 2010). Short-term cultures (≤ 4 weeks) have been demonstrated with neurons dissociated from honey bee pupal antennal lobes (Kriessl and Bicker, 1992; Barbara et al., 2008). Long-term cultures have been initiated using pre-gastrula embryos (36 – 40 h after oviposition) that remained mitotically active for 3 months (Bergem et al., 2006). The limited duration of cell survival and absence of lines gave rise to the tenet that honey bee cells were refractory to continuous *in vitro* growth. Difficulty in adapting honey bees cells to *in vitro* conditions may be the result of selecting donor tissues whose age or origin is unsupportive of long-term growth. Recently, gene transfer technology has been used to evade these limitations, where insertion of the green fluorescent protein gene by lentivirus transduction (Chan et al., 2010) and the human *c-myc* proto-oncogene by lipofection (Kitagishi et al., 2011) into embryonic honey bee cells was performed to demonstrate if activation of the transgenes was feasible and could promote long-term proliferation and survival. The latter method resulted in the establishment of a cell line that remained viable during an 8-month follow-up period; however, subsequent evidence to support claims of a continuous line has not been forthcoming. Our objective was to use standard insect cell culture techniques without the use of retroviruses or transfection of human oncogenes to isolate honey bee cell lines. Herein, we report the isolation and characterization of a cell line, which we

have named AmE-711, from primary cell cultures derived from fragmented honeybee embryonic tissues. At the time this manuscript was submitted, the AmE-711 line has been passaged 18 times and remains in culture.

3.2 MATERIALS AND METHODS

3.2.1 Ethics Statement

No specific permits were required for the described field studies. Observations were conducted at the University of Minnesota apiary; therefore, no specific permissions were required for this location. The apiary is the property of the University of Minnesota and not privately-owned or protected in any way. Field studies involved observing the European honey bee (*Apis mellifera* L.), which is neither an endangered or protected species. The honey bee cell line reported below is an original description of a line that was developed by the authors at the University of Minnesota from honey bee embryos. The cell line was isolated from an insect; no institutional review board or ethics committee approval was needed.

3.2.2 Mass Collection of Honey Bee Eggs

A honey bee colony was visually inspected for the absence of signs of brood diseases (i.e., American foulbrood, European foulbrood, and Chalkbrood) before it was selected for the collection of eggs. An empty frame of drawn-out comb was placed in the center of a selected brood box within the colony for 24 h to allow the worker bees to clean the comb cells in preparation for the queen to lay eggs. After 24 h, the queen from

the colony was restricted to one side of the empty frame for 24 h using a metal cage that covered the entire side of the frame. Queen-attending nurse bees were small enough to pass freely between the wire mesh of the cage. After being restricted for 24 h, the cage was removed and the queen released. The frame was examined for the presence of eggs and subsequently returned to the colony for incubation. Between 48 and 72 h after oviposition (i.e., after the release of the queen), the frame was removed from the colony and brought to the laboratory.

In the laboratory, the frame was struck at an acute angle several times against a tabletop covered with clean packing paper similar to the method of Evans et al. (2010). The side of the frame that contained the eggs faced down as it was struck, which allowed the eggs to fall onto the paper. Macroscopic debris, such as wax flakes, was removed and the eggs deposited into a sterile 15 mL conical tube. This method allowed for efficient collection of several dozen to hundreds of eggs per frame.

3.2.3 Culture Medium and Supplements

The basal medium was Leibovitz's L15 medium (Life Technologies, Grand Island, NY) (Leibovitz, 1963) modified according to Munderloh and Kurtti (1989). Briefly, the modifications included the addition of glucose, organic acids, vitamins, trace minerals and amino acids to the L15 base. This basal medium has been used previously to culture insect and tick cell lines (Munderloh and Kurtti, 1989; Sagers et al., 1996; Munderloh et al., 1999). The complete medium (HB-1) for honey bee cells was prepared by mixing 3 parts of the basal medium with one part of cell culture grade distilled water

and supplementing this with fetal bovine serum (FBS; 10%), tryptose phosphate broth (5%; BD Biosciences, Franklin Lakes, NJ), bovine lipoprotein-cholesterol concentrate (0.1%; MP Biomedical, Aurora, OH), HEPES (10 mM), and $NaHCO_3$ (0.9 mM). The pH was adjusted to 7.0 – 7.2 with 1 N NaOH.

3.2.4 Primary Culture

Honey bee eggs were surface sterilized with sequential washes of 0.525% sodium hypochlorite containing Tween 80, 0.5% benzalkonium chloride, and 70% ethanol followed by several rinses with sterile water. The water was removed and ≤ 200 µL of HB-1 medium was added to the tube. The eggs were homogenized in medium by gently pressing a sterile 1.5 mL pestle (Kimble Chase, Vineland, NJ) against the eggs to disrupt the chorion, releasing the inner embryonic fragments into the medium. The homogenate was then transferred to a Nunclon flat-sided culture tube with an effective growth area of 5.5 cm^2 and non-ventilating screw cap (Thermo Fisher Scientific, Inc., Waltham, MA) containing 500 µL medium with the addition of 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin (Life Technologies, Grand Island, NY). The sides of the conical tube were washed 1 or 2 times with ≤ 500 µL medium to collect any residual embryonic fragments and then transferred to the culture tube. The screw cap was then tightened and the culture containing ≤ 2.0 mL total volume of medium was moved to a non-humidified incubator set at 32°C. Culture medium was replaced 1 or 2 times a week or when a sharp drop in pH was noted by a change in phenol red indicator.

3.2.5 Subculture and Preservation of the Cell Line

Transfer or passage was initiated when the monolayer of a primary culture (or subculture) was ≥ 80% confluent. At the time of transfer, the medium was removed and the cell layer was trypsinized (0.25% trypsin-EDTA; Life Technologies, Grand Island, NY) for several minutes at 32°C to dissociate the cell layer from the flask substrate. Trypsinization was stopped with fresh medium and the cell suspension was transferred at a split ratio of 1:2 or 1:3. However, a ratio as low as 1:10 allowed for continual expansion of the AmE-711 line but extended the interval until the next subculture.

Starting at the 3rd transfer, cultures were selected for cryopreservation to assure retention of characteristics and a banked source of the subsequent line. Once cultures were ≥ 80% confluent, cells were dissociated as above. The cells were then pelleted by centrifugation at 400 g for 5 min at 4°C. The cells were resuspended in chilled freezing medium comprised of L15 base with 20% FBS and 10% dimethyl sulfoxide. The suspension was aliquoted into cryotubes and frozen at a rate of $^{-}$1°C per min using a Handi-freeze tray (Union Carbide, Houston, TX) or CoolCell alcohol-free freezing container (BioCision, LLC, Mill Valley, CA). Cryotubes were transferred to liquid nitrogen for long-term storage. Cells stored in liquid nitrogen were regenerated successfully. The AmE-711 line is currently cultured in HB-1 medium without antibiotics and has been screened and found negative for *Mycoplasma* sp. using a LookOut detection kit (Sigma-Aldrich, Co., St. Louis, MO) (Curt Nelson unpublished data) and by light microscopy of cells stained with Giemsa.

3.2.6 Conventional Polymerase Chain Reaction

We used PCR sequencing of amplicons to confirm the species identity of the AmE-711 line. Genomic DNA was extracted from the whole abdomen of a 20-day old adult honey bee, honey bee embryos, a larva of the Common Eastern bumblebee, *Bombus impatiens*, AmE-711 cells, and HL-60 human promyelocytic leukemia cells using a DNAeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. DNA yield was determined by spectrophotometry prior to PCR.

Each PCR reaction contained ~14 – 18 ng of template, 0.2 µM of previously published forward (5'-ttaagatccccaggatcatg-3') and reverse (3'-gttatccacgtcataaacgt-5') primers (Hall and Smith, 1991) specific for the amplification of *A. mellifera* mitochondrial-encoded cytochrome c oxidase subunit I gene (*Cox 1*), puReTaq Ready-to-Go beads (GE Healthcare, Piscataway, NJ), and 22 µL of nuclease free water. Reactions were run using a thermal profile consisting of an initial step of 94°C for 5 min, followed by 40 cycles of a 3-step protocol consisting of 94°C for 20 sec, 40°C for 1 min, and 72°C for 1 min, and a final step of 72°C for 5 min. Amplified products were resolved on a 1% agarose gel by electrophoresis, stained with a 1:5,000 dilution of Gel Green fluorescent nucleic acid stain (Biotium, Inc., Hayward, CA), and visualized with UV light.

Honey bee embryo and AmE-711 amplicons for *Cox 1* were purified by Diffinity Rapid Tip 1 purification tips (MidSci, St. Louis, MO) according to the manufacturer's protocol. Purified products were checked by spectrophotometry and ~60 ng of template and 10 pM of forward-only or reverse-only primers used for PCR as above were diluted with nuclease free water and submitted to the University of Minnesota Biomedical

Genomics Center for Sanger Classic automated sequencing. Sequences were manually aligned to remove primer artifacts and queried against the BLAST database. The partial *Cox 1* sequence for the AmE-711 line was deposited in Genbank (Entry: KC921208).

3.2.7 Karyology

AmE-711 cells in log phase growth were exposed to 12.5 µM colchicine in fresh medium and incubated at 32°C for 24 h. After 24 h, the medium was removed and the cells were dissociated with 0.25% trypsin for < 10 min at 32°C. Dislodged cells were pelleted by centrifugation at 270 g for 8 min and then resuspended in 4 mL of 75 mM KCl and incubated at 34°C for 60 min. The cells were pelleted by centrifugation at 270 g for 8 min and resuspended after the addition of 3:1 methanol:acetic acid fixative. The cells were pelleted again and resuspended in fresh fixative and incubated at room temperature for 30 min. The cells were pelleted a third time and resuspended in 200 µL of fresh fixative. Fixed cells were dropped onto slides pre-chilled at ⁻20°C and allowed to air dry. Slides were stained with 3.2% Giemsa in Sorenson's buffer, pH 6.8, for 60 min at 34°C. Chromosome spreads were photographed using a DXM 1200 image capture device attached to an Eclipse E400 phase contrast microscope with 100X oil objective (Nikon, Inc., Melville, NY). Chromosome counts were determined from 126 cells in metaphase arrest.

3.2.8 Growth Analysis

AmE-711 cells were dissociated as above and the suspension was used to inoculate three 12.5 cm^2 culture flasks at a density of 3.0 x 10^5 cells/mL. The flasks were placed in an incubator at 32°C and HB-1 medium was replaced every 3 days. Starting at 24 h after inoculation and every 24 h thereafter until the cultures reached confluence, the number of cells in 10 random fields from each of the 3 flasks was counted manually using phase contrast microscopy. Average cell density ± SD was plotted per cm^2 on a linear scale against time (days).

3.2.9 Effect of Temperature on Cell Growth

AmE-711 cells were dissociated as above and the suspension used to inoculate 15 flat-sided culture tubes (5.5 cm^2) with 2.25 x 10^5 cells per tube. Tubes were randomly assigned to the baseline condition (i.e., harvest within 24 h at 32°C after inoculation) or one of four temperatures after inoculation: 25, 28, 32, and 34°C. HB-1 medium was replaced once during a 7-day incubation period. After 7 days, the medium was aspirated and the cell layer was washed twice with 1X DPBS (without Ca or Mg; Mediatech, Inc., Manassas, VA). Tubes were incubated overnight at room temperature with 0.5 mL of 0.5 N NaOH to solubilize proteins and stored at 4°C until analysis. 250 µL of Quick Start Bradford 1X dye (Bio-Rad, Hercules, CA) was added to 5 µL of solubilized protein to separate wells of a sterile flat bottom microtiter plate (Sarstedt, Inc., Newton, CA). Samples were thoroughly mixed and absorbance read at 595 nm using a VersaMax

microplate reader (Molecular Devices, LLC, Sunnyvale, CA). Three replicate tubes were established for each treatment and 3 replicate wells were assayed from each tube. Data were normalized against a reagent blank and bovine serum albumin (BSA) standard set (Bio-Rad, Hercules, CA). Mean total protein per unit surface area of tube substrate (cm^2) was analyzed by one-way ANOVA and post-hoc comparisons for temperature were carried out by Tukey's HSD test ($\alpha = 0.05$).

3.2.0 Effect of Commercial Medium on Cell Growth

AmE-711 cells were dissociated as above and the suspension used to inoculate 18 flat-sided culture tubes with 2.0×10^5 cells per tube. Tubes were incubated for 48 h at 32°C in HB-1 medium (baseline). After 48 h, the medium was aspirated and the cell layer was washed once with 1X DPBS. Tubes were then randomly assigned to receive complete medium containing a different commercially available base: Grace's, ILP-41, L15 (i.e., HB-1), Schneider's, or Shield's. All media, except L15, were purchased from Sigma-Aldrich, Co. (St. Louis, MO). Tubes were incubated for an additional 5 days and processed for protein as above. Three replicate tubes were established for each treatment. Mean total protein was analyzed and post-hoc comparisons determined for commercial media as above.

3.3 RESULTS

3.3.1 Features of Primary Cultures and Young Cell Lines

Nearly 100 primary cultures were prepared from fragmented honey bee embryonic tissues with the method described (or with slight variation in procedures). Inoculum size varied, but as little as 25 embryos was sufficient to inoculate one 5.5 cm^2 tube and initiate a primary culture. A 5.5 cm^2 flat-sided culture tube with a non-ventilating cap was the preferred plasticware for establishing primary cultures as the relative surface area and volume were small and minimized evaporation.

Explants of embryonic tissue became loosely adherent within 24 h after inoculation. There was difficulty in distinguishing the morphology of the cells, and it was not possible to determine the origin of the rudimentary tissues, in these fragments. Cells in these explants were undifferentiated, appearing large and round similar to those described by Bergem et al. (2006) (Figure 3.1A). However, cells that migrated away from the tissue explants were firmly attached and had a differentiated morphology, unlike Bergem et al. (2006). In the initial days of culture, an aggregation of squamous-shaped epithelial-like cells was often noted as the first distinguishable cell type that migrated away from, and formed a periphery around, the tissue explants. The formation resembled an epithelial sheet of tightly packed cells (Figure 3.1B). The epithelial-like cells had a high nuclear–cytoplasmic ratio and contained several prominent nucleoli. Within the first week to one month, most primary cultures showed cells that had migrated away from the tissue explants and were highly heterotypic (Figure 3.1C). However, fibroblast-type cells became the dominant cell type in most advanced cultures (Figure 3.1D). Fibroblast-type

cells were spindle-shaped, had a densely staining nucleus by Giemsa, and a low nuclear–cytoplasmic ratio. In addition, thin, elongated cytoplasmic extensions of some fibrous cells in an advanced state of differentiation were capable of exhibiting undulating contractions.

3.3.2 Isolation of the AmE-711 Line

Most of the primary cultures that we were able to subculture reached confluence within 3 months of inoculation. We were able to subculture approximately one-third of all primary cultures. After the first transfer, the frequency of subsequent transfers or passages was unpredictable and only one line (AmE-711) was taken past 5 transfers (Figure 3.2). Enzymatic dissociation by trypsin, as opposed to mechanical techniques such as sloughing, tapping the flask, or scraping, has been suggested as effective for dislodging strongly adherent insect cells from the flask substrate prior to transfer (Lynn, 2002; Genersch et al., 2013). We also observed that trypsinization for < 10 min at 32°C allowed for a homogenous suspension to be obtained for re-seeding and that the cells to be transferred were insensitive to the proteolytic activity of trypsin exposure. However, incubation periods with trypsin > 10 min led to cells that were slow to re-attach and/or injured, likely through irreversible damage of protein function (Huang et al., 2010).

3.3.3 Characterization of the AmE-711 Line:

3.3.3.1 Karyotype

The expected haploid and diploid karyotypes for *A. mellifera* are n = 16 and 2n = 32, respectively (Hoshiba and Kusanagi, 1978; Hoshiba et al., 1981). From 126 chromosome spreads, the range in chromosome number we observed was 6 – 133 (Figure 3.3). The modal chromosome number was 32, and 50% of the spreads had 26 – 36 chromosomes. Chromosomes were small (< 2 μm), making it difficult to identify individual chromosomes by diagnostic characters such as banding pattern and arm length. Some of the variability in chromosome number that we report can be explained, in part, to this small size, which could be resolved with higher magnification or other staining techniques. However, all cells in our culture system have a karyotype indicative of *A. mellifera* origin (Figure 3.4A). Polyploidy was rare and was observed in only two sets (< 2.0%) that had chromosome numbers of 92 and 133. Moreover, 3.2% and 4.0% of the sets we observed had a karyotype that was haploid or tetraploid (4n = 64) (Figure 3.4B), respectively.

3.3.3.2 Species Identification by Polymerase Chain Reaction

Polymerase chain reaction products amplified with primers specific for the *A. mellifera Cox 1* gene showed a positive signal for DNA from adult honey bee abdomen, honey bee embryos, and AmE-711 cells (Figure 3.5). The amplicon matched closely the 1044 bp amplicon for *Cox 1* reported by Hall and Smith (1991) and Corona et al. (1999). No signal was observed for the larva of the Common Eastern bumblebee, *B. impatiens*

(negative control), HL-60 human promyelocytic leukemia cells (negative control), or PCR controls. Comparative sequence analysis showed that the amplicon for the AmE-711 line was identical to that of the honey bee embryo and to a 956 bp sequence of the complete *Cox 1* gene (sequence position, 416 – 1371; Genbank Entry: M23409.1) (Crozier et al., 1989) and *A. mellifera ligustica* mitochondrial genome (sequence position, 1952 – 2907; Genbank Entry: L06178.1) (Crozier and Crozier, 1993).

3.3.3.3 Cell Growth

AmE-711 cells were split at a 1:3 ratio and maintained in the presence of 10% FBS at 32°C. Under these conditions, the population doubling time was estimated to be approximately 4 days (y = 3.14 + 0.76x; r^2 = 0.99) during the exponential growth phase (Figure 3.6). To gauge the mitotic index, cells were incubated with 0.125 mM colchicine in hypotonic KCl solution for 40 min according to the method described by Brito and Oldroyd (2010). The frequency of cells in metaphase arrest, or those cells with nuclei that had clearly visible chromosomes, after this pulse of colchicine was 0.8% (40 out of 5,000 spreads examined).

A one-way ANOVA was conducted to compare the effect of incubation temperature on total protein expression, which served as a proxy for cell proliferation. There was a statistically significant difference in mean total protein per cm^2 of tube substrate for cultures incubated at different temperatures ($F_{4,10}$=16.00, p=0.0002). Interestingly, cultures that had been incubated at 25ºC for 7 days had protein levels (2.70 ± 0.67 μg/cm^2) that were significantly lower compared to cultures incubated at 28ºC

(12.08 ± 5.88 µg/cm^2), 32ºC (13.76 ± 0.78 µg/cm^2), and 34ºC (14.89 ± 1.27 µg/cm^2) (Figure 3.7). There was no difference in total protein for cultures incubated at 28, 32, and 34ºC. Genersch et al. (2013) suggest that incubation temperature has no effect on *in vitro* growth of honey bee cells. These authors report no difference in growth between cultures maintained at room temperature (18 – 25ºC) and those maintained in the range of 27 – 31ºC (Genersch et al., 2013). Although we saw considerable variability in protein content for replicate cultures at 28ºC, our finding would suggest that there is a range at which cultures will display greater proliferation.

We tested the adequacy of other commercially available media in addition to L15 on the maintenance of the AmE-711 line and found a statistically significant difference in cell growth based on mean total protein per cm^2 of tube substrate ($F_{5,12}$=13.64, p=0.0001) (Figure 3.8). Proliferation was greater for cultures grown in L15 (i.e., HB-1) (15.04 ± 0.58 µg/cm^2) compared to Grace's (11.35 ± 0.16 µg/cm^2), ILP-41 (11.36 ± 0.85 µg/cm^2), and Schneider's (12.40 ± 0.48 µg/cm^2), but not Shield's (14.05 ± 0.95 µg/cm^2). Interestingly, Bergem et al. (2006) reported poorer growth outcomes with L15 as a base as opposed to Grace's, but this could be due to other factors such as differences in supplementation of the complete medium, initial seeding density, or type of cultureware used. We are conducting further experiments to determine if the medium developed by Hunter (2010) and others sustain growth similar to what we have observed with L15 and whether the AmE-711 line can adapt to Shield's as a base.

3.4 DISCUSSION

Cell culture is a remarkable technique, in that populations of cells from tissues explanted from a whole animal or plant can continue to grow when inoculated into a sterile flask containing a physiologically-relevant medium. Cells in primary culture represent a simplified environment that approximates the *in vivo* condition; however, these cells normally undergo senescence resulting from finite proliferation and differentiation capability (Hayflick and Moorhead, 1961). Therefore, the time frame to use primary cultures in applications may be limited. Interestingly, some cells in primary cultures can undergo alteration, either naturally through the accumulation of mutations or artificially by transfection. Altered cells have the capability to divide continuously and become confluent within the growth medium. In cultures that have cells that continuously divide, it is possible to transfer some of this material to daughter or subcultures to create lines where the number of cells expands and the lifespan of the culture is prolonged indefinitely (i.e., immortalized).

We have developed an *in vitro* system that allows for long-term maintenance of primary cultures and young cell lines derived from honey bee embryonic tissues. We found that it was not unusual for several of our primary cultures to remain mitotically active for more than a year after they were initiated, and this allowed for the eventual subculture and isolation of a line that has undergone 18 transfers (at the time of submission of this manuscript). From growth cycle and protein expression analyses, we estimate that the AmE-711 line undergoes at least 2.4 doublings between passages. This would equate to a minimum of 43 generations since its isolation, which is in the range of

the hypothetical threshold of 40 – 50 generations that delineates when most mammalian cell lines cease mitotic activity and enter senescence and a few lines become altered with the potential for immortalization (Hayflick and Moorhead, 1961). We have taken the necessary steps to ensure a supply of cells through cryopreservation, especially at early passages. We have successfully recovered cells from cryopreservation and these cells are viable and mitotically active. This hallmark in cell line development will allow long-term maintenance of the AmE-711 line and permit the potential distribution of this resource with other laboratories. Furthermore, our low success rate in establishing lines (1 line from ~ 100 primary cultures) and the long period of adaptation (~ 1 year) is not unexpected for insect cell culture (Brooks and Kurtti, 1971; Bergem et al., 2006). However, it alludes to the fact that more work is needed, using the AmE-711 line as a platform, to increase success and shorten the period of adaptation, as well as promote selection of different cell types similar to what is reported for dipteran and some other insect lines (Fallon and Kurtti, 2005).

Cell culture is imperative for infection and axenic growth of microbes that are causative agents of disease. Many factors such as growth medium, culture age, and cell type and surface structure limit host range (Chen et al., 2009); therefore, future research will need to ascertain the susceptibility of the fibroblast-type cells in the AmE-711 line to specific pathogens. The utility of an *in vitro* culture system comprised of honey bee cells has recently been demonstrated with research aimed at elucidating virulence factors of the bacterium that causes American foulbrood, *Paenibacillus larvae*. Poppinga et al. (2012) used pupal midgut cells in primary culture to identify a shape-determining protein

of *P. larvae*, whose predicted function permits adhesion to the host cell. Two other pathogens of honey bees, *Nosema apis* and *N. ceranae*, are obligate intracellular fungi that are widespread in beekeeping operations (Cox-Foster et al., 2007). *Nosema ceranae* is considered an emerging pathogen and debate persists as to whether it is more virulent than *N. apis* (Higes et al., 2007; Paxton et al., 2007), which has been recognized in honey bee colonies since the early twentieth century. Remarkably, it has been demonstrated that both species of Microsporidia can infect the heterologous lepidopteran cell line, IPL-LD-65Y, derived from ovarian tissues, which is neither tissue nor host specific for these highly-evolved fungi (Gisder et al., 2011). This finding broadens the potential use of the AmE-711 line for studying host cellular responses to not only these, but other honey bee pathogens, such as the many viruses to which we have limited knowledge of their infectivity and pathogenicity (Chen and Siede, 2007). Moreover, efforts have been initiated in our laboratory to determine whether AmE-711 cells can be infected with *Nosema* sp. spores.

3.5 CONCLUSIONS

Declining honey bee populations in North America and Europe (United Nations Environment Programme, 2010) has been the impetus for considerable research efforts aimed at mitigating the many challenges thought to be responsible for this crisis. This decline has resulted in increased inputs (e.g., labor, expenses) by beekeepers to monitor and treat colonies in crisis, as well generates uncertainty in the supply of colonies for commercial pollination or other bee-derived products. In tandem with the recently

sequenced genome of *A. mellifera* (Honeybee Genome Sequencing Consortium, 2006), an *in vitro* system comprised of honey bee cells will facilitate studies in functional genomics and/or target mechanisms of pathogenesis through infection with viruses, for example. Honey bees are also host to a plethora of non-pathogenic, horizontally acquired microbes (Gilliam, 1997); a host-derived culture system could cast light on mechanisms of host tolerance and nutritional consequences of harboring these symbionts. In addition, the introduction of foreign gene constructs or knockdown of specific gene targets through RNA interference are attractive applications that would permit questions to be asked about subcellular and molecular changes involved with honey bee development or metabolic responses to pesticides and other xenobiotics.

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Authors' Contributions

MG and TK conceived and designed the experiments. MG carried out the experiments and performed the statistical analyses. MG and TK drafted the manuscript. TK and MS contributed materials and participated in the coordination of the study. All authors read and approved the final manuscript.

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3.7 FIGURE LEGENDS

Figure 3.1 Phase contrast micrographs of honey bee cells.

Cell characteristics depict different stages in the culture process: **A.** Round, undifferentiated cells (white arrowhead) of an unattached tissue fragment from a newly inoculated primary culture. **B.** Outgrowth of tightly packed epithelial-like cells (white arrowhead) from a tissue explant in an early stage primary culture. **C.** A remnant tissue explant (white arrowhead) in a primary culture at 1 month that is nearly confluent with heterotypic cell arrangements, most notably elongated fibroblast-type cells. **D.** A monolayer of mainly fibroblast-type cells that are loosely packed (~60% confluent) in the AmE-711 cell line that had been transferred 14 times. White arrowhead indicates a rounded, refractile cell undergoing mitosis, enlarged in the lower right corner inset. Scale bar = 10 μm.

Figure 3.2 Passage number and cumulative days of AmE-711 cells in culture.

The black arrowhead indicates the date when AmE8-11 (inoculation date: 7/8/11; culture day 0) and AmE9-11 (inoculation date 7/22/11; culture day 14) primary cultures were combined. Several additional primary cultures were isolated between 10/20/11 and 11/10/11, and non-adherent material (i.e., tissues fragments, single cells) collected within days after inoculation of these cultures was added to the combined AmE8-11 and AmE9-11 flask. The combination of these two primary cultures, along with material from other primary cultures, would later become the AmE-711 cell line. † indicates the lifespan of many primary cultures and young cell lines isolated using the method described.

Figure 3.3 Distribution of chromosomes of AmE-711 cells.

The AmE-711 line had been transferred 8 times. A total of 126 metaphase spreads were counted. Haploid, diploid, and tetraploid conditions are demarcated by n, 2n, and 4n, respectively. Chromosome counts greater than 64 (4n) were considered as polyploid.

Figure 3.4 Chromosomes of the AmE-711 cell line.

Chromosomes were prepared from a brief (40-min) incubation with 0.125 mM colchicine in hypotonic KCl solution. **A.** Two representatives of diploid (2n = 32) chromosome spreads in nuclear matrix. **B.** A tetraploid (4n = 64) chromosome spread in nuclear matrix. Scale bar = 10 μm.

Figure 3.5 Approximate 1050-bp region within the mitochondrial-encoded cytochrome c oxidase subunit I gene (*Cox 1*) from samples of genomic DNA amplified by PCR.

Lane 1, marker (100 bp); lane 2, *Apis mellifera* adult abdomen; lane 3, *A. mellifera* embryo; lane 4, AmE-711 cells; lane 5, *Bombus impatiens* larva; lane 6, HL-60 human promyelocytic leukemia cells; lane 7, *Taq* polymerase and primers without DNA; lane 8, *Taq* polymerase and AmE-711 DNA without primers.

Figure 3.6 Proliferation of AmE-711 cells *in vitro*.

The number of cells was counted from 10 random fields from cultures that had been transferred 12 times. Values are expressed as the mean number of cells per cm^2 surface area ± SD of 3 replicate cultures.

Figure 3.7 Expression of proteins in AmE-711 cells at different temperatures.

The AmE-711 line had been transferred 12 times and incubated for 7 days at different temperatures. Mean total protein per unit surface area of tube substrate (cm^2) was determined from 3 replicate cultures per temperature using a Bradford assay. Baseline data represent the amount of protein in cells from 3 replicate cultures harvested within 24 h after inoculation. All cultures were run in triplicate and the data were normalized against a reagent blank (0.5 N NaOH) and BSA standard curve. Mean total protein was significantly different between cultures grown at different temperatures ($F_{4,10}=16.00$, $p=0.0002$). Columns with different letters are significantly different by Tukey's HSD ($\alpha = 0.05$).

Figure 3.8 Expression of proteins in AmE-711 cells grown in different media. AmE-711 cells had been transferred 15 times and incubated for 5 days in different commercially available media. Mean total protein per unit surface area of tube substrate (cm^2) was determined from 3 replicate cultures per base medium using a Bradford assay. Baseline data represent the amount of protein in cells from 3 replicate cultures harvested 48 h after inoculation and before the HB-1 media of the remaining cultures was replaced with a random assignment of the following base media: Grace's, ILP-41, L15, Schneider's, and Shield's. All cultures were run in triplicate and the data were normalized against a reagent blank (0.5 N NaOH) and BSA standard curve. Mean total protein was significantly different between cultures grown in the presence of different base media ($F_{5,12}$=13.64, p=0.0001). Columns with different letters are significantly different by Tukey's HSD ($\alpha = 0.05$).

Figure 3.1

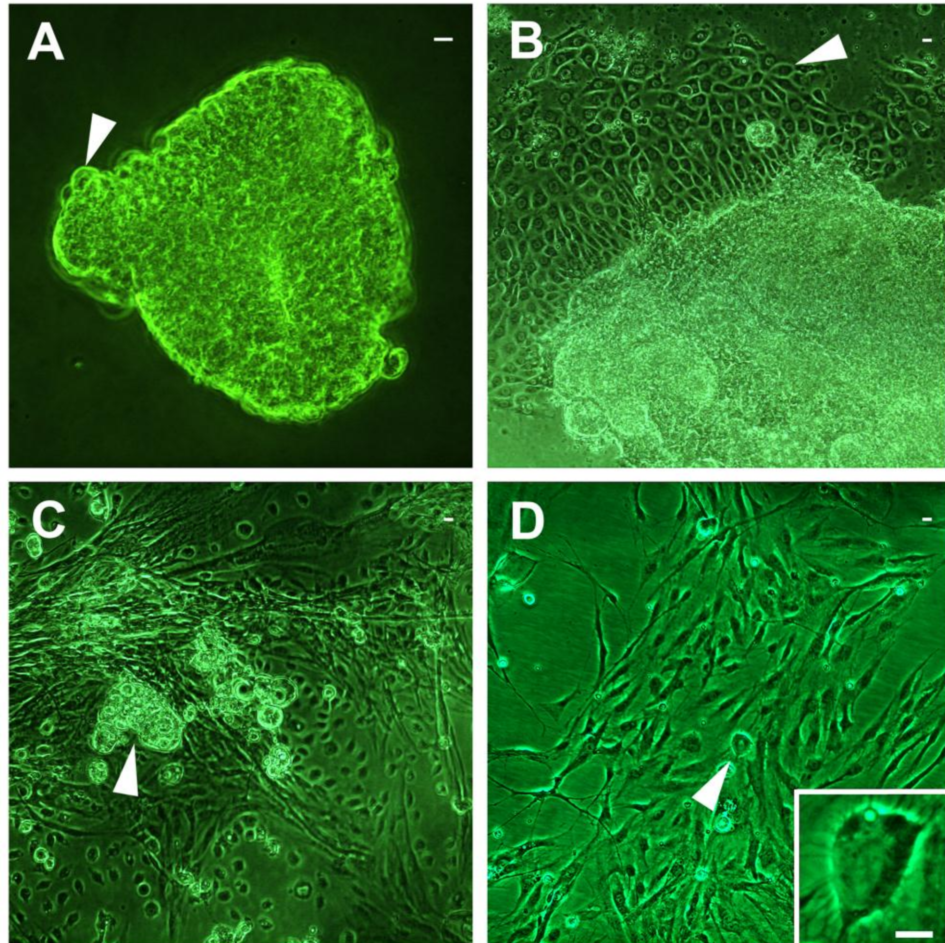


Figure 3.2

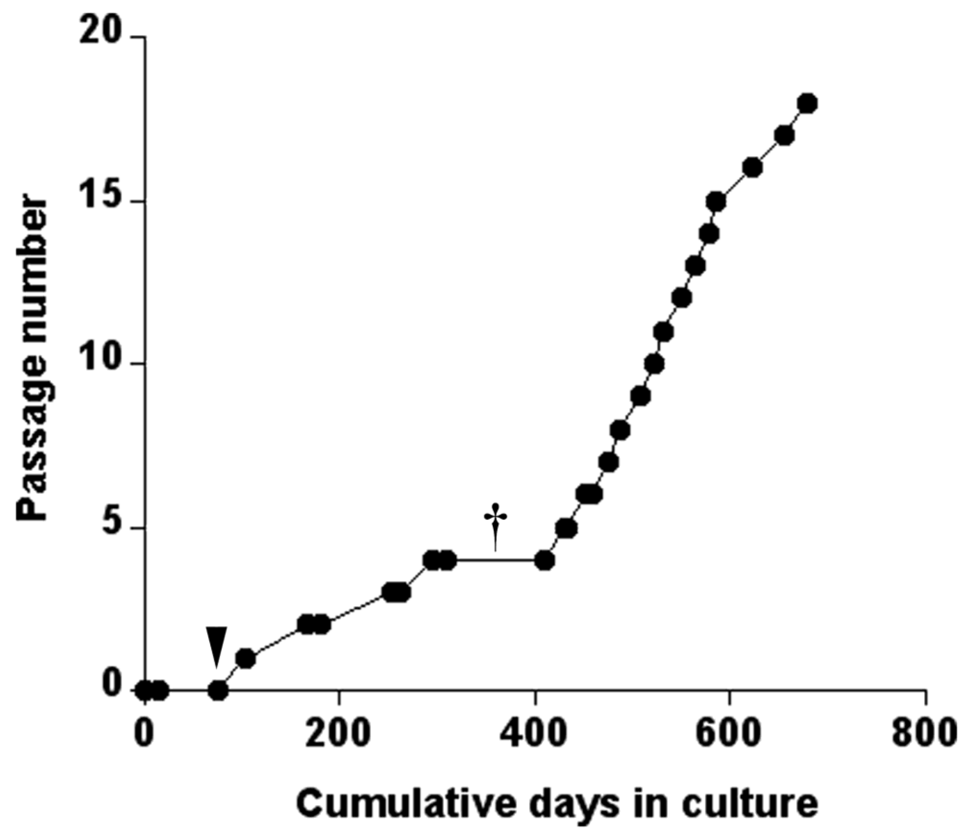


Figure 3.3

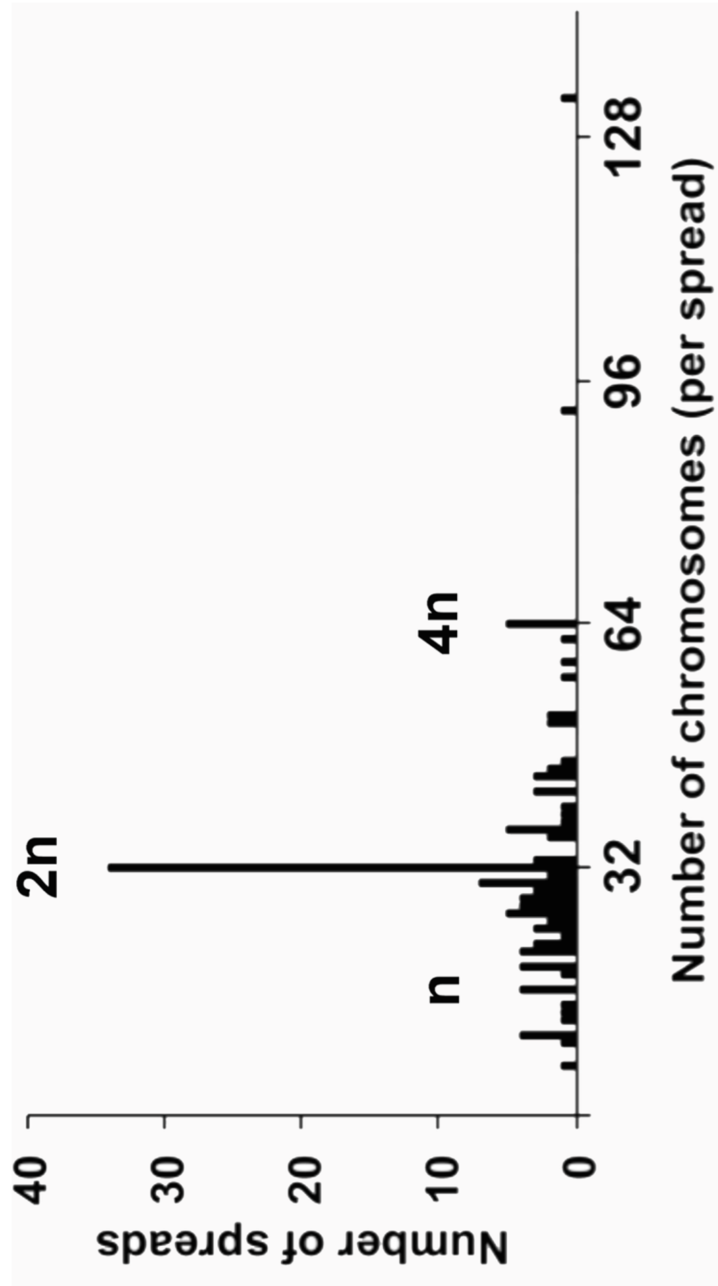


Figure 3.4

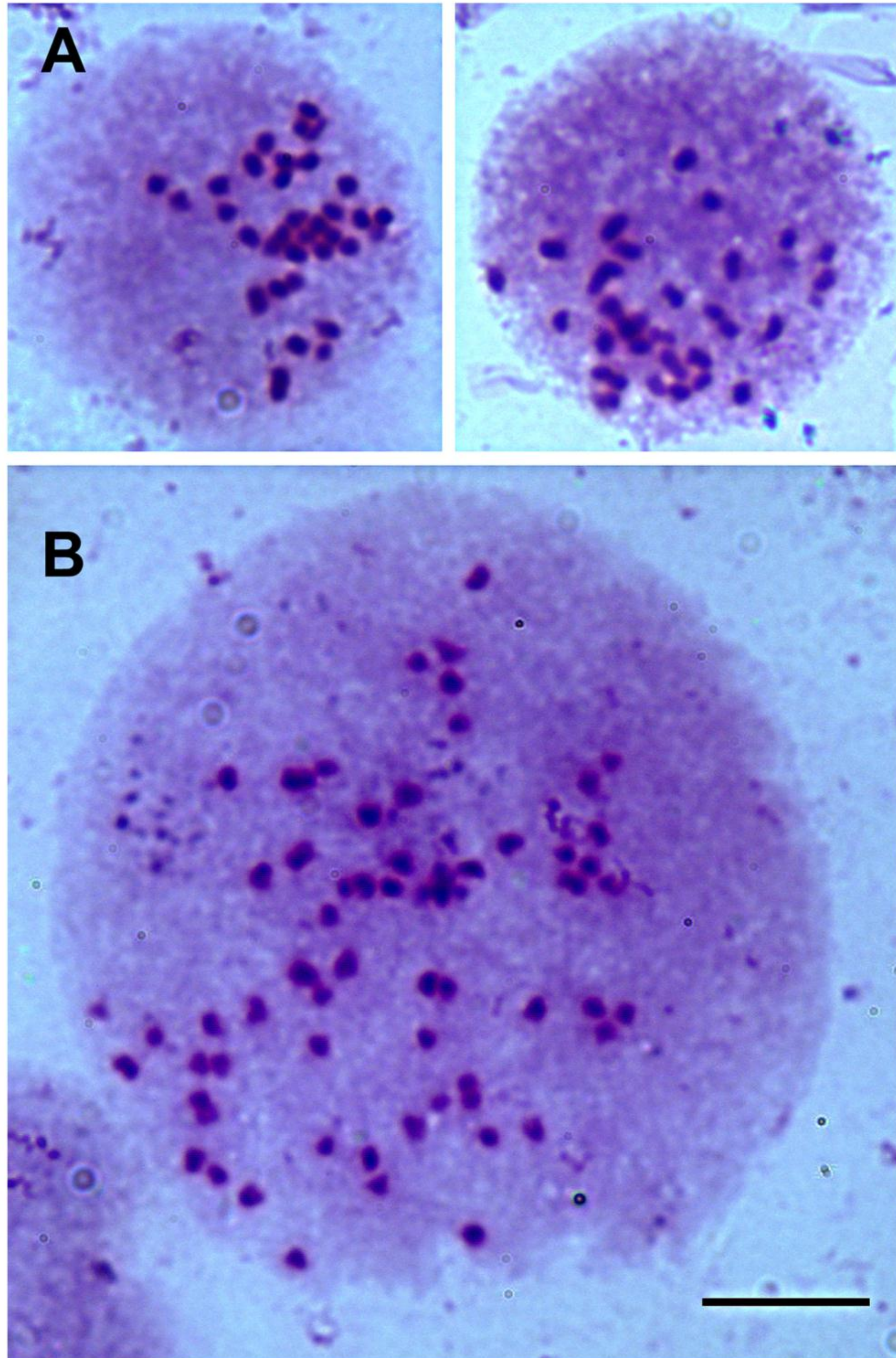
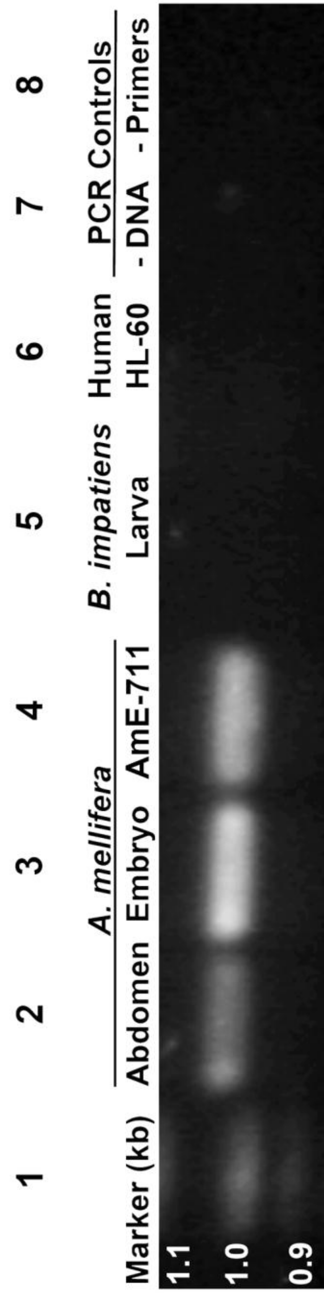


Figure 3.5



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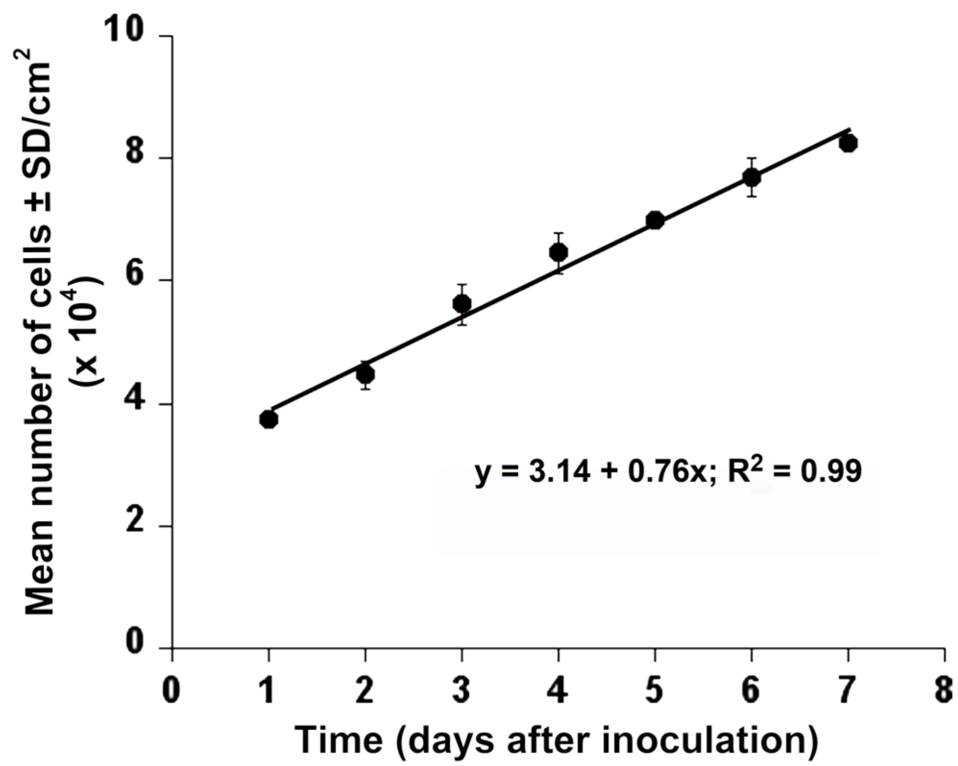


Figure 3.7

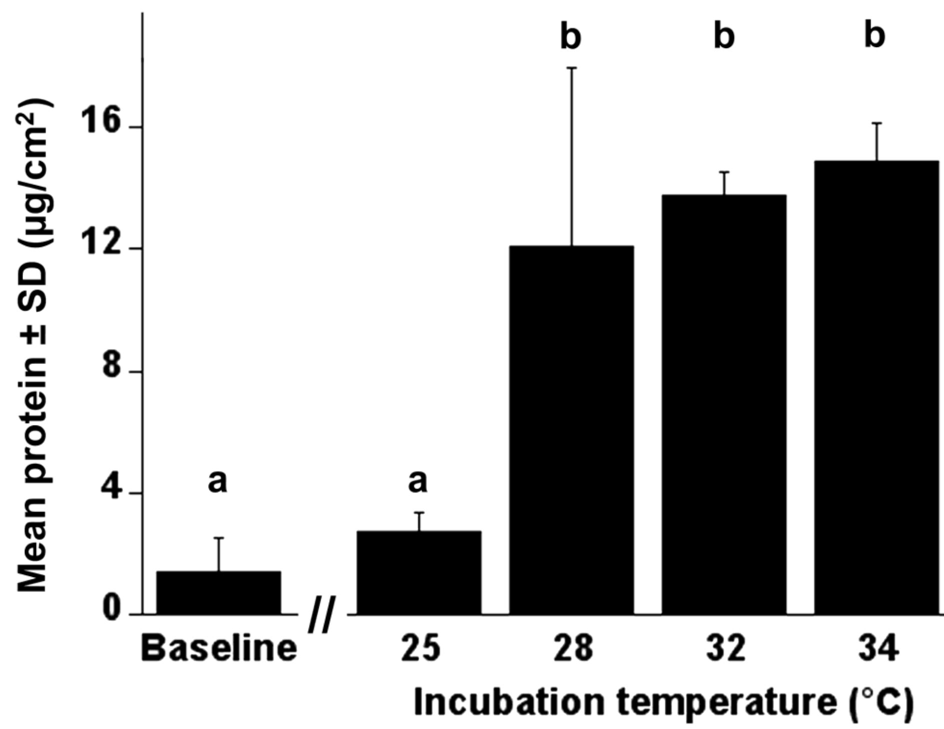
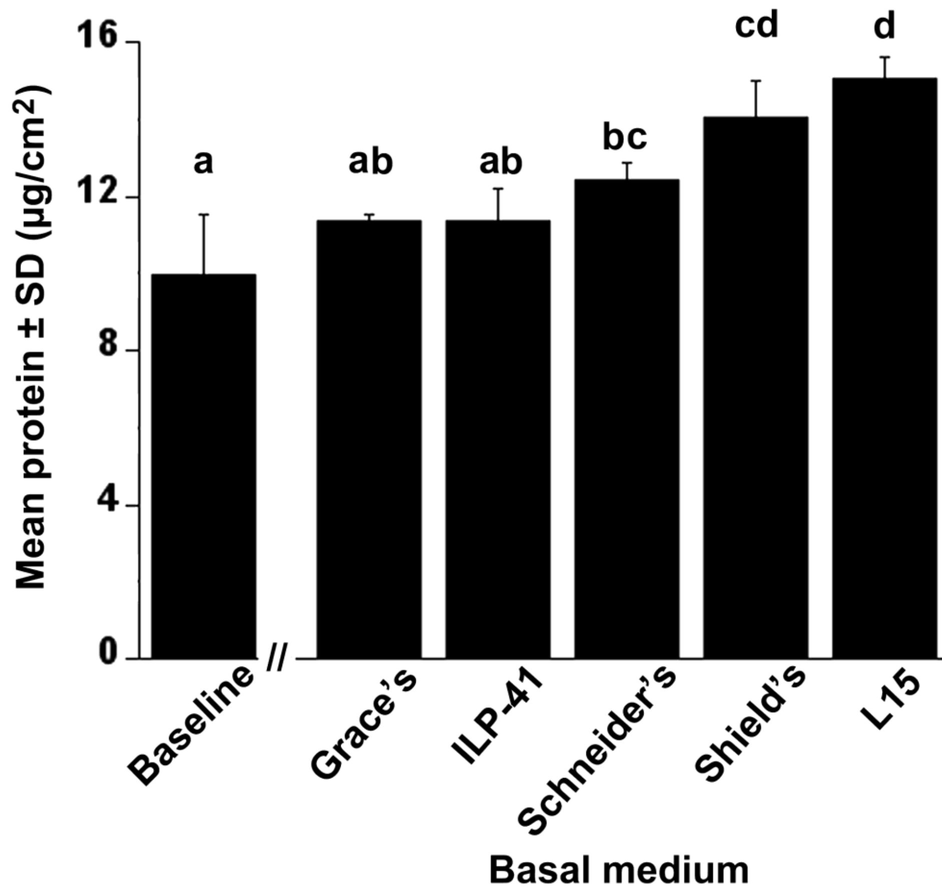


Figure 3.8



CHAPTER 4

The Influence of Medium Supplements and Culture Condition on AmE-711 Cell Growth

Michael J. Goblirsch and Timothy J. Kurtti

SUMMARY

The availability of honey bee cell cultures would provide a greatly needed resource to evaluate and understand obligate intracellular pathogens and other detractors to honey bee health at the cellular level as well promote strategies against infectious diseases. Despite our long history of interacting with honey bees, the development of *in vitro* systems from these beneficial insects has lagged in comparison to other insects that have the potential to significantly impact human and animal welfare. The paucity of honey bee cell lines may be explained in part to a need for the optimization of culture conditions that sustain *in vitro* growth of bee cells. We used the continuous honey bee cell line, AmE-711, as a platform to assess the effects of different supplements (e.g., FBS, TPB, royal jelly, and carbohydrates) to commercial L15 medium versus modified L15 medium that is the standard formulation used for the routine maintenance of the line. Modified L15 was superior to commercial L15 at stimulating the growth of AmE-711 cells under all conditions tested. AmE-711 cells required serum for proliferation, and royal jelly as a supplement was capable of supporting growth. Furthermore, AmE-711 cells cultured in medium where bicarbonate buffer was removed or with free gas exchange at a slightly higher than normal temperature outperformed other conditions in

terms of growth. These experiments provide a foundation for ongoing experiments that seek an optimal culture environment for the growth of honey bee cells.

KEYWORDS: colony collapse disorder, cytology, honey bee, intracellular pathogen, invertebrate cell line

4.1 INTRODUCTION

It has become a normal occurrence for beekeepers in the U.S., parts of Europe, and several other countries to experience high levels of honey bee colony mortality on an annual basis (van Engelsdorp et al., 2008; van der Zee et al., 2012; OPERA, 2013; Spleen et al., 2013). Of the multitude of factors that are thought to contribute to the large scale decline and death of honey bee colonies, pathogenic microorganisms such as viruses and Microsporidia elicit concern due to their high prevalence and burden in beekeeping operations (Cox-Foster et al., 2007; van Engelsdorp et al., 2009). In response, research tools such as diagnostic assays for the detection and enumeration of pathogens have been developed to expand upon their role and/or interaction with other factors such as pesticides to see how they contribute to honey bee decline (Aronstein et al., 2013; Yan et al., 2014). One resource that would complement detection assays by providing a substrate for the growth of intracellular pathogens in a simplified, homogenous host environment is continuous cell lines derived from honey bee (i.e., host) tissues.

Despite efforts to establish continuous cell lines from honey bee tissues, few successes have been reported except for the recent establishment of the MYN9 cell line from 3 – 5 day old larvae (Kitagishi et al., 2011) and the AmE-711 cell line from embryonic tissues (Goblirsch et al., 2013). The MYN9 cell line was obtained by transforming honey bee cells with the human oncogenic *c-myc* gene (Kitagishi et al., 2011). Transformation with exogenous DNA is used extensively for applications such as creating “immortalized” cell lines, in the production of recombinant proteins, or for gene function studies. However, the introduction of foreign DNA may alter endogenous

biochemical processes through epigenetic mechanisms (e.g., silencing genes by enhanced methylation of cellular DNA) that could produce distinctly different phenotypes from the host (Heller et al., 1995; Remus et al., 1999; Doerfler et al., 2001). In some instances, the altered phenotype may be incompatible to the host (Sigmund, 2000), which may be a drawback for infection studies examining pathogen-induced changes to host biochemical processes. The MYN9 cell line demonstrates the potential for *in vitro* systems derived from honey bee cells as models of transgenesis, but cell lines that are obtained through natural transformation or without the introduction of foreign DNA may be more desirable in certain applications.

The AmE-711 cell line was established using traditional techniques where embryonic tissues were mechanically disaggregated and the tissue fragments used as inocula for primary cultures. After a long adaptation period of several months, which is a typical trajectory in the transition from primary culture to young cell line using tissues derived from insects (Brooks and Kurtti, 1971), the growth rate of AmE-711 cells from early subcultures increased and allowed for the establishment of a continuous cell line. Although the AmE-711 cell line remains in continuous culture and is passaged on a regular basis; it represents one success from a relatively large number of primary cultures and young cell lines established by Goblirsch et al. (2013) that eventually ceased to proliferate.

The paucity of cell lines established from the honey bee, and Hymenoptera overall, relative to other orders of insect (e.g., Diptera and Lepidoptera; Lynn 2001) suggests that there are factors yet to be identified that could be applied to expanding the

number of existing honey bee cell lines, or establishing new cell lines composed of a specific cell type (e.g., epithelial, fibroblast, haemocyte), from a specific tissue (e.g., nervous, reproductive, digestive), or stage of development (e.g., larva, pupa, adult). Successful adaptation (i.e., attachment, growth, and proliferation) of cells *in vitro* requires appropriate culture conditions that approximate the physiological milieu where the cells were derived from. Determining the medium and culture environment can be a daunting task, especially if there has been limited previous success at establishing cell lines from a given taxa like the honey bee. One study reported the establishment of bee cell cultures from larval and pupal tissues using a medium that had been modified from its original use for the culture of hemipteran cells, named WH2 (Hunter, 2010). WH2 medium used Schneider's as the basal medium supplemented with 10% FBS, L-histidine, inorganic salts, and a proprietary mixture of lipids, vitamins, and trace minerals (Insect Medium Supplement; Sigma-Aldrich, St. Louis, MO). WH2 medium supported the growth of a heterogeneous population of honey bee cells that had a doubling time of at least 9 days (Hunter, 2010). Similar to honey bee cells cultured in WH2, the AmE-711 cell line was isolated and is maintained in a medium that was developed specifically for tick cell culture (Munderloh and Kurtti, 1989) and is not commercially available. The medium used to maintain the AmE-711 cell line uses Leibovitz's L15 as a base that is modified by adding known quantities of vitamins, trace minerals, amino acids, α-ketoglutaric acid, and glucose. These compounds are not present in L15 but are utilized by insect/invertebrate cells for energy, metabolism, and osmoregulation. Modification of L15 by Munderloh and Kurtti (1989) has been shown to support the maintenance of

several tick and other insect cell lines (Sagers et al., 1996, Munderloh, et al., 1999). However, its preparation requires a substantial commitment in time, reagents, and labware which may deter some researchers from using it for the culture of AmE-711 cells in their own laboratory or for the isolation and maintenance of other cell lines derived from honey bee tissues or Hymenoptera in general.

The objective of this research was to test permutations of the existing growth medium and culture environment to determine: 1) what modifications and supplements to commercial L15 are needed for AmE-711 cell growth; 2) if undefined supplements such as FBS and TPB could be removed, reduced, or partially replaced with royal jelly, a product, albeit undefined, that is derived from honey bees and used to feed brood; 3) if incubating AmE-711 cells with free gas exchange in 3% CO_2 at a slightly higher than normal temperature had a positive effect on cell growth.

4.2 MATERIALS AND METHODS

4.2.1 Cell Line

The honey bee AmE-711 cell line was originally isolated by Goblirsch et al. (2013) from embryonic tissues of *Apis mellifera*. The AmE-711 cell line is routinely maintained using Leibovitz's L15 medium (Leibovitz, 1963) as the basal medium and modified according to Munderloh and Kurtti (1989). The complete medium is supplemented with 10% fetal bovine serum (FBS) and 5% tryptose phosphate broth (TPB). AmE-711 cells are maintained in sealed vessels at 32°C in a non-humidified

incubator. Experiments performed below used the AmE-711 cell line when it had been passaged 36 – 40 times.

4.2.2 Preparation of Experimental Media

Leibovitz's L15 with L-glutamate was purchased from Gibco (Grand Island, NY) and prepared in sterile tissue culture tested water. The medium was either left as basal L15 without modification and designated as commercial L15 or modified according to Munderloh and Kurtti (1989) except without the addition of lipoprotein concentrate and designated as modified L15. The pH was adjusted to 6.8 – 6.9 with either 1N HCl or 1N NaOH prior to the addition of the supplements. Sterile filtered FBS of USDA origin was purchased from BioWest (Kansas City, MO) and heat-inactivated in a warm water bath at 55°C for 30 min prior to use. Tryptose phosphate broth was purchased from Gibco (Grand Island, NY) and prepared according to the manufacturer's instructions. In addition to tryptose, TPB contains dextrose, sodium chloride, and disodium phosphate.

4.2.3 Royal Jelly

Royal jelly was purchased from the Golden Queen Store (Beijing, China). Royal jelly was aliquoted into 1.5 mL microcentrifuge tubes and frozen. Aliquots of royal jelly were then dehydrated using a Savant SS21 speedvac system (Thermo Fisher Scientific Inc., Waltham, MA). Dehydrated royal jelly was kept at -20°C until use. Royal jelly (0.1 g) was dissolved in 50 mL of commercial L15 to make a 0.2% stock solution. The royal jelly stock solution was passed through a 0.22 μM Millex GP low binding syringe filter

(Millipore, Billerica, MA). Modified and commercial L15 were supplemented with 5% FBS and 5% TPB and different concentrations of royal jelly (0, 0.0002, 0.002, and 0.02%) to test whether its addition to the culture medium was sufficient for stimulating the growth of AmE-711 cells.

4.2.4 Carbohydrate Source

Leibovitz's L15 medium was developed for the culture of mammalian cells in free gas exchange and uses galactose instead of glucose as a carbohydrate source. Sodium pyruvate and alanine are also added to Leibovitz's L15 as a source of energy and bicarbonate used for synthesis reactions (Barngrover et al., 1985). One step in the modification of commercial L15 used for the cultivation of insect and tick cell lines involves the addition of glucose to achieve a final concentration of 1.8% (Munderloh and Kurtti, 1989). AmE-711 cells were cultured in commercial L15 supplemented with 10% FBS and 5% TPB and different concentrations of glucose to test whether the addition of this carbohydrate alone was sufficient for stimulating cell growth. D-glucose (3.6036g; Sigma, St. Louis, MO) was dissolved in 50 mL of commercial L15 to make a 7.2% stock solution. The glucose stock solution was filter sterilized as above and diluted in commercial L15 to prescribed concentrations for use in subsequent experiments (0, 0.45, 0.9, and 1.8%).

The diet of a honey bee colony is derived mainly from the pollen (protein) and nectar/honey (carbohydrates) its foragers gather from flowering plants. The major components of honey are the monosaccharides, fructose and glucose, and are found at

relatively consistent levels regardless of geographic origin or sampling date (White and Doner, 1980). Fructose and glucose are metabolized in different ways by cells, with the former potentially resulting in an increase in fatty acid synthesis (Sun and Empie, 2012). Fatty acids are utilized by cells as energy stores or in the formation of membranes; therefore, we wanted to test whether the mixture of sugars typically found in honey showed an advantage over glucose alone in stimulating AmE-711 cell growth when added to commercial L15. Non-crystallized liquid honey was obtained from a local beekeeper and was determined to be 17% water using a hand refractometer. Using White (1992) as a reference, the composition of the remaining 83% was assumed to be fructose (~38%) and glucose (~30%), with minor contributions of di- and oligosaccharides such as sucrose (~10%), and vitamins, minerals, and other constituents (~5%). Based on this assumption, 4.34 mL of honey was pipetted into and dissolved in 45.66 mL of commercial L15 to make a 7.2% stock solution of mixed carbohydrates (and vitamins and trace minerals) derived from honey. The stock solution of mixed carbohydrates was sterile filtered as above and diluted in commercial L15 and reported as percentage carbohydrate equivalents (g solute per 100 g solution) for use in subsequent experiments (0, 0.45, 0.9, and 1.8%). All experimental media were stored at 4°C until use.

4.2.5 Growth Assay

Cell growth was quantified by comparing the amount of protein produced per culture against a baseline established from cultures harvested 24 hours after inoculation. AmE-711 cells from seven 25 cm^2 culture flasks that were 80 – 100% confluent were

dissociated by incubating in trypsin for <10 min at 32°C. The cells were then combined into a 50 mL sterile conical tube and the suspension was thoroughly mixed and used to inoculate flat-sided culture tubes (5.5 cm^2) with a final volume of 1 mL modified L15 containing approximately 3.75 x 10^5 cells per mL. Tubes were randomly assigned to the baseline condition (i.e., harvest 24 hours after inoculation) or experimental conditions that tested the effects of supplementation with FBS, TPB, royal jelly, carbohydrates, and sodium bicarbonate buffer. An additional experimental condition tested the effect of free gas exchange in a 3% CO_2-enriched atmosphere at a higher than normal incubation temperature (34°C). Twenty-four hours after seeding, the complete medium was aspirated from each culture tube and replaced with the assigned experimental medium. The experimental medium from each tube was exchanged with fresh medium every third or fourth day during a 14-day incubation period. Tubes were inspected by phase contrast microscopy and a representative of each condition was photographed at day 7 and 14, prior to the harvest of protein, to approximate the confluency of the cultures and confirm the data obtained from the protein assay.

At the end of the 14-day incubation period, the medium was aspirated from each tube and the cell layer was washed twice with 1X DPBS (without Ca or Mg; Mediatech, Inc., Manassas, VA). Tubes were incubated in 0.5 mL of 0.5 N NaOH for 48 hours at room temperature to solubilize proteins and then stored at 4°C until analysis. 250 µL of Quick Start Bradford 1X dye (Bio-Rad, Hercules, CA) was added to 5 µL of solubilized protein from each tube to separate wells of a sterile flat bottom microtiter plate (Sarstedt, Inc., Newton, CA). Samples were thoroughly mixed and absorbance read at 595 nm

using a VersaMax microplate reader (Molecular Devices, LLC, Sunnyvale, CA). Three replicate tubes were established for each condition and 3 replicate wells were assayed from each tube. One of the 3 tubes containing modified L15 supplemented with 0.02% royal jelly became contaminated during the 14-day incubation period; therefore, data for this condition represent n = 2. Data were normalized against a reagent blank and bovine serum albumin (BSA) standard set (Bio-Rad, Hercules, CA). Data are reported as the mean fraction of total protein ± standard deviation against the baseline. After correcting for the dilution factor, the average amount of protein for the baseline condition was estimated to be 5.86 $\mu g/cm^2$.

4.3 RESULTS

4.3.1 The Effect of Fetal Bovine Serum

AmE-711 cells were cultured in either commercial or modified L15 supplemented with 5% TPB and increasing concentrations of FBS (0, 2.5, 5, and 10%). The addition of FBS to modified L15, but not commercial L15, had a stimulating effect on the growth of AmE-711 cells (Fig 4.1A). Cells cultured for 14 days in modified L15 showed a positive linear relationship in protein yield with increasing concentrations of FBS. Cells from cultures treated with modified L15 supplemented with 10% FBS had the highest mean protein yield, which increased by 2.5- and 4-fold compared to the protein yield from cells cultured in modified L15 supplemented with the next lowest concentration of FBS (5%) or cells harvested 24 hours after seeding (baseline), respectively. The addition of FBS to commercial L15 did not support the growth of AmE-711 cells using any of the

concentrations tested. Although the protein yield for cells cultured for 14 days in commercial L15 supplemented with 5% FBS were slightly above baseline on average, it was no different than the protein yield of cells harvested 24 hours after inoculation.

Cells cultured in commercial or modified L15 without FBS appeared healthy, consisting of fibroblast-type morphology, which is typical for the AmE-711 cell line (Figure 4.1B). Cells cultured without FBS had a rounded or ovoid soma with conspicuous nucleus and spindle-like projections of the cytoplasm that terminated as thin filopodia. These projections were not as elongate as observed in cultures supplemented with FBS and may be better characterized as fibrocyte-like, a metabolically inactive form of fibroblast. Although cells cultured in commercial L15 without FBS were fibroblast-like, these cells had cytoplasmic extensions that were uneven, appearing bead-like or scalloped along their longitudinal axis, and the nuclear area of some of these cells showed the presence of several small vesicles. However, cells cultured in either commercial or modified L15 without FBS had a healthy appearance, but the lack of increase in protein yield suggests that mitogenic and/or cytokinetic factors present in serum are needed for AmE-711 cell growth.

4.3.2 The Effect of Tryptose Phosphate Broth

AmE-711 cells were cultured in either commercial or modified L15 supplemented with increasing concentrations of TPB (0, 2.5, 5, and 10%). Fetal bovine serum was also added to the medium but the concentration was reduced from 10% to 5% to observe the influence of TPB. Modified L15 supplemented with TPB and reduced serum stimulated

the growth of AmE-711 cells with the average protein yield greatest for cultures supplemented with 10% TPB (Figure 4.2A). The maximum yield realized decreased by 1.5-fold compared to cells cultured in modified L15 supplemented with 10% FBS and 5% TPB, which is the standard formulation used for routine cultivation of the AmE-711 cell line. The protein yield of cells cultured in modified L15 supplemented with 2.5 and 5% TPB were slightly greater than baseline but were no different than the protein yield from cells cultured without TPB but 5% FBS. This finding shows that modified L15 supplemented with 5% FBS was sufficient at maintaining a basal level of cell growth regardless of TPB concentration.

There was a positive linear relationship in protein yield for AmE-711 cells cultured in commercial L15 with reduced serum and supplemented with increasing concentrations of TPB. Despite the positive trend in the amount of protein obtained, the highest yield was no different than baseline. The average amount of protein harvested from AmE-711 cells cultured in commercial L15 supplemented with 10% TPB was indistinguishable from baseline and decreased by 30-fold compared to the average protein yield for cells cultured in the complement medium that consisted of modified L15 supplemented with 10% TPB. Unlike what was observed for AmE-711 cells cultured in modified L15 and reduced serum, commercial L15 supplemented with 5% FBS was not sufficient at maintaining a basal level of cell growth, regardless of TPB concentration.

AmE-711 cells cultured in modified L15 and supplemented with TPB concentrations less that 5% were fibroblast-type but had polar cytoplasmic extensions that were longer and thinner with a compressed soma compared to cells cultured in

complete medium. Moreover, several cells had the presence of a large, often crescent-shaped, vacuole abutting the nucleus (Figure 4.2B). The presence of one to several large vacuoles was also found in cultures grown in commercial L15 supplemented with different concentrations of TPB and 5% FBS. The presence of these vacuoles may indicate the activation of programmed cell death processes such as autophagy as a response to nutrient deprivation.

4.3.3 Serum is Necessary for Proliferation of AmE-711 Cells

AmE-711 cells were cultured in modified L15 without FBS or TPB. The absence of these two supplements in the medium inhibited the growth of AmE-711 cells (data not shown). The average protein yield for cells cultured without FBS and TPB was equivalent to the yield from cells harvested 24 hours after seeding. Similar to cells cultured in modified L15 with 5% TPB but without FBS, cells cultured without FBS and TPB appeared healthy and had fibroblast-like morphology typical of the AmE-711 cell line.

4.3.4 The Effect of Royal Jelly

The influence of the amount of royal jelly on AmE-711 cell growth was evaluated using either commercial or modified L15 supplemented with 5% FBS and 5% TPB and increasing concentrations of royal jelly (0, 0.0002, 0.002, and 0.02%). The presence of royal jelly in modified L15 stimulated the growth of AmE-711 cells in a concentration dependent manner that plateaued at the highest concentration tested (0.02%; Figure 4.3).

The average protein yield increased by 2-fold for cells cultured in modified L15 supplemented with concentrations of royal jelly $\geq$ 0.002% compared to cells cultured in modified L15 that contained no royal jelly but was supplemented with 5% FBS and 5% TPB only. However, even the maximum protein yield obtained for cells cultured in modified L15 supplemented with any of the concentrations of royal jelly tested was 1.6-fold less than the protein yield from cells cultured in the standard medium used to maintain the AmE-711 cell line.

Protein levels for AmE-711 cells cultured in commercial L15 supplemented with 5% FBS and 5% TPB and increasing concentrations of royal jelly were unchanged and no different than baseline. Cells cultured in modified L15 supplemented with royal jelly were normal in appearance and showed mitotic activity throughout the incubation period, whereas cells cultured in commercial L15 contained numerous large vacuoles, which was more pronounced than what was observed for cells cultured in commercial L15 supplemented with different concentrations of TPB and 5% FBS.

4.3.5 Influence of Carbohydrates

The effect of glucose and/or mixed carbohydrates, typically found in honey, as supplements to commercial L15 were evaluated to determine their ability to stimulate the growth of AmE-711 cells. Commercial L15 was supplemented with 10% FBS and 5% TPB in addition to either glucose or a mixed carbohydrate solution made from honey. Glucose supplementation had a negligible affect on cell growth during the 14-day incubation period (Figure 4.4A). There was a positive trend in the amount of protein

harvested from cells cultured in commercial L15 and increasing concentrations of glucose. The highest yield was obtained when AmE-711 cells were cultured in medium supplemented with 1.8% glucose and was only slightly greater than baseline. Moreover, the maximum yield was 3.5-fold lower than the yield obtained from cells cultured in modified L15 supplemented with 10% FBS and 5% TPB, which is the standard medium used to maintain the AmE-711 cell line.

Similar to the effect of supplementing commercial L15 with glucose, the addition of increasing concentrations of a mixed carbohydrate solution made from honey had a negligible affect on the growth of AmE-711 cells. Although the protein yield from cells cultured with commercial L15 supplemented with 0.9 and 1.8% honey solution was greater than 2-fold above the lowest concentration of honey solution tested (0.45%), these levels were at least 4-fold lower than the standard medium used to maintain the AmE-711 cell line.

Qualitatively, the condition of AmE-711 cells cultured in commercial L15 supplemented with either glucose or a mixed carbohydrate solution made from honey was poor. Most cells were rounded with one to several large vacuoles, and the cytoplasm was granulated. Furthermore, cells either lacked cytoplasmic extensions typical for fibroblast-type morphology, or if present, these extensions were granular and/or detaching (Figure 4.4B).

4.3.6 Removal of the Bicarbonate Buffer Stimulated AmE-711 Cell Growth

AmE-711 cells were cultured in modified L15 with 10% FBS and 5% TPB but without the addition of sodium bicarbonate. The average protein yield from cells cultured in medium without bicarbonate was almost 7-fold greater than the yield obtained from the baseline condition (Figure 4.5). In addition, the protein yield from cells grown in modified L15 without sodium bicarbonate increased by approximately 2-fold compared to the yield from cells grown in the standard medium (i.e., modified L15 supplemented with 10% FBS, 5% TPB, and sodium bicarbonate) used for routine maintenance of the AmE-711 cell line. This finding suggests that the addition of the bicarbonate buffer may have an inhibitory affect on AmE-711 cell growth.

4.3.7 Culturing Cells in 3% CO_2 at 34°C Improved AmE-711 Cell Growth

AmE-711 cells were cultured in modified L15 with free gas exchange in a culture environment regulated at 3% CO_2 and 34°C. Under these conditions, the protein yield obtained from AmE-711 cells increased by 6-fold compared to the yield obtained from the baseline condition (Figure 4.5). Furthermore, the protein yield was comparable to cells cultured with standard medium without sodium bicarbonate in a sealed vessel at 32°C. This finding shows that culturing cells at a slightly higher than normal temperature (34°C) with free gas exchange in a 3% CO_2 atmosphere expands the range of culture conditions that sustain, and possibly enhance, the growth of AmE-711 cells.

4.4 DISCUSSION

One limitation to finding new treatment strategies that are active against the numerous intracellular pathogens currently plaguing beekeeping operations in the U.S. and other parts of the world is a shortage of available of cell lines derived from honey bee tissues that can be used for axenic growth of these pathogens. To our knowledge, only two continuous honey bee cell lines have been reported in the literature (Kitagishi et al., 2011; Goblirsch et al., 2013). Of these two, the AmE-711 cell line is freely available but difficult to maintain (Goblirsch, personal observation). Several factors that may contribute to the fastidious nature of AmE-711 cells may lie in the fact that it is a nascent line and requires further optimization of the growth medium or other culture conditions currently employed for the maintenance of the line. In this work, the influence of some of the supplements to the L15 medium on the growth of AmE-711 cells was investigated as a step towards further optimizing culture conditions. We analyzed the effects of different concentrations of several supplements on the growth of AmE-711 cells at their current passage (36 – 40) using medium comprised solely of commercially available L15 or modified L15 that is routinely used to maintain the cell line. We also examined the effect of culturing AmE-711 cells with free gas exchange in a 3% CO_2-enriched atmosphere at a higher than normal temperature (34°C).

The addition of FBS to modified L15 medium was necessary for the growth of AmE-711 cells in culture. AmE-711 cell growth, as measured by protein yield, was greatest at 10% FBS and decreased linearly with the reduction in serum concentration. Moreover, supplementing modified L15 with 5% FBS without TPB maintained a basal

level of cell growth and cells appeared healthy with fibroblast-type morphology. As expected, one benefit of supplementing culture medium with FBS is that it provides factors (e.g., proteins, hormones, lipids and fatty acids, vitamins, trace minerals, carbohydrates, amino and nucleic acids) required for attachment, growth, and proliferation. Serum is a mammalian-derived supplement commonly added to growth medium used for the culture of insect cells and is considered a substitute for insect-derived supplements such as hemolymph or other extracts (e.g., egg extract) that may be difficult to obtain in sufficient quantities. Much progress has been made in the identification of the constituents of serum that show biological activity in processes such as cell proliferation and differentiation; however, it is still considered an undefined supplement.

Royal jelly is secreted by hypopharyngeal glands of adult workers and is used to feed larvae, with larvae destined to become queens receiving significantly greater amounts compared to larvae destined to become workers or drones. The composition of royal jelly consists of proteins, amino acids, carbohydrates, fatty acids, vitamins, and trace minerals (Krell, 1996). Royal jelly is marketed as a health product for human consumption, and can be obtained easily unprocessed in large quantities. Since queens are long-lived, have larger bodies, and have larger, highly prolific ovaries relative to workers, I wanted to test whether the addition of this undefined insect-derived supplement stimulated the growth of AmE-711 cells, and potentially serve as a replacement for mammalian-derived FBS and TPB. AmE-711 cells exposed to modified L15 supplemented with royal jelly showed improvement in growth compared to cells

exposed to modified or commercial L15 that did not contain royal jelly. Although growth did not reach the same level as that obtained with the standard medium used to maintain the AmE-711 cell line, cells cultured in modified L15 supplemented with royal jelly appeared normal with adherent fibroblast-type morphology. The tolerance of AmE-711 cells to culture medium containing royal jelly may provide a system to study the role of royalactin and epigenetic modification of the honey bee methylome, which are hypothesized to be factors that maintain queen and worker phenotypes from a common genome (Foret et al., 2009; Lyko et al., 2010; Kamakura, 2011).

Eliminating or reducing undefined supplements derived from mammals such as FBS or TPB to growth medium for the culture of AmE-711 cells has distinct advantages. From a practical standpoint, removal reduces cost, issues with supplier and lot variability, or the potential for contamination with adventitious agents. Moreover, many transfection protocols have been optimized for use under serum-free conditions. Although several insect cell lines can be cultured in serum-free medium (Brunner et al., 2010), the AmE-711 cell line failed to proliferate in medium that did not contain FBS and/or TPB. However, AmE-711 cells did appear healthy at least through 14 days of incubation in medium that did not contain FBS and/or TPB. Healthy, non-dividing AmE-711 cells have utility. For example, they could be used for assays that evaluate metabolic stress, cytotoxicity, or genotoxicity due to exposure to field-relevant doses of agrochemicals that honey bee colonies may encounter in their environment. In tandem with other cells lines derived from vertebrates and invertebrates, this information could be important for cell-

based screens of agents that have high specificity against pests yet are safe to honey bees, humans, and other non-target species (O'Neal et al., 2013).

Serum deprivation may also lead to synchronization of the division cycle of the cell population (Hatt et al., 1994; Langan and Chou, 2011). Synchronized, non-dividing AmE-711 cells could be used to characterize the kinetics of viral entry, replication, and cytopathogenesis of the many of picornaviruses that cause disease in honey bees and how these parameters compare to a population of comprised of proliferating cells. Further studies are needed to determine if the reintroduction of FBS to serum-deprived AmE-711 cells reinstates mitotic activity.

The major sugar found in commercial L15 is galactose, and does not contain glucose; therefore, glucose was added by Munderloh and Kurtti (1989) because it is the principal sugar of tick hemolymph (Bassal, 1986) and was shown previously to support the growth of tick cells *in vitro* (Řeháček and Brzostowski, 1969). Although the major sugar of honey bee hemolymph is trehalose (20 mg/mL), a disaccharide formed from two units of α-glucose, it also contains significant levels of glucose (16 mg/mL) and fructose (11 mg/mL) (Fell, 1990). We found that AmE-711 cells showed minimal growth when glucose alone or the mixture of carbohydrates typically found in honey, mainly fructose and glucose, were added to commercial L15 supplemented with 10% FBS and 5% TPB. This finding suggests that other compounds in addition to glucose are needed to stimulate the growth of AmE-711 cells. For example, the addition of trace minerals (e.g., Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mo^{6+}, Se^{2-}, Zn^{2+}) above levels supplied in FBS may be a necessary

addition to commercial L15 to support AmE-711 cell growth as it provides cofactors for reactions that metabolize glucose and other carbohydrates.

The AmE-711 cell line was isolated and is maintained in sealed vessels at 32°C. AmE-711 cell growth was improved when incubated with free gas exchange in a 3% CO_2-enriched atmosphere at 34°C. Reports by Hunter (2010) and Goblirsch et al. (2013) suggest that the growth rate of honey bee cells *in vitro* is retarded when cultured at temperatures below 32°C. When larvae and pupae are present, honey bees (i.e., *Apis mellifera*) maintain nest temperature within a relatively narrow range (33 - 36°C; Seeley, 1985). The rearing of brood at temperatures outside of this range can have negative affects on task performance, learning, and memory consolidation later during adult worker life (Tautz et al., 2003; Jones et al., 2005). Further studies are needed to determine how temperatures that bees are exposed to during development are linked to biochemical processes that promote AmE-711 cell growth and proliferation *in vitro*.

Sodium bicarbonate is added to the medium for the maintenance of the AmE-711 cell line in sealed vessels. The increase in CO_2 and H_2O as byproducts of respiration from increased cell proliferation acidifies the medium towards non-physiological. By adding bicarbonate, excess H^+ can react with the bicarbonate ions (HCO_3^-) forming carbonic acid (H_2CO_3) to stabilize the pH. Interestingly, AmE-711 cells cultured in bicarbonate-free medium obtained more growth compared to cells grown in medium that contained bicarbonate. One explanation for increased cell growth in bicarbonate-free medium may pertain to the frequency of the medium exchange during the 14-day incubation period. Medium was replaced with fresh medium every third or fourth day

during the experiments. The pH increased noticeably when medium that contained bicarbonate was exchanged, possibly because the bicarbonate ions reacted with H^+ to inflate the pH until it could be offset by a rebound in cell respiration. A pH shift towards alkaline may have been prevented when medium was replaced without bicarbonate. The transient shift toward alkaline may have an affect on enzymes that catalyze substrates for cell growth. L-glutamine is added to the modified L15 formulation used for the maintenance of the AmE-711 line. Glutamine is catalyzed into glutamate and ammonia by glutaminase. It has recently been shown that cell growth is greatest when glutaminase activity is elevated at or below physiological pH and that the ammonia produced may act to neutralize the pH in cancer cells (Huang et al., 2013).

Demonstrating the utility of *in vitro* systems derived from honey bees rests on the availability of “robust” cell lines (Li et al., 2014). The shortcoming in isolating and maintaining honey bee cells in culture has been partly due to a failure to provide an appropriate culture environment that promotes *ex situ* adaptation. The experiments performed above represent a step towards refining and identifying the range of culture conditions capable of supporting growth of honey bee cells using the AmE-711 continuous cell line as a model. This fundamental knowledge will aid other attempts seeking to expand upon this much needed resource that could be used for honey bee conservation.

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Authors' Contributions

MG and TK conceived and designed the experiments. MG carried out the experiments and performed the statistical analyses. MG drafted the manuscript. The authors read and approved the final manuscript.

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4.6 FIGURE LEGENDS

Figure 4.1 The effect of fetal bovine serum concentration on the growth of *Apis mellifera* cells (AmE-711) in culture.

A. The yield of cell protein realized after 14 days using modified L15 (solid line) and commercial L15 (dashed line) supplemented with different concentrations of FBS compared to the yield of cell protein from baseline cultures after 24 hours. Each point represents the mean value obtained ± standard deviation.

B . Morphology of AmE-711 cells cultured in the absence of serum. AmE-711 cells cultured in modified L15 (upper panel) or commercial L15 (lower panel) that did not contain FBS had fibroblast-type morphology with rounded or ovoid soma, a conspicuous nucleus, and spindle-like projections of the cytoplasm that terminated as thin filopodia. Cytoplasmic extensions of cells cultured in commercial L15 without FBS were uneven along their longitudinal axis (arrow) and several cells had a nuclear area that showed the presence of several small vesicles (asterisk). Scale bar = 10 µm.

Figure 4.2 The effect of tryptose phosphate broth concentration on the growth of *Apis mellifera* cells (AmE-711) in culture.

A. The yield of cell protein realized after 14 days using modified L15 (solid line) and commercial L15 (dashed line) supplemented with different concentrations of TPB compared to the yield of cell protein from baseline cultures after 24 hours. Each point represents the mean value obtained ± standard deviation. The dotted line represents the yield of cell protein realized after 14 days using modified L15 supplemented with 10% FBS and 5% TPB, which is the standard formulation used for the routine culture of AmE-711 cells.

B. Morphology of AmE-711 cells cultured with varying concentrations of TPB. AmE-711 cells were cultured in modified L15 (upper panel) or commercial L15 (lower panel) supplemented with varying concentrations of TPB. Large vacuoles, with some crescent-shaped and abutting the nucleus, were observed in cells cultured in modified L15 with 0 and 2.5% TPB at day 7 (white arrow) and 14. The presence of one to several large vacuoles was also found in cultures grown in commercial L15 supplemented with different concentrations of TPB. Scale bar = 10 µm.

Figure 4.3 The effect of royal jelly concentration on the growth of *Apis mellifera* cells (AmE-711) in culture.

The yield of cell protein realized after 14 days using modified L15 (solid line) and commercial L15 (dashed line) supplemented with different concentrations of royal jelly compared to the yield of cell protein from baseline cultures after 24 hours. Each point represents the mean value obtained ± standard deviation. The dotted line represents the yield of cell protein realized after 14 days using modified L15 supplemented with 10% FBS and 5% TPB, which is the standard formulation used for routine culture of AmE-711 cells.

Figure 4.4 The effect of carbohydrate concentration on the growth of *Apis mellifera* cells (AmE-711) in culture.

A. The yield of cell protein realized after 14 days using commercial L15 supplemented with different concentrations of glucose (solid line) and honey (dashed line) compared to the yield of cell protein from baseline cultures after 24 hours. Each point represents the mean value obtained ± standard deviation. The dotted line represents the yield of cell protein realized after 14 days using modified L15 supplemented with 10% FBS and 5% TPB, which is the standard formulation used for routine culture of AmE-711 cells.

B. Morphology of AmE-711 cells cultured with varying concentrations of glucose or honey. AmE-711 cells cultured in commercial L15 supplemented with either glucose (1.8%; upper panel) or a mixed carbohydrate solution made from honey (1.8%; lower panel) were in a state of deterioration, regardless of concentration. Cells appeared rounded with one to several large vacuoles, and the cytoplasm was granulated. Furthermore, cells either lacked cytoplasmic extensions typical for fibroblast-type morphology, or if present, these extensions were granular and/or detaching. Scale bar = 10 μm.

Figure 4.5 The effect of supplement removal and culture environment on the growth of *Apis mellifera* cells (AmE-711).

The yield of cell protein realized after 14 days using modified L15 supplemented with 10% FBS and 5% TPB but without sodium bicarbonate buffer (gray bar) and incubated with free gas exchange in a 3% CO_2-enriched atmosphere at 34°C (white bar) compared to the yield of cell protein from baseline cultures after 24 hours. Each bar represents the mean value obtained ± standard deviation. The dotted line represents the yield of cell protein realized after 14 days using modified L15 supplemented with 10% FBS and 5% TPB, which is the standard formulation used for routine culture of AmE-711 cells.

Figure 4.1A

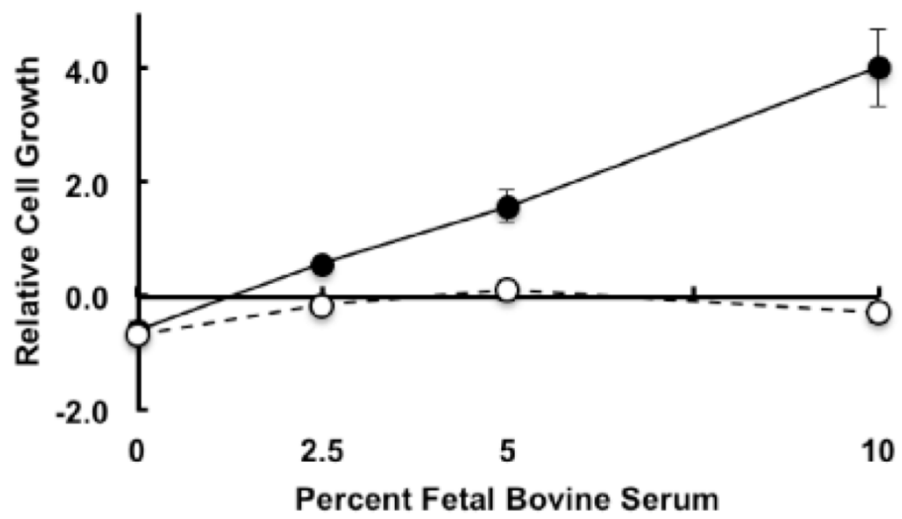


Figure 4.1B

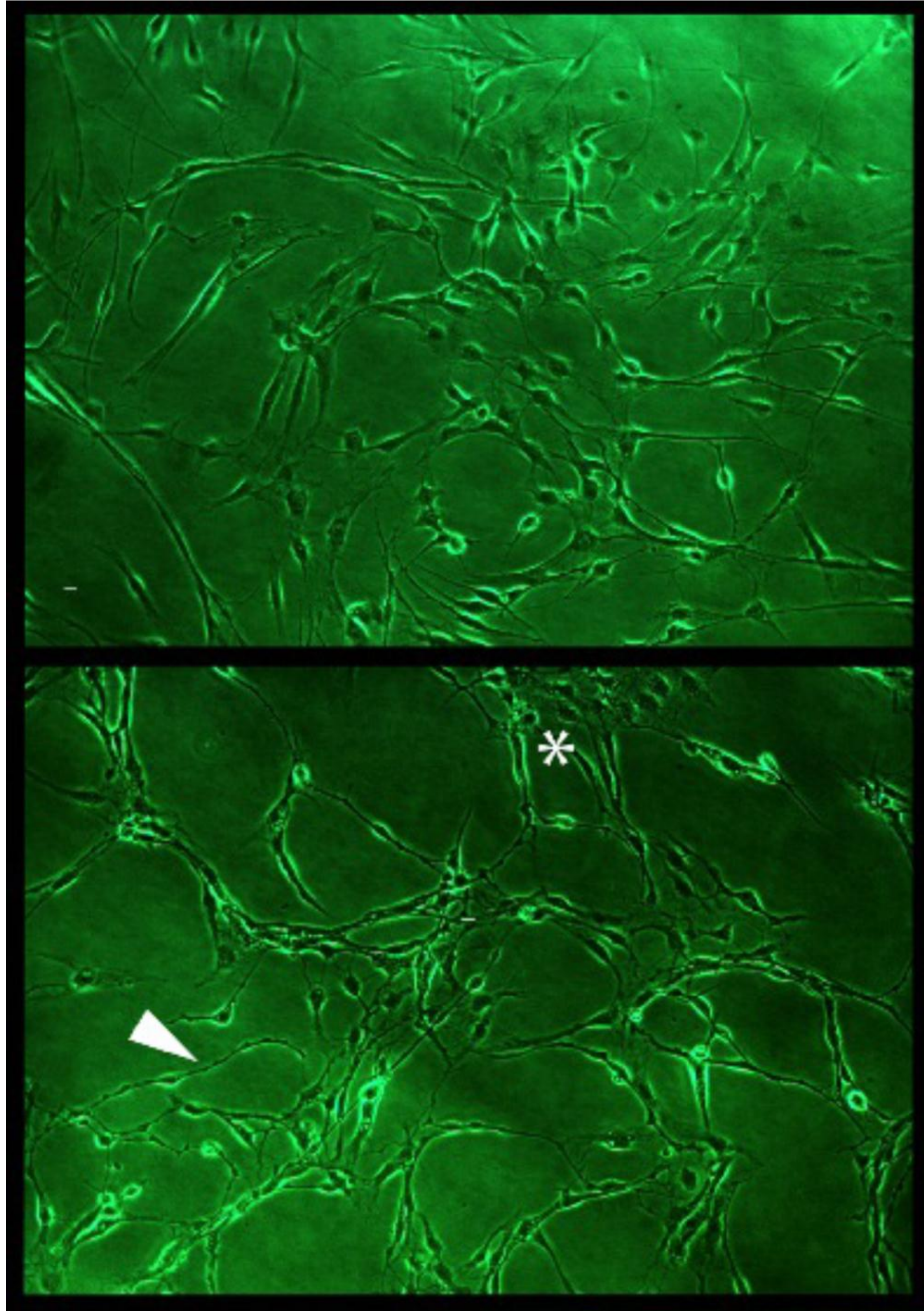
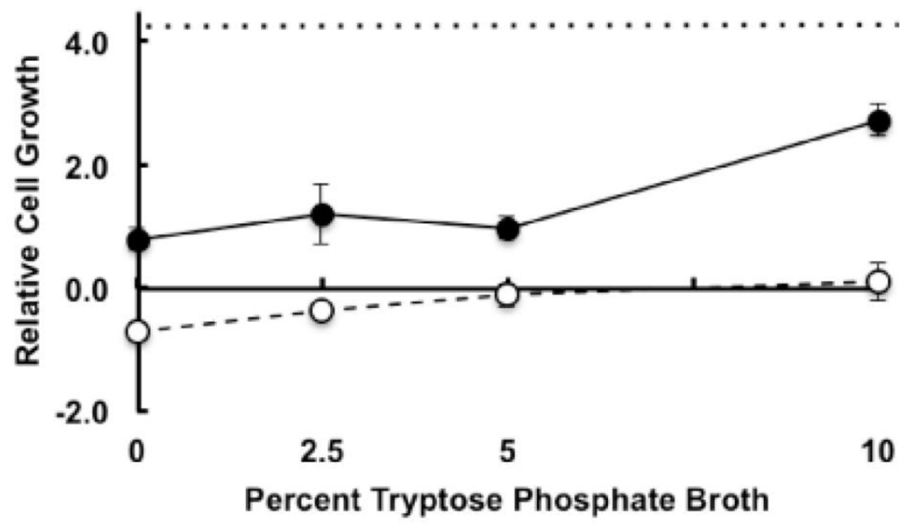


Figure 4.2A



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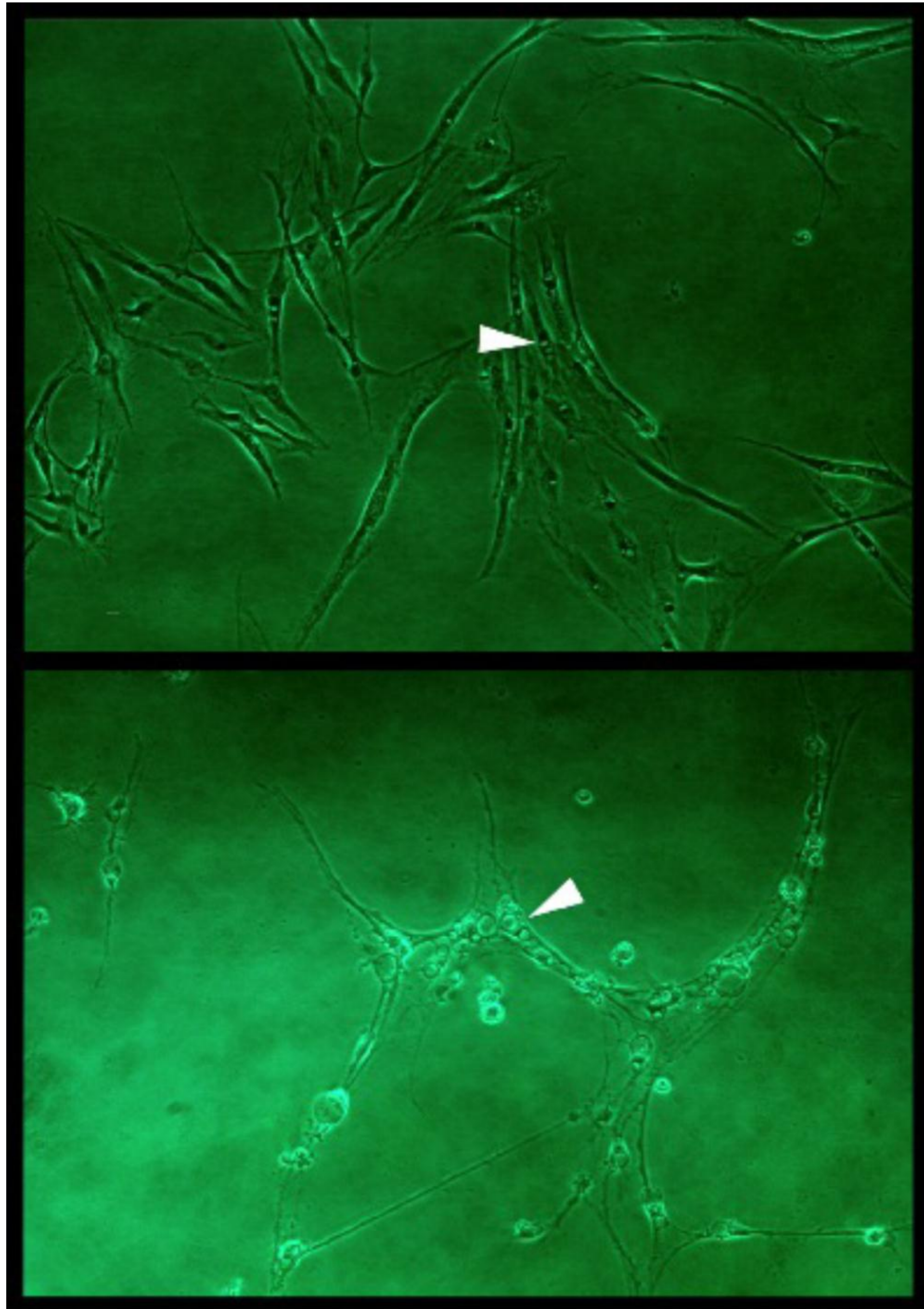


Figure 4.3

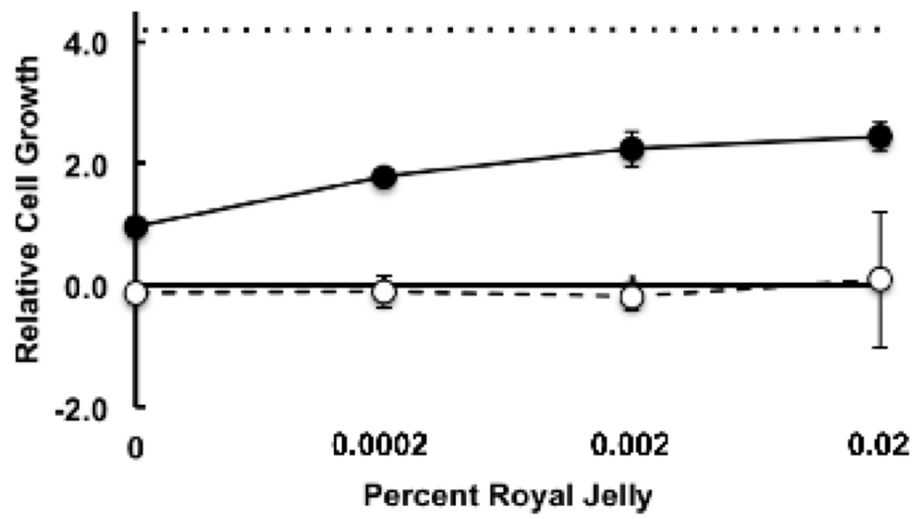
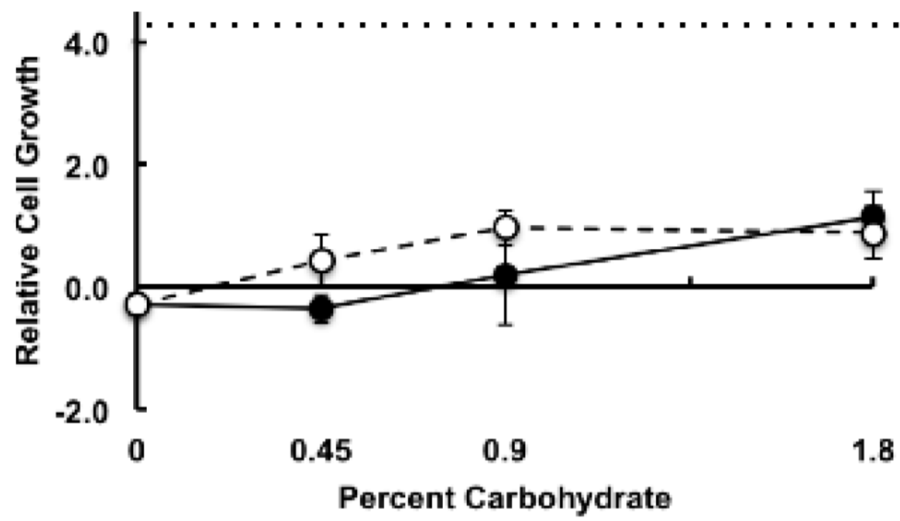


Figure 4.4A



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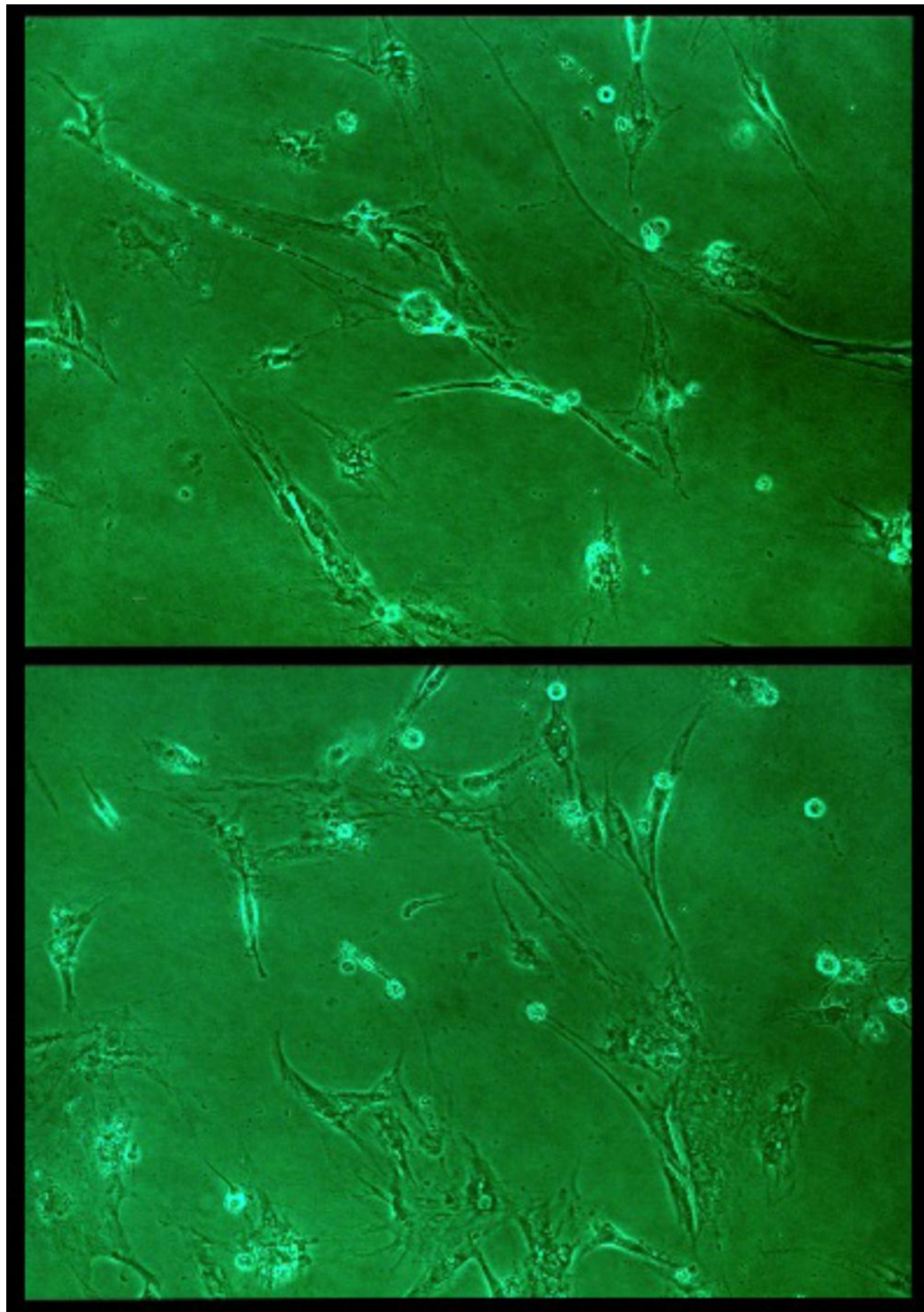
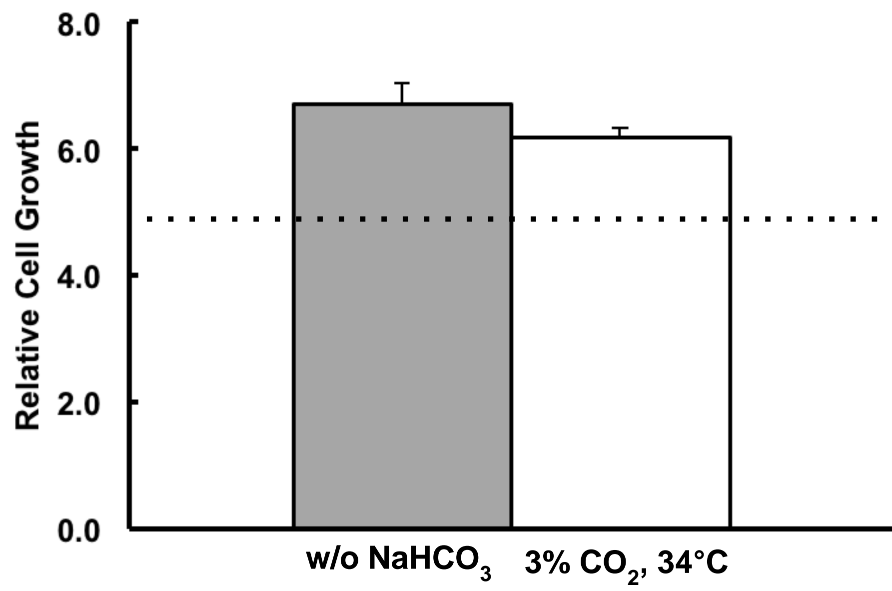


Figure 4.5



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APPENDIX 1. Table A1. Comparison of the number of marked pollen foragers that had presence/absence of spores.

It was possible that some of the control bees observed at the entrances during the foraging studies conducted for Chapter II were infected naturally after being introduced into colonies, as most honey bee colonies now have *N. ceranae*. It was also possible that some of the bees that were inoculated with *N. ceranae* never became infected. To make sure that the number of foragers observed during the precocious window of foraging was due to infection, only those bees that had evidence of spores by microscopy from the *Nosema*-infected group were compared to control bees that showed no evidence of spores. Counts between rows within category followed by different letters are significant for the marked pollen forager data (Chi-squared test, $p<0.05$).

Table A.1

Trial	Marked pollen foragers			
	Control	Infected	χ^2	*p*-value
1. July, 2010	19[a]	35[b]	4.31	0.0379
2. August, 2010	11[a]	24[b]	4.21	0.0402
3. July, 2011	15[a]	45[b]	14.45	<0.0001
Total	45[a]	104[b]	23.24	<0.0001

APPENDIX 2. Considerations for future research on infection studies with *Nosema* using honey bee workers in cages or colonies.

Individual level infection – In order to diagnose infection with *Nosema*, the ventriculus (i.e., site of infection) must be included in the sample that is to be inspected. Dissecting the alimentary canal intact is not an easy task. As the alimentary canal is teased from the body at the distal end of the abdomen, it is possible to sever the canal at the thin membranous connection between the ventriculus and the small intestine, leaving the ventriculus behind in the abdomen. To avoid incomplete dissection of the alimentary canal, it may be more appropriate to detect infection and quantify spores using PCR-based methodologies with DNA/RNA extracted from whole abdomens. The integrity of DNA/RNA is preserved for the long-term when stored at -80°C and can be used for other downstream analyses such as measuring a gene of interest.

Inoculating bees – *Nosema* spores should be used within 1 month of purification to avoid a decrease in viability and infectivity. When preparing the inoculum, spores should be suspended in 50% w:v sucrose solution. The same sucrose solution without spores should be used as an inoculum for the control bees. Spores should be administered orally and on an individual basis. By gently holding each bee between the thumb and forefinger, a pipette tip can be applied near the mouthparts for the bee to consume the inoculum. Inoculating bees individually allows for control of the volume (i.e., dose) that each target bees receives. Five µL, administered with a narrow pipette tip, is a sufficient volume for a bee to consume in its entirety in a timely manner. Selecting the age of bee

to inoculate can be matched to correspond to the research question at hand. One advantage of using newly-emerged bees is that they likely have an empty honey crop, have low activity, and their cuticle is not hardened, rendering the sting apparatus ineffective. However, inoculating bees at different ages may provide a more thorough picture of host-pathogen dynamics between honey bees and *Nosema*.

Putting bees in cages – In addition to inoculated bees, adding bees that did not receive an inoculum to cages will keep a relatively high number of bees as inoculated bees are sampled (i.e., removed from the cages). This will minimize any effects that may result from changing the interaction rate between individuals due to a diminishing population may have on the behavioral or physiological patterns of those bees that remain in the cages. Bees that did receive an inoculum can be paintmarked to distinguish them from the experimental bees.

Putting bees in colonies – Unlike cages where it is best to keep experimental groups segregated to avoid infecting control bees, when performing colony-level studies it may be best to introduce both *Nosema*-inoculated and sucrose-inoculated control bees into the same colonies. Introducing both groups into the same colonies ensures that they are exposed to the same conditions.

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