Presence of the microsporidian *Nosema ceranae* in the honeybee *Apis mellifera* and the effect on the earwig *Euborellia annulipes'* ovarian basal follicle length and return time to egg clutch

By
Kelsey Braun
April, 2010



Allegheny College
Department of Biology

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

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Pledge

Approval by the Senior Thesis Committee:

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Dr. Susan Rankin

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ABSTRACT

The presence of the microsporidian *Nosema ceranae* in honeybees *Apis mellifera* is strongly correlated with the worldwide problem of Colony Collapse Disorder (CCD). To observe its effect on ovary development and possible disorientation effects, spores were obtained from honeybees and applied at 4 different concentrations to carrot for *Euborellia annulipes* earwigs to consume. Each earwig was given carrot, as needed, with 100 μL of either purified water ("Untreated"), crushed up bee solution without spores ("Bee Gut No Spore"), 1:10 diluted *Nosema ceranae* bee solution ("Low Spore Count"), or original concentration of bee solution containing spores ("High Spore Count"). An initial 7-day mortality test was conducted with 0-day females, and their ovaries were dissected at 17-days after treatment initiation. The group treated with "Bee Gut No Spore" had significantly smaller ovaries, which did not support my hypothesis that those infected with the higher concentration of *N. ceranae* would have the shortest basal follicles. Those from the "Low Spore Count" and "High Spore Count" had similar follicle lengths, and the earwigs ingesting the lower concentration of spores ate significantly more carrot than the others. I also tested the behavioral effects of *N. ceranae* in earwigs by contaminating food as mentioned above and observing the amount of time taken to return to their egg clutches when separated from them. A physical barrier was used in half of the trials, and it significantly increased the earwig's time to return to her egg clutch, as I hypothesized. There was no significant difference in return time between the treatment groups, refuting my hypothesis that those from the higher concentration would take the longest to return to their clutches.

INTRODUCTION

In recent years, Colony Collapse Disorder (CCD) has become a major worldwide problem. Honeybees (*Apis mellifera*) have been disappearing from their hives, and 29% of beekeepers in the USA reported losing 75% of their bees in 2006-2007 (Winfree et al. 2007; Higes et al. 2008). These substantial losses pose a serious threat to the global economy because honeybees are used for the pollination of many fruit crops. In the US alone, they pollinate 95 different fruits and vegetables, and the total contribution of pollination in terms of added value to fruit crops exceeds $15 billion per year; the worldwide CCD-related losses have been estimated at $75 billion (Stokstad 2007b; Maori et al. 2009).

Although bees occasionally abandon their hives if disturbed, there have been unusual observations of collapsed hives. The queen usually remains, surrounded by untended brood. Other insects, such as moths or hive beetles, don't rob the abandoned hives of honey or nectar, suggesting that the hives are contaminated. Unfortunately, the potential source of contamination or the exact cause of these collapses is unknown and expected to be a complex array of factors. The most novel suspects include bacteria, fungi, parasites, and viruses (Cox-Foster et al. 2007).

A prominent virus strongly associated with Colony Collapse Disorder is the Israeli acute paralysis virus (IAPV). It was first described in 2004 in Israel, where infected bees presented with shivering wings, progressed to paralysis, and then died outside the hive (Blanchard et al. 2008; Palacios et al. 2008; Cox-Foster et al. 2007). The first detection of IAPV occurred in France, and it was characterized as a member of the *Dicistroviridae* family. Only one complete genome sequence of IAPV has been reported, and in the US metagenomic survey, IAPV was found only in CCD operations, yielding a positive predictive value of 96.1% and a specificity of 95.2%. The prevalence of this virus in CCD indicates that IAPV is a significant marker for CCD

(Cox-Foster et al. 2007). Scientists have not been able to establish a causal relationship between IAPV and CCD. Bees in hives suffering from CCD tend to accumulate many secondary diseases, so IAPV infection could be a consequence rather than a cause of the disorder (Blanchard et al. 2008; Stokstad 2007a).

Another problem associated with Colony Collapse Disorder is infestation by *Varroa destructor* mites. They influence the flight behavior of forager bees, possibly preventing return to the colony. Colony breakdown caused by this parasite is characterized by a rapid loss of foragers until the colony only contains the queen and a few workers. A significantly larger proportion of foragers departing the colony were infected, compared to foragers returning to the colony. This could be because foragers lose a considerable number of mites by unloading them during foraging or while temporarily visiting other colonies. The median time outside the colony was 1.7 times higher in the infected workers compared to the uninfected workers (Kralj and Fuchs 2006).

The extended returning times or lack of return of infested foragers might be due to impaired orientation, rather than a change of motivation. The changed flight and orientation behavior may be interpreted as an adaptive response. The colony could benefit from the infested bee failing to return to the colony, because that would then help to prevent transmission from the infested workers to the rest of the hive (Kralj and Fuchs 2006).

Research on microsporidians, such as *Nosema ceranae,* has continued to progress, and there is a direct correlation between infection and the death of honeybee colonies (Higes et al. 2008). Microsporidia are a very specialized group of intracellular parasites related to fungi (Antúnez et al. 2009) found in many invertebrate (mainly in arthropods) and vertebrate (mainly in fishes) species. The parasites can be transmitted either exclusively horizontally (many

species), both horizontally and vertically, or exclusively vertically (rare), but they do not disturb the course of oogenesis (Świątek and Górkiewicz 2006). Microsporidians are very severe on their hosts because they lack mitochondria and therefore have little metabolic ability themselves (Mayack and Naug 2009). *Nosema* disease is one of the most prevalent adult honey bee diseases and is caused by two described species of microsporidia: *Nosema apis* and *N. ceranae* (Chen et al. 2008). In fact, the overall prevalence of any *Nosema* species in the US metagenomic survey was 94.1% (100% CCD; 85.7% non-CCD) (Cox-Foster et al. 2007).

The genus *Nosema* has more than 150 species, usually infecting invertebrate hosts, including at least 12 orders of insects, and they are most commonly found in Lepidoptera and Hymenoptera (Higes et al. 2006). In the honeybee, *Nosema* infection causes impaired protein metabolism, indicated by the lower proteolytic activity of the midgut. The lower amount of amino acids results in a reduced hypopharengeal gland and altered fatty acid composition in the hemolymph. Additionally, infection can cause premature aging and reduced longevity, which alter the honeybee's task division and lead to precocious foraging, thus affecting the bee's flight behavior (Kralj and Fuchs 2010; Mayack and Naug 2009). Pebrine disease in silkworms, caused by *Nosema bombycis*, and dysentery in honeybees, caused by *N. apis*, are some of the well known diseases attributed to *Nosema* (Higes et al. 2006). It also affects various species of beetles, moths, budworms, wasps, locusts, crickets, and grasshoppers but has not been reported in earwigs (Van Frankenhuyzen et al. 2004).

N. apis was the original pathogen associated with *A. mellifera*, and *N. ceranae* was originally the main pathogen of *Apis ceranae* (Huang et al. 2007). However, the finding of *N. ceranae* in *A. mellifera* led to the conclusion that *N. ceranae* most likely jumped host to *A. mellifera*, probably within the last decade. Host colonies and individuals may now be co-

infected by both microsporidia (Klee et al. 2007), but *N. ceranae* is the primary colonizer of *A. mellifera* and is detected all over the world in both healthy and weak honeybee colonies (Higes et al. 2008).

N. apis and *N. ceranae* are phylogenetically quite distinct, but they have similar morphologies under an optic microscope, and SSUrRNA sequence identity is 92%. This 16SrRNA identity is low compared to other hymenopteran species that infect the same or similar hosts (Huang et al. 2007), so partial amplification of the 16S (SSU) rRNA gene sequence using PCR is a reliable method used to differentiate between *N. apis* and *N. ceranae* (Klee et al. 2007).

N. ceranae is more capable of adapting to high temperatures than *N. apis*. In a recent study, when *N. ceranae* was kept at 60°C for 2, 3, 4, and 6 hours, the viability of the spores did not significantly decrease, and the viability was maintained after 1 month as well. However, heating *N. apis* spores for at least 15 minutes at 60°C kills the parasite. When *N. ceranae* was kept at 4°C, however, a significant reduction in spore viability to 87.9% was observed in the third month, suggesting that care must be taken in order to optimize the spore conservation in laboratory settings (Fenoy et al. 2009).

Transmission of *Nosema* in honeybee colonies is mainly via the fecal-oral route, in which pathogens are spread by transferring feces of diseased hosts to uninfected hosts via ingestion. Adult bees ingest *Nosema* spores when they are eating contaminated food and when they are cleaning up fecal material from infected bees. The spores germinate within the midgut and release polar tubes that transfer their sporoplasm into midgut epithelial cells where they generate more spores. Millions of new spores can be found inside of a bee's midgut a few weeks after initial infection, and the spores voided with feces become new sources of the infection in the colonies. The bees do not exhibit obvious external disease symptoms, but *Nosema* infections

cause digestive disorders, shortened life spans, decreases in population sizes of honeybee colonies, and reduction of honey production and crop products that rely on bees for pollination (Chen et al. 2008).

When honeybees are infected with *N. ceranae*, the infection appears as isolated islands of single infected midgut epithelial cells. The parasites multiply well within the basal cells of the epithelium, and the autoinfective capacity of the spores rapidly spreads the infection. Under normal circumstances the high regenerative capacity of healthy digestive epithelium commonly compensates for partial cellular destruction, but when the parasites destroy the basal cells, alterations are irreversible and the infected bee dies. The high mortality observed after *N. ceranae* infection demonstrates a high pathogenicity of the microsporidian to *A. mellifera* (Higes et al. 2006). When experimentally inoculated into the honeybee, colony depopulation is 6 times higher in infected colonies than in uninfected ones (Higes et al. 2008).

Foragers were always more infected than interior bees, and there are four phases of natural *N. ceranae* infection. In Phase 1, 'asymptomatic' phase, from the outside, infected colonies looked similar to those of uninfected hives, and fewer than 60% of foragers were infected. Phase 2, 'replacement' phase, started when unusual colony behaviors were detected, as when the queen continued to lay eggs throughout the winter months. The third phase, 'false recovery' phase, started when the colonies built up rapidly and the queens laid so many eggs that almost every comb was full of brood. Phase 4, 'depopulation' phase, was observed when fewer than 40% of frames were covered with bees. In this phase, the bees were very active but 2 months later the queen, surrounded by only young bees, was found dead, and a larger proportion of interior bees were infected (Higes et al. 2008).

Since *Nosema* sp. and *V. destructor* mites inhibit the bees' abilities to return to their colony by affecting their flight behavior I wanted to see if infection by *Nosema ceranae* had the same effect in other organisms. Due to the risk of injury to other students with possible bee allergies, I was unable to set up live bee colonies at Allegheny College. Therefore, my inability to manipulate live honeybees lead me to use the earwig, *Euborellia annulipes*, to observe whether *N. ceranae* infection of mated female earwigs affected their ability to return to their egg clutch when separated from it. Earwigs are widely used for research in maternal care because they invest a lot of time in guarding their eggs and cleaning them from fungi and other pathogens to enhance larval survival (Radl and Linsenmair 1991; Rankin et al. 1995, 1996; Kölliker 2007; Kölliker and Vancassel 2007). They also recognize their own nests by marking them with chemicals to prevent provisioning foreign offspring with food by mistake (Radl and Linsenmair 1991). These characteristics make the earwig a good organism to use because they have high motivation to get back to their nest and do not easily mistake their nest for another earwig's.

A physical barrier was also used to accurately simulate the earwigs' separation from their clutches in the environment and prevent them from being able to constantly see their clutches. I hypothesized the physical barrier to cause the earwigs to take longer to return to their egg clutches. For those with no barrier, I hypothesized the earwigs infected with the higher concentration of *N. ceranae* spores would have a longer return time to their egg clutches. This hypothesis was also applied to the group of earwigs that had a physical barrier. Since *N. ceranae* infection can cause oocyte degeneration in queen honeybees (Liu 1992), or enhance the queen's egg laying in other cases (Higes et al. 2008), ovary development characteristics were also observed, using earwigs separate from the time trials. I hypothesized that the ovaries would be smaller in females that were infected with the higher concentration of *N. ceranae* spores.

METHODS

Preliminary bee dissections

I obtained honey bees (*A. mellifera*) from a local beekeeper in the Meadville, PA area and stored them in a wooden bee cage with wire netting on two sides. The bees were fed 1:2 water:sugar solution, with some molasses in it as well. When ready to perform the midgut dissection, I moved the bees to a cold room at 4ºC for 30 minutes or until all of them were immobile. The midguts were dissected while in the cold room to prevent getting stung and then placed on a microscope slide in saline. I cut along the length of the midgut to make sure that the contents were sususpended in the saline and viewed the contents under a microscope (40X) after staining with methylene blue.

N. ceranae preparation

I was unable to isolate *N. ceranae* from the bee dissections. Therefore, *A. mellifera* that were known to have *N. ceranae* spores present were obtained from the Pennsylvania Department of Agriculture. I also obtained honeybees that did not have spores present. The samples were sent in 50 mL conical tubes and preserved in 70% ethyl alcohol. Using a mortar and pestle, I ground up 10 honeybees containing spores in 10 mL of purified Milli Q water, and the liquid was transferred to a 15 mL conical tube. This sample was used for the "High Spore Count" treatment and was diluted (1:10) with purified water to obtain the "Low Spore Count" treatment. I determined the *N. ceranae* spore concentration by injecting 10 µL of the diluted sample into both sides of a hemocytometer, counting the spores in the middle 25 squares, and averaging the count obtained from both sides. The concentration was calculated, and I multiplied by 10 to get the concentration of the original "High Spore Count" mixture (Appendix). I then ground up 10

honeybees without spores in the same manner, but I did not dilute the solution. All samples were stored at 4°C.

Earwig maintenance

All earwigs (*E. annulipes*) were incubated at 28ºC during a 12h light:12h dark photoperiod. They had shelter in 200-mL containers and were provided with either cat food or carrot and a 2-dram shell vial for water. When a carrot slice was given for food, each piece was around 0.2 g, and the mass of every new piece given was always recorded. After testing was complete and earwigs were sacrificed, I measured the mass of the remaining carrot to obtain the total carrot mass ingested by each earwig. The remaining carrot mass from only the tested earwigs was measured.

Effects of *N. ceranae* on mortality and basal follicle length of *E. annulipes*

Sixty 0-day female earwigs were separated into 4 treatment groups (N=15): "Untreated", "Low Spore Count", "High Spore Count", and "Bee Gut No Spores". I used purified Milli Q water for the "Untreated" group. Each earwig was given a disc of carrot (around 0.2 g), and 100 μL of the respective treatment solution was pipetted onto the carrot. The solutions given to the "Low Spore Count" and "High Spore Count" groups contained 15,900 and 159,000 spores, respectively. They were reared individually in 200-mL containers and incubated at 28°C during a 12h light:12h dark photoperiod. I provided them with a 2-dram shell vial for water. I weighed them daily for 7 days to make sure that they were eating and gave them food as needed. For every new piece of carrot, 100 μL of the treatment solution was applied. I dissected the ovaries after chilling the females on day 17. The basal follicle length was determined using a dissecting microscope with an ocular micrometer.

Return time to egg clutch

Earwig Mating

Males and females (all 0-day) were separated by sex and reared under the same conditions as above, but they were given cat food. At day 7, the males were allowed to mate with the females individually. Copulation lasting at least 1 minute ensured that the mating was successful. Immediately after a successful copulation, the female was separated from the male and reared individually at the above conditions, but I gave her a disc of carrot for food and applied 100 μL of a treatment solution to the carrot. Food was given as needed, and I continued the matings until I obtained 25 mated females for each of the 4 treatment groups. After oviposition, I waited 3 days to set them up in the arena so that they could be tested on the 4th day. In previous studies (Sutton, unpublished), mothers with 4-day old clutches returned to the egg clutch faster than those of a different clutch age. Any earwig that did not oviposit after 20 days was sacrificed.

Arena preparation

The arena was a 17 x 17 x 6.5 cm plastic container, and I put 1 cup of Quikrete Premium Play Sand on the bottom to simulate the natural environment so that the mothers could burrow if they wanted. Half of all the trials contained a physical barrier, and the other half did not. I used gravel for the barrier, and spread it in the center along the entire width of the arena, making sure that the earwig would not be able to see her clutch over the barrier when she was separated. Each "3-day" mother earwig was given a 24h acclimation period in the arena. I removed the cotton from the 2-dram shell water vial and placed it on one side of the arena, near the perimeter, and the female and her eggs were placed on the cotton. A piece of the remaining carrot and a new water vial were also placed in the arena, on the same side of the egg nest. I used fresh

gravel and sand for every test. The arenas were kept in a controlled room at 28°C and 12h light:12h dark cycle, and testing was done here as well.

Absence of barrier

After the 24h acclimation period, I observed the earwig's behavior and timed her to make sure that she stayed near her eggs for 1 minute before I separated her from her egg clutch. I placed her on the opposite side of the arena and timed how long it took for her to get back to the egg clutch. Each earwig was given 30 minutes, and I only considered it to be a successful return if she stayed in direct contact with her eggs for 1 minute. If she buried her eggs under the cotton, I watched for when she got back to the cotton and could see that she was digging to find her eggs. Females from the "Untreated" (N=12), "Bee Gut No Spore" (N=10), "Low Spore Count" (N=10), and "High Spore Count" (N=10) treatment groups were tested, and they were sacrificed along with their eggs afterward.

Presence of barrier

Because earwigs encounter environmental barriers when they leave their egg clutches and may not always be able to easily see their clutches, I wanted to determine if the presence of a physical barrier significantly affects the earwig's ability to return to its clutch. Using new mothers with 3-day-old clutches, the same treatment groups were used: "Untreated" (N=14), "Bee Gut No Spore" (N=10), "Low Spore Count" (N=10), and "High Spore Count" (N=11). I set each one up in an arena as above, but I placed gravel across the arena. They were given a 24h acclimation period, and I moved the "4-day" mother to the other side of the barrier. All of the testing restrictions (see above) were applied, and the same conditions had to be met in order to consider the return successful. I took note of the time if the earwig stayed under a rock for a long period, and after the 30 minutes I checked the area to see if she had moved her egg clutch

underneath the rocks. If she had, this previously-noted time was considered to be the clutch return time.

Statistical Analysis

Data were analyzed for significant main effects using a 1-way analysis of variance (ANOVA). A 2-way ANOVA was used when comparing the return time to the egg clutches between the treatment groups and the presence and absence of a barrier.

RESULTS

Basal follicle length

I was unable to obtain measurements of 15 basal follicles in each group due to deaths, inability to find ovaries because of underdevelopment, and having accidental males due to sexing error. There was only 1 death, and it was from the "High Spore Count" group. There was a difference in follicle length between the "Untreated" (N=15) and spore count treatments ("Low" (N=14) and "High" (N=12)), but it was not statistically significant (ANOVA, P=0.12 and P=0.19, respectively). The basal follicle lengths of females from the "Bee Gut No Spore" group (N=11) were significantly smaller (P=0.001) than those from the other 3 treatment groups (Fig. 1). Almost all of the females from the "Bee Gut No Spore" groups had clear, undeveloped ovaries rather than yellow ones seen in most of the females from the other groups.

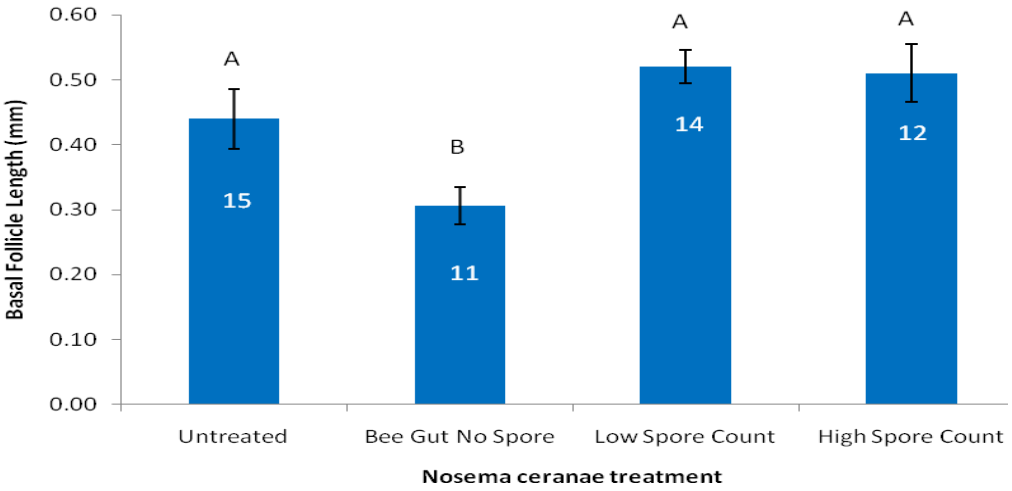

Figure 1. Mean length of basal follicles from ovaries of 17-day *E. annulipes* after *N. ceranae* ingestion. Sample sizes are indicated within the bars, and error bars represent standard error. Means with the same letter above the bars are not significantly (P<0.05) different.

After measuring the amount of carrot leftover in each dissected earwig's container and determining the total amount of carrot ingested after 17 days, individuals from the "Low Spore Count" ate significantly more (P<0.0001) carrot than those from the other treatment groups (Fig. 2). All of the earwigs ate the center of the carrot first, regardless of treatment, and then work their way out to the perimeter of the carrot disc.

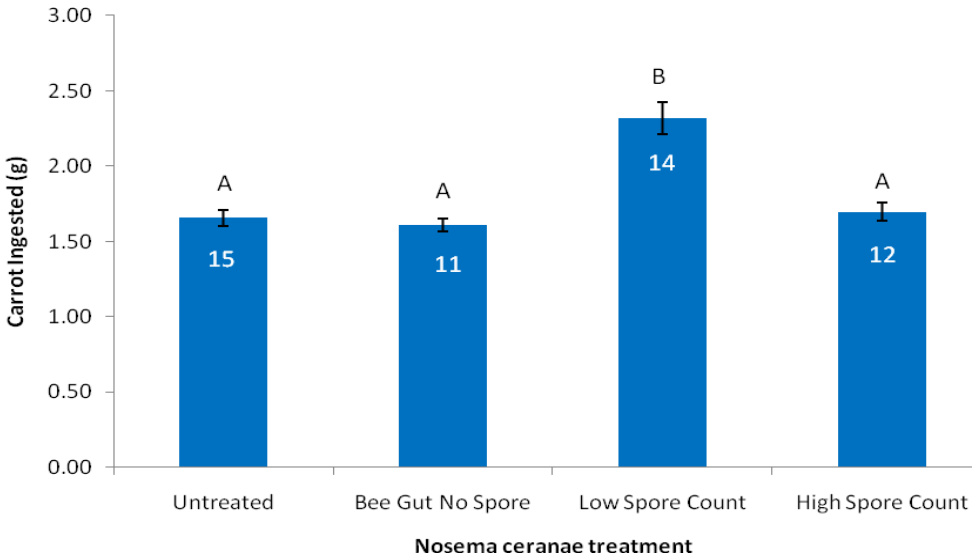

Figure 2. Mean carrot mass ingested after 17 days of *N. ceranae* application. Sample sizes are indicated inside each bar, and error bars represent standard error. Means with the same letter above the bar are not significantly (P<0.05) different.

Return time to egg clutch

After mating 130 females with 130 males, only 87 time trials were conducted due to the females either not ovipositing or eating their eggs. Females ate their eggs most when treated with the "High Spore Count" solution. Those females also had the shortest mean time taken to return to the egg clutch when no physical barrier was present, but the highest return time when the barrier was present; however, these observations were not statistically significant, and there was no other significant difference between return times among the treatments within the barrier categories (P=0.93) (Fig. 3). The mean clutch return time for all of the earwigs with the barrier present, regardless of treatment group, was significantly longer (P=0.006) than those with no barrier present (Fig. 3). If the earwigs took a much longer time to find their egg clutches or did not find them at all, I found that they were not consistently looking for their eggs. When the barrier was present, most often the females would go underneath a rock after 2 minutes if they had not found their clutch by then. Only 1 female moved her entire clutch underneath a rock during the 24h acclimation period the night before, and a few moved their clutches to the water vial. Some of the earwigs would travel along the perimeter of the arena and eventually find their clutch by being guided by the wall.

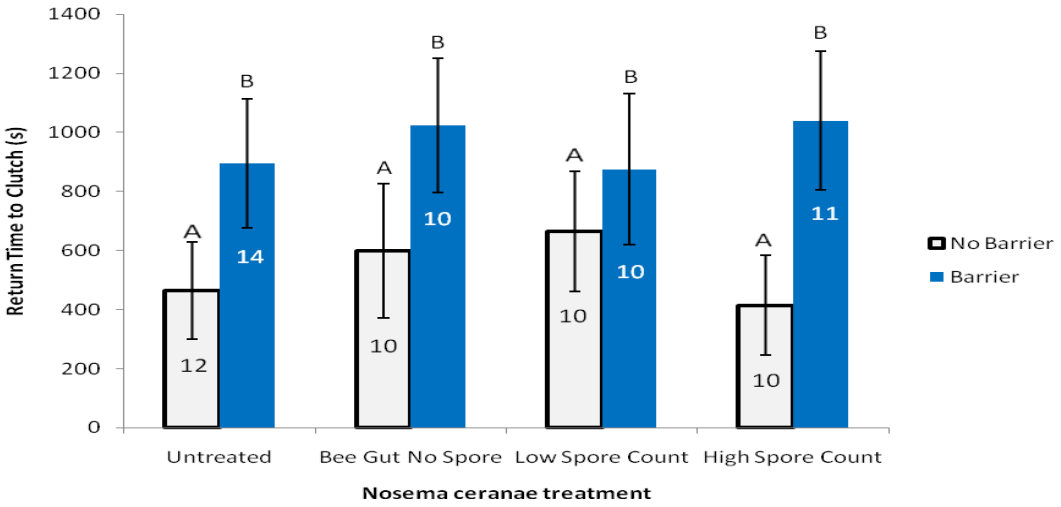

Figure 3. Mean egg clutch return time for 4-day mother earwigs after separation and *N. ceranae* ingestion. Females were given a 30-minute time period to return to their clutch, and times measured include the 1-minute period at the egg clutch needed to consider the return successful. Sample sizes are indicated inside each bar, and error bars represent standard error. Means with the same letter above the bar are not significantly ($P<0.05$) different.

The amount of carrot eaten among the tested mothers was also determined. Individuals from the "Low Spore Count" ate significantly more carrot, but only when compared to the "Bee Gut No Spore" group ($P=0.03$) and "High Spore Count" group ($P=0.03$) (data not shown); however, not all of the females were the same age when their clutches were 4-days old, and they did not all have the same exposure time to the *N. ceranae*. The mothers also ate the center of the carrot first and worked their way out to the perimeter of the carrot.

DISCUSSION

Basal follicle length and carrot consumption

After ingestion of *N. ceranae* for 17 days, the significantly lower mean basal follicle length of the *E. annulipes* in the "Bee Gut No Spore" treatment group and the highest mean basal follicle lengths in both the "Low" and "High" spore concentrations (Fig. 1) did not support my hypothesis that the ovaries would be smaller in females that were infected with the higher concentration of *N. ceranae* spores. These findings contrast to the oocyte degeneration observed when mated queen honeybees were infected by *N. apis* spores for 7 days, in which the infected

females' yolk granules went into various stages of breakdown and ooplasm underwent autolysis. This breakdown is thought to indicate the resorption taking place in the ovarioles, which is usually associated with starvation, lack of available protein, or a lack of the juvenile hormone (Liu 1992).

My findings also do not support the significantly decreased reproductive potential in the Indian tasar silkworm *Antheraea mylitta*, in which the ovary weight was reduced by 31.6% after *Nosema* sp. infection (Rath et al. 2003). Additionally, Świątek and Górkiewicz (2006) found that the presence of microsporidia in *Liophloeus lentus* (Insecta, Colepotera) did not disturb the course of oogenesis, and no detectable differences in ovariole morphology were detected. However, in the previtellogenic oocytes, those that were infected were much larger and oval in shape, with tubular cristae; the oocytes from the non-infected females were smaller, with lamellar cristae. Overall, they concluded that microsporidia infects oocytes via the outer ovariole sheath (Świątek and Górkiewicz 2006).

Since the earwigs in the current study were fed pieces of carrot rather than the more-fattening and nutritious cat food, my food choice may explain why I observed more underdeveloped basal follicles in the 17-day females. The significantly higher carrot ingestion of the "Low Spore Count" earwigs when infected with *N. ceranae*, and the lowest mean carrot mass associated with the "Bee Gut No Spore" (Fig. 2) may also explain why the "Bee Gut No Spore" earwigs had significantly lower follicle lengths (Fig. 1) since ovary breakdown is associated with the lack of available protein. Because *Nosema* is capable of releasing a factor to replace the juvenile hormone normally produced by the host, it remains to be determined whether oocyte degeneration is due to the direct influence by *Nosema* or due to the parasite altering the endocrine activities (Liu 1992).

From previous research, during the interclutch interval of the second gonadotrophic cycle, the basal follicle lengths increased and were longer than that of newly oviposited eggs (Rankin et al. 1995). Since I measured the ovaries of virgin females, it would be interesting to see if *N. ceranae* significantly affects the difference in follicle length if the females were allowed to mate and go through multiple periods of oviposition.

The significantly higher carrot ingestion of the "Low Spore Count" solution of *N. ceranae* (Fig. 2) is supported by previous observations obtained when honeybees infected with *N. ceranae* became more responsive to the sucrose provided to them as food and were less inclined to share the food with other bees. Since parasites compete with hosts for nutrition and exert an energetic stress on them, the bees infected with *Nosema* attempt to compensate for the imposed energetic stress by feeding more, indicating that they have a higher hunger level in response to the pathogenic infection (Mayack and Naug 2009; Naug and Gibbs 2009). This increase in hunger was seen in the earwigs infected with *Nosema* (Fig. 2). Since foraging in honeybees is regulated by colony demand and hunger level of the individuals, this increase could increase foraging rates, thereby increasing the potential for horizontal transmission via flowers (Mayack and Naug 2009). Food consumption also increased in the silkworm *A. mylitta* when infected with *Nosema* sp. (Rath et al. 2003). The preference for the low spore concentration of *N. ceranae* rather than the high concentration may be due to the earwigs' possible aversion from the higher amount of bee debris in the more highly concentrated solution.

Mayack and Naug (2009) also looked at the midguts of the *Nosema*-infected honeybees and found that those of the infected individuals were smaller than those of the uninfected bees. These findings suggest that regulatory pathways could be involved in increasing the hunger level. Therefore, it would be interesting to dissect the midguts out of earwigs after *N. ceranae*

infection to see if the spores penetrate the midguts and cause them to be smaller, thereby helping to explain their higher ingestion of carrot when infected with a spore solution.

Return time to egg clutch

Within the trials where a physical barrier was either present or absent, no significant differences in the earwigs' return time the clutches between the treatment groups were observed (Fig. 3). These findings did not support my hypothesis that females infected with the higher *N. ceranae* concentration would take longer to return to their clutches. Since there is little knowledge about the effect of *N. ceranae* on the honeybee's ability to return to its hive, my hypothesis was based on previous observations of impaired orientation of honeybee foragers infested by *Nosema* sp. (Kralj and Fuchs 2010). My findings indicate that there is no impaired orientation when earwigs are infected with *N. ceranae* (Fig. 3), which is in contrast to the finding that a higher proportion of *A. mellifera* infected with *Nosema* sp. did not return to their hive when released 30 m away from the colony. These observations indicate that *Nosema* influences the honeybee's flight behavior (Kralj and Fuchs 2010), which is also supported by Woyciechowski and Kozlowski (1998), because they showed that bees infected by *Nosema* sp. foraged more frequently in detrimental weather conditions than did healthy honeybees. It is still unknown whether this response to *Nosema* infection is a physiological response or an adaptation, because the bees could be purposefully avoiding their own hives so that they prevent horizontal transmission to the rest of the colony (Kralj and Fuchs 2010). Since it is unknown whether specifically *N. ceranae* causes this change in flight behavior, further studies in which *A. mellifera* and *N. ceranae* are tested together are needed to convincingly show that *N. ceranae* does not aid in Colony Collapse Disorder by impairing the honeybees' ability to successfully return to their own hives.

The significantly shorter return time to the clutches between all of the earwigs in the absence of a barrier compared to those with the barrier supported my hypothesis that the barrier would cause the earwigs to have a longer return time to their clutches (Fig. 3). Many of the earwigs went underneath the rocks after being separated from their egg clutch. These observations support the idea that insects recognize visual cues in order to identify their nest (Wcislo 1992), because after separation, the earwigs may remember that they moved their eggs either underneath or near the original nest. They may have mistaken the rocks for being their nest, so they continually tried to search underneath the rocks. *Forficula auricularia* earwigs will accept foreign objects similar in size to their eggs (Rankin et al. 1996). Upon misidentifying the rocks as being their nest, the earwigs in my study may also have thought that the sand granules underneath the rocks were their eggs, resulting in the longer return time to their own true clutch. To eliminate this possibility, my study would need to be repeated, but a different substance that does not resemble their eggs in any shape, size, or color would need to be used on the floor of the testing arena.

The females in each of the treatment groups did not all have the same exposure time to the *N. ceranae* infected carrot since the oviposition times after mating were different. Therefore, in future studies, the females should be infected with the *Nosema* spores when they are 0-days, or any time before mating is done. Because I could not be sure how many spores were inside the earwigs at the time of testing, an infection method similar to that of experimental honeybee infection could be used instead as well. In this previous study, bees were force-fed a solution containing 125,000 spores by holding open their mouthparts and touching a droplet of the spore solution at the tip of a micropipette until the entire droplet was consumed (Higes et al. 2006). If this method, or an adaptation of it, could be used with earwigs, there would be more certainty in

how many spores were infecting the earwigs.

Due to time constraints, when I was done testing the females, they were sacrificed along with their eggs before they had time to hatch. Therefore, another study could be done to see if the egg and/or offspring viability is affected by *N. ceranae* infection. Since microsporidians can pass transovarially from mother to offspring in egg-laying animals (Świątek and Górkiewicz 2006), one could also determine if this transmission occurs in earwigs by crushing up the eggs and trying to identify the presence of the spores by microscopy and/or PCR amplification (Klee et al. 2007).

Even though *Nosema* usually infects at least 12 orders of insects, *Nosema* sp. are most commonly found in Lepidoptera and Hymenoptera. Infection is mostly common in honeybees (Hymenoptera), silkworms (Lepidoptera), locusts (Orthoptera), and crickets (Orthoptera) (Higes et al. 2006), but has not been reported in earwigs (Van Frankenhuyzen et al. 2004). Therefore, it is difficult to determine the likelihood of earwigs becoming naturally infected with *Nosema*. After infecting earwigs with various concentrations of *N. ceranae* and determining the effect on the females' ovary size, those in the "Bee Gut No Spore" group had the smallest ovaries, and those infected with the "Low Spore Count" and "High Spore Count" solutions had the largest ovaries. These findings were unexpected due to oocyte degeneration in queen honey bees after *Nosema apis*, but the finding that the earwigs ingesting the "Low Spore Count" solution ate significantly more carrot is in correlation with the increase in hunger level when honeybees are infected with *Nosema* spores (Naug and Gibbs 2009). After observing the time taken to return to the egg clutches, the different *N. ceranae* treatments had no significant effect on the return times, but the presence of a physical barrier significantly decreased the return times, possibly due to the earwigs mistaking the sand granules as their own eggs while they were searching. Even

though my study did not find any correlation between *N. ceranae* infection and the ring-legged earwig's ability to return to their egg clutch, further studies with *A. mellifera* are needed because risk-prone foraging by bees that are already in lower energetic state due to *Nosema* infection may play a role in Colony Collapse Disorder because such bees have a lower likelihood of making it back to the hive.

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APPENDIX

Concentration calculation of "Low Spore Count" *N. ceranae* solution:

$$\frac{(number\ of\ cells\ counted)}{(proportion\ of\ chamber\ counted)\times(volume\ of\ chamber)} \times \frac{(volume\ of\ sample\ dilution)}{(volume\ of\ original\ mixture\ in\ sample)}$$

1st chamber: $\frac{174\ \text{spores}}{\left(\frac{1}{9}\right)\times(0.009\ \mu\text{L})} \times \frac{10\ \mu\text{L}}{10{,}000\ \mu\text{L}} = 174\ \text{spores}/\mu\text{L}$

2nd chamber: $\frac{144\ \text{spores}}{\left(\frac{1}{9}\right)\times(0.009\ \mu\text{L})} \times \frac{10\ \mu\text{L}}{10{,}000\ \mu\text{L}} = 144\ \text{spores}/\mu\text{L}$

Average spore concentration = $\frac{174\ \text{spores}/\mu\text{L} + 144\ \text{spores}/\mu\text{L}}{2} = 159\ \text{spores}/\mu\text{L}$

Therefore 15,900 spores in 100 µL administered to carrot

Concentration of "High Spore Count" *N. ceranae* solution:

15,900 spores/100 µL × 10 = 159,000 spores in 100 µL administered to carrot