



# Effects of pollen dilution on infection of *Nosema ceranae* in honey bees



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## ABSTRACT

Multiple stressors are currently threatening honey bee health, including pests and pathogens. Among honey bee pathogens, *Nosema ceranae* is a microsporidian found parasitizing the western honey bee (*Apis mellifera*) relatively recently. Honey bee colonies are fed pollen or protein substitute during pollen dearth to boost colony growth and immunity against pests and pathogens. Here we hypothesize that *N. ceranae* intensity and prevalence will be low in bees receiving high pollen diets, and that honey bees on high pollen diets will have higher survival and/or increased longevity. To test this hypothesis we examined the effects of different quantities of pollen on (a) the intensity and prevalence of *N. ceranae* and (b) longevity and nutritional physiology of bees inoculated with *N. ceranae*. Significantly higher spore intensities were observed in treatments that received higher pollen quantities (1:0 and 1:1 pollen:cellulose) when compared to treatments that received relatively lower pollen quantities. There were no significant differences in *N. ceranae* prevalence among different pollen diet treatments. Interestingly, the bees in higher pollen quantity treatments also had significantly higher survival despite higher intensities of *N. ceranae*. Significantly higher hypopharyngeal gland protein was observed in the control (no *Nosema* infection, and receiving a diet of 1:0 pollen:cellulose), followed by 1:0 pollen:cellulose treatment that was inoculated with *N. ceranae*. Here we demonstrate that diet with higher pollen quantity increases *N. ceranae* intensity, but also enhances the survival or longevity of honey bees. The information from this study could potentially help beekeepers formulate appropriate protein feeding regimens for their colonies to mitigate *N. ceranae* problems.

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## 1. Introduction

Honey bees (*Apis mellifera*) are critical pollinators of many important agricultural crops and currently face several stressors, including pests, parasites and pathogens (Klein et al., 2007; Bromenshenk et al., 2010; Potts et al., 2010; Calderone, 2012). The microsporidian *Nosema ceranae* is one such parasite that was first reported in the European honey bee in 2006 (Higes et al., 2006; Huang et al., 2007; Chen et al., 2008). *Nosema* spores ingested by honey bees germinate inside the host midgut and reproduce there intracellularly. *N. ceranae* infection has been shown to promote precocious foraging (Goblirsch et al., 2013), modify vitellogenin titres and queen mandibular pheromones in queens (Alaux et al., 2011), reduce longevity (Eiri et al., 2015),

decrease immune functions (Antúnez et al., 2009) and increase colony loss (Higes et al., 2008, 2009).

Reduced longevity may be observed in infected bees as the presence of *N. ceranae* in the midgut disrupts protein metabolism and causes energetic stress. This obligate parasite obtains its energy from the bees' midgut cells and impairs midgut epithelial cells and midgut development during replication (Higes et al., 2007; Holt et al., 2013). In doing so, *Nosema* spp. infections negatively affect the midgut proteolytic enzyme activity (Liu, 1984; Malone and Gatehouse, 1998). Additionally, the presence of *N. ceranae* alters the expression of some portions of the midgut proteome responsible for energy production, protein regulation and antioxidant defense (Vidau et al., 2014). As a result of the energetic stress caused by such disruptions to nutrient digestion and metabolism, infected honey bees commonly demonstrate pronounced hunger (Mayack and Naug, 2009) and are less likely to share food with nestmates via trophallaxis (Naug and Gibbs, 2009). This, in turn, may have detrimental effects on colony health.

A growing body of literature supports the notion that *N. ceranae* infection also affects hypopharyngeal gland structure and function. In honey bees, hypopharyngeal glands secrete major components of royal jelly and larval brood food (Patel et al., 1960), as well as

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synthesize enzymes involved in the conversion of sucrose to simple sugars and honey production (White et al., 1963; Ohasi et al., 1999). Explorations into the effects of *N. ceranae* parasitism on honey bee hypopharyngeal glands have demonstrated that infection induces gland atrophy (Alaux et al., 2010a). The parasite, however, has not been detected in the hypopharyngeal gland tissue (Huang and Solter, 2013). However, functional development and activity of hypopharyngeal glands in honey bees is reliant on the ability to ingest and digest pollen (Haydak, 1970; Mohammadi et al., 1996). Thus, changes in the hypopharyngeal glands of *N. ceranae*-infected honey bees are potentially indirect manifestations of the effects infection has on protein digestion and metabolism.

In the wake of deteriorating honey bee colony health and unsustainable colony declines (vanEngelsdorp et al., 2012; Steinhauer et al., 2014; Lee et al., 2015; van der Zee et al., 2015), bee nutrition has garnered more attention and attained greater importance (DeGrandi-Hoffman and Chen, 2015; Fleming et al., 2015; Vaudo et al., 2015). The honey bee, as with any animal, requires adequate nutrition to thrive (Haydak, 1970). Honey bees get the vast majority of their required protein, lipids and vitamins from pollen (Hrassnigg and Crailsheim, 1998; Brodschneider and Crailsheim, 2010). However, the pollen nutrition obtained by the honey bee is plant-dependent (Crailsheim, 1990; Huang, 2012). Diminished pollen quantity and diversity within the forage available to honey bees leads to a nutritional deficit that negatively impacts colony survival (Naug, 2009) by hindering brood production, inhibiting gland development, facilitating the presence of diseases, reducing individual bee weight, decreasing immunocompetence and shortening individual life span (Schmidt, 1984; Schmidt et al., 1987, 1995; Alaux et al., 2010b; Avni et al., 2014; Wang et al., 2014). Pollen quantity, quality and diversity have also been shown to affect the survival of honey bees parasitized by *N. ceranae* (Eischen and Graham, 2008; Di Pasquale et al., 2013).

Protein and carbohydrates are critical macronutrients that influence growth, performance and survival of insects, and most insects regulate nutrient intake when provided an opportunity (Behmer, 2009). The geometric framework (GF) has been used in many organisms to determine optimal balance of nutrients (Raubenheimer and Simpson, 1999; Behmer, 2009). Recently, some studies have used the geometric framework for nutrition in social insects to determine their intake targets and have examined how ratios of protein to carbohydrate or essential amino acids to carbohydrates affect the survival and physiology. Paoli et al. (2014) reported that honey bee nutritional requirements are dependent on age and behavioral role of adult bees, and bees receiving high amino acid diets had poor longevity. Pirk et al. (2010) found that honey bee adults survived longer on lower protein to carbohydrate ratio diets, and the survival was longest on a pure carbohydrate diet. In black garden ants, Dussutour and Simpson (2012) demonstrated that diets with high protein and low carbohydrate reduced ant worker lifespan by up to 10-fold due to higher intake of protein. Interestingly, in another study, Archer et al. (2014) found that honey bees fed with high protein diet (1:3 P:C) had lower mortality when exposed to stressors such as low temperatures and nicotine. In this study the authors speculated that protein conferred a survival benefit in the stress challenged bees by enhancing their ability to mount a stress response.

Resistance to pathogens can heavily depend upon nutrition (Behmer, 2009; Alaux et al., 2010b; Mao et al., 2013). Thus, understanding the relationship between bee nutrition and pathogen infection (intensity and prevalence) is paramount. Much of the research regarding bee nutrition and pathogen infection has focused on *Nosema apis* because our knowledge of *N. apis* infections in *A. mellifera* significantly predates that of *N. ceranae* (Bailey, 1955). For example, Rinderer and Elliot (1977) reported that feeding protein to honey bees infected with *N. apis* increased the spore devel-

opment of the pathogen, but also improved the longevity of infected bees. But Mattila and Otis (2006) reported contradictory findings. In their field experiment, Mattila and Otis found there was no increase in the longevity of workers from *N. apis*-inoculated colonies that had received protein supplements. In recent years, however, researchers have documented many significant differences between *N. apis* and *N. ceranae* regarding infectivity, epidemiology, biology, phylogeny, pathology, genetics and distribution (Chen et al., 2013; Milbrath et al., 2015). Furthermore, in temperate climates *N. ceranae* has been reported to be the predominant species with occasional mixed infections of both *N. ceranae* and *N. apis* (Paxton et al., 2007; Fries, 2010; Natsopoulou et al., 2015). Some bee research has focused on the interactions between *N. ceranae* infection and pollen nutrition. Porrini et al. (2011) found that the presence of pollen in honey bee diets increased the intensity of *N. ceranae* spores. Zheng et al. (2014) reported that increased pollen feeding intensified *N. ceranae* spore loads in bees; and longevity of infected bees was lower than that of uninfected bees—with or without pollen feeding. In another study, Basualdo et al. (2014) found higher levels of hemolymph protein and survival in bees fed with bee bread when compared to protein substitutes, despite higher parasite development. More recently Fleming et al. (2015) reported higher *Nosema* levels in bees fed with commercial protein substitute diets than bees fed with wildflower pollen.

While these studies have explored how *N. ceranae* infection levels and infected bee longevity are influenced by protein/pollen nutrition, a great deal of uncertainty remains regarding the role of pollen quantity consumption on *N. ceranae* infection and survival of honey bees. To our knowledge, currently there is a dearth of comprehensive studies that have explored the effects of pollen quantity consumption on *N. ceranae* infection, prevalence, and survival of infected bees while simultaneously examining critical physiological parameters such as hypopharyngeal gland protein content and midgut enzyme activity. Here we hypothesize that *N. ceranae* infected bees with access to higher pollen quantities will have lower *N. ceranae* intensity, lower prevalence, higher hypopharyngeal gland protein, higher midgut enzyme activity and higher survival than infected bees receiving less pollen in their diet. To test these hypotheses, we examined the effects of diets containing different quantities of pollen on: (a) the prevalence and intensity of *N. ceranae* and (b) longevity and physiological parameters (hypopharyngeal gland protein and midgut proteolytic enzyme activity) of bees inoculated with *N. ceranae*.

## 2. Materials and methods

### 2.1. *N. ceranae* prevalence, intensity and survival analyses

In June 2014, capped combs with emerging bees were obtained from eight honey bee colonies headed by sister queens at Oregon State University apiaries (Corvallis, OR, USA). Sister queen colonies were used to control any variation in *Nosema* infection attributed to genetics of the bees. These combs with emerging bees were placed in an incubator under simulated hive conditions (33 °C, 55% RH) for bee emergence. Twenty four hours later we gently brushed newly emerged bees into a large container and mixed them thoroughly by hand. After the bees were mixed, 250 individual bees were placed inside cylindrical wire cages (3069 cm<sup>3</sup>) and returned to the incubator. To provide caged bees with *ad libitum* access to water and 50% sucrose solution, two 25-ml glass vials (one containing each liquid) were covered with two layers of cheesecloth and then secured, inverted, to the top of each cage. On alternate days, we measured the consumption of both water and sucrose solution and replaced the vials. Varying dilutions of pollen diet were also provided to the bees in experimental cages.

To obtain diets with different quantities of pollen, the pollen diets were diluted using a cellulose powder similar to methods described in Waddington et al. (1998), Willard et al. (2011) and Lewis et al. (2012). The consumable, non-nutritious alpha-cellulose lowers the concentration of pollen in the diet and presumably leads to a higher digestive demand relative to the nutrients obtained (Willard et al., 2011). The advantage of using cellulose is that we can quantitatively reduce nutritional content of the diet without reducing the total weight of the diet assigned for each treatment. Treatments consisted of ground wild flower pollen (The Pollen Man, OR, USA) and  $\alpha$ -cellulose powder (Sigma Aldrich product C8002, MO, USA) in the following pollen:cellulose ratios: (a) 1:0 (b) 1:1 (c) 1:2 (d) 1:3 and (e) 0:1. We also included a control diet group that was provided pollen similar to 1:0 pollen:cellulose treatment but did not receive *N. ceranae* spores. To hold the diet blend (pollen and cellulose) together, 35 ml of 33% sucrose solution was mixed into 300 g of each diet. The pollen/sucrose mixture was then measured out to 25 g and packed into Petri dishes (VWR, 150 × 15 mm) and placed at the bottom of corresponding cages. In all, there were 6 treatments and 6 replicates (cages) of each treatment, for a total of 36 cages. Prior to the experiment, wildflower pollen used in this study was sent for pesticide analysis to assess presence of any pesticide residues. The estimated protein (P) and carbohydrate (C) ratios of the five pollen:cellulose diets are shown in Table 1. These estimates are based on the protein and carbohydrate percentages reported in bee collected pollen in the literature (Human and Nicolson, 2006; Szczesna, 2007; Di Pasquale et al., 2013; Nicolson, 2013; Yang et al., 2013; Komosinska-Vashev et al., 2015).

Five days after *ad libitum* access to the diet treatments, bees in each cage were mass-inoculated with *N. ceranae* spores similar to Pettis et al. (2012). Prior to inoculation, the *Nosema* species identification was performed following the PCR methods described by Hamiduzzaman et al. (2010). The *N. ceranae* spore solution was formulated following the centrifugation method described in Fries et al. (2013). Briefly, the contents of infected gut samples were collected and centrifuged at 5000 rpm for five minutes at room temperature to produce a pellet of spores. After discarding the supernatant, the pellet was resuspended in distilled water by vortexing. This process was repeated 2–3 times. The concentrate was calculated to inoculate 250 bees with 10,000 spores per bee. The spore inoculant was formulated in 30 ml of 50% sucrose solution and inverted on top of cages for *ad libitum* access for twenty-four hours. After twenty-four hours of *ad libitum* feeding, most of the spore-containing sucrose solution had been consumed. The remaining solution was then topped up with regular 50% sucrose solution (without spores) to prevent the bees from going hungry before the next feeding, but ensuring that the full dosage was consumed by the bees in each cage.

Once a week we measured consumption of the diet (pollen or pollen plus cellulose) patties by reweighing the remaining patties and replaced them with fresh patties. Bee mortality was recorded every other day; but for the sake of convenience, dead bees were removed at the time of diet replacement. On a few occasions when there were too many dead bees to record an accurate count, the dead bees were removed and counted at times other than at diet replacement. Sixteen days after spore inoculation, 30 bees were randomly removed from each cage for analysis; however, consumption and survival measurements continued for another 2 weeks. The abdomens of 20 bees were used to estimate the *Nosema* prevalence and intensity as determined by observer-blind light microscopy techniques described by Cantwell (1970). Briefly, each abdomen was macerated using a mortar and pestle in 1 ml of distilled water. Further, a 10  $\mu$ l drop of this solution was placed on a hemocytometer (Cat# 3200, Hausser Scientific, PA, USA) and *Nosema* spores were counted at 400 $\times$  magnification.

**Table 1**

Protein to carbohydrate ratios of the treatments (pollen:cellulose diets).

Pollen:cellulose diets (treatments)	P:C Ratio
1:0	1:2.19
1:1	1:2.38
1:2	1:2.58
1:3	1:2.77
0:1	0:11.6

Each bee abdomen was checked individually for *N. ceranae* infection. The heads of the same 20 bees were used for dissecting and estimating hypopharyngeal gland protein content. The remaining 10 bees were used to analyze midgut proteolytic enzyme activity.

## 2.2. Hypopharyngeal gland protein analysis

The hypopharyngeal gland protein content was measured using a standard BCA Assay (Pierce™ Biotech BCA Assay Kit, Thermo-Scientific, IL, USA). We removed pairs of hypopharyngeal glands from twenty bees. The glands from each individual bee were stored in 1.5 ml microcentrifuge tubes containing 5  $\mu$ l PBS (Phosphate Buffered Saline, Sigma–Aldrich) and stored at  $-80^{\circ}\text{C}$  until time of analysis. Glands were homogenized with a plastic micropestle. Once glands were homogenized thoroughly, 25  $\mu$ l of PBS was added to each tube. Samples were then vortexed for a few seconds and centrifuged at room temperature for 2 min at 13,300 rpm. The supernatant from each tube was used for analysis.

We quantified the protein content of each sample following the microplate procedure of the Pierce™ Biotech BCA Assay Kit Instructions (Thermo-Scientific). Our samples were homogenized in PBS; therefore, PBS was used as the diluent for the Bovine Serum Albumin (BSA) standards and the blank. BSA standard solutions were stored at  $-20^{\circ}\text{C}$  and used for several consecutive days. We loaded 10  $\mu$ l of each sample, standard and blank into duplicate wells of a 96-well plate kept on ice. Plating this volume allows the assay to have a protein detection range of 125–2000  $\mu\text{g}/\text{ml}$ . After addition of the BCA working reagent (200  $\mu\text{l}/\text{well}$ ), the plate was shaken for 30 s in a microplate spectrophotometer (BioTek Synergy 2, Gen5 2.00 software, VT, USA). The uncovered plate was then incubated at  $37^{\circ}\text{C}$  for 30 min and again at  $4^{\circ}\text{C}$  for 12 min while covered. The absorbance at 562 nm was measured and protein concentration in  $\mu\text{g}/\text{ml}$  was calculated from the resulting standard curve.

## 2.3. Total midgut proteolytic enzyme activity analysis

Bees that were removed from cages for analysis were kept at  $-20^{\circ}\text{C}$  until the time of analysis. The abdomens of the bees were removed and placed in a separate 2-ml microcentrifuge tube containing 90  $\mu$ l of chilled 0.1 M Tris buffer (pH 7.9). The abdomens were homogenized with one 3 mm tungsten-carbide bead in a Qiagen® TissueLyser II at (30 oscillations/s., 2 × 45 s.), then centrifuged at room temperature for 6 min at 13,000 rpm. Three 1.5-ml tubes containing 25  $\mu$ l of the chilled Tris buffer were prepared for each sample. For a given sample, 5  $\mu$ l of the resulting supernatant was added to each of the three tubes – 1 tube to serve as sample blank, and 2 as replicate measurements used to calculate the mean of each sample. We added 60  $\mu$ l of 2% Azocasein (Sigma–Aldrich) to all tubes and vortexed each gently. Sample blanks were left at room temperature while the other tubes were incubated at  $37^{\circ}\text{C}$  for 4 h.

After incubation, all tubes were placed on ice to stop the reaction and 300  $\mu$ l of chilled 10% trichloroacetic acid (TCA, Macron) were added to all tubes. Each tube was vortexed well and then centrifuged at room temperature for 4 min at 13,300 rpm. We

transferred 350 µl of the resulting supernatant to a separate plastic 1-cm cuvette containing 200 µl of 50% ethanol in an ice bath. Each cuvette was vortexed gently before reading absorbance at 440 nm on a Beckman DU 64 spectrophotometer. For each bee abdomen analyzed, there were a total of three cuvettes. The first cuvette contained the sample blank, which was incubated at room temperature and used to zero the spectrophotometer before measuring absorbance of the 2 replicate cuvettes. Total midgut proteolytic enzyme activity was calculated from the mean of the replicate readings for each sample and expressed in terms of OD<sub>440</sub>.

#### 2.4. Data analysis

All data analysis was carried out using SAS 9.3. After statistical analysis, means were back-transformed as needed for presentation herein. Data pertaining to consumption of pollen, water, and sucrose syrup were analyzed using repeated measures while *Nosema* intensity, *Nosema* prevalence, hypopharyngeal gland protein quantity, and total midgut proteolytic enzyme activity were analyzed by generalized linear mixed model analysis for pairwise comparisons of the treatments. In these analyses, cage was considered a random effect. Mean separations were performed using Fisher's protected least significant difference (LSD) test ( $P \leq 0.05$ ). To meet normal distribution assumptions, we applied a logarithmic transformation to *Nosema* intensity data and a square root transformation to *Nosema* prevalence data. For hypopharyngeal gland protein estimation, data points which were outside of the limits of the BSA standards were removed and considered as missing observations. Survival analysis was conducted using PROC LIFETEST and Kaplan–Meier estimates were computed and survival curves were generated for various treatments (Le, 1997). For this, survival probabilities were plotted against number of days from the initiation to the end of experiment. Survival curves were compared using log rank tests (Allison, 1998). A Cox proportional hazards model was also used to model the survival data and calculate hazard ratios. Bees that survived until the end of the experiment (Day 28) and those that were removed for midgut enzyme analysis, hypopharyngeal gland protein content and *Nosema* intensity and prevalence were treated as censored cases.

### 3. Results

#### 3.1. Consumption of pollen diet, sucrose solution and water

The average consumption of pollen, syrup and water are presented in Table 2.

The pollen diet consumption in control was significantly higher when compared to 1:1, 1:2, 1:3 and 0:1 pollen:cellulose treatments ( $F_{5,120} = 3.81$ ;  $P = 0.0031$ ), but there was no significant difference in pollen diet consumption between control (received pollen but not inoculated with *Nosema*) and 1:0 pollen:cellulose treatments. The mean amount of pollen diet consumed by bees in all treatments was significantly higher in the first week of the experiment (9.25 g) when compared to the second (6.0 g), third (5.8 g), or fourth weeks (5.33 g), which did not differ significantly ( $F_{3,120} = 39.53$ ;  $P < 0.0001$ ). There were significant differences in

sucrose syrup consumption among treatments ( $F_{5,419} = 15.47$ ;  $P < 0.0001$ ) with significantly higher sucrose syrup consumption observed in the control, 1:0 and 1:1 pollen:cellulose treatments compared to the 1:2, 1:3 and 0:1 pollen:cellulose treatments. Further, the water consumption was also significantly different among treatments ( $F_{5,419} = 6.71$ ;  $P < 0.0001$ ) with significantly higher water consumption observed in the control, 1:0 and 0:1 pollen:cellulose treatments. The treatments 1:2 and 1:3 pollen:cellulose had significantly lower water consumption when compared to all the other diets.

#### 3.2. *Nosema* prevalence and intensity

The effects of treatment on *N. ceranae* intensity (spore load) are summarized in Fig. 1.

Among the treatments that received *Nosema* spore inoculations, there were significant differences in *Nosema* spore intensities ( $F_{5,188} = 5.75$ ;  $P < 0.0001$ ). Significantly higher spore intensities were observed in the 1:0 and 1:1 pollen:cellulose treatments when compared to the remaining treatments. Treatments 1:3 and 0:1 pollen:cellulose showed the lowest *Nosema* spore intensities. There were no significant differences in *N. ceranae* prevalence among treatments ( $F_{5,29} = 1.26$ ;  $P = 0.3066$ ) with all treatments falling within a range of 23.3–35.8% infection.

#### 3.3. Survival analysis

Kaplan–Meier survival curves were used to plot the survival data (Fig. 2) and log rank tests were used to compare the survival curves of the various treatments. Log rank tests indicated that there were significant differences in survival among bees fed different ratios of pollen and cellulose ( $\chi^2 = 1062.8$ ;  $df = 5$  and  $P < 0.0001$ ). The highest survival was observed in the control group followed by 1:0, 1:1, 1:2, 1:3 and 0:1 pollen:cellulose ratio diets. The hazard ratios estimated from Cox regression for 1:0, 1:1, 1:2, 1:3 and 0:1 pollen:cellulose ratio diets were 1.29, 1.57, 2.16, 2.66 and 3.59 respectively.

#### 3.4. Hypopharyngeal gland protein content

The effects of diet on the amount of protein from adult hypopharyngeal glands are summarized in Fig. 3.

The hypopharyngeal gland protein content was significantly different among treatments ( $F_{5,494} = 133.91$ ;  $P < 0.0001$ ). Significantly higher hypopharyngeal gland protein content was observed in the control (29.29 µg/bee) followed by 1:0 pollen:cellulose treatment (23.34 µg/bee). All other remaining treatments had significantly lower concentrations of hypopharyngeal gland protein in the range of 5.9–9.0 µg/bee and were not significantly different from each other.

#### 3.5. Total midgut protease activity

The mean midgut proteolytic enzyme activity was significantly different among treatments ( $F_{5,354} = 103.07$ ;  $P < 0.0001$ ) and the highest enzyme activity was observed in the control and 1:0

**Table 2**

Average consumption of diet, syrup and water per sampling event (total study period: 4 weeks) of treatment bees infected with *Nosema ceranae*. (Control bees were not infected). 1:0, 1:1, 1:2, 1:3 and 0:1 refer to ratios of pollen and cellulose respectively.

Type	Control	1:0	1:1	1:2	1:3	0:1
Diet (g/week)	7.79 <sub>a</sub>	6.83 <sub>ab</sub>	6.08 <sub>b</sub>	5.88 <sub>b</sub>	6.38 <sub>b</sub>	6.63 <sub>b</sub>
Syrup (ml/2 days)	23.13 <sub>a</sub>	22.77 <sub>ab</sub>	23.64 <sub>a</sub>	20.68 <sub>bc</sub>	19.85 <sub>cd</sub>	17.54 <sub>d</sub>
Water (ml/2 days)	57.71 <sub>a</sub>	55.32 <sub>ab</sub>	51.49 <sub>bc</sub>	49.28 <sub>cd</sub>	45.30 <sub>d</sub>	53.46 <sub>abc</sub>

For each row, values followed by different letters are significantly different at  $P \leq 0.05$  (least significant difference test).

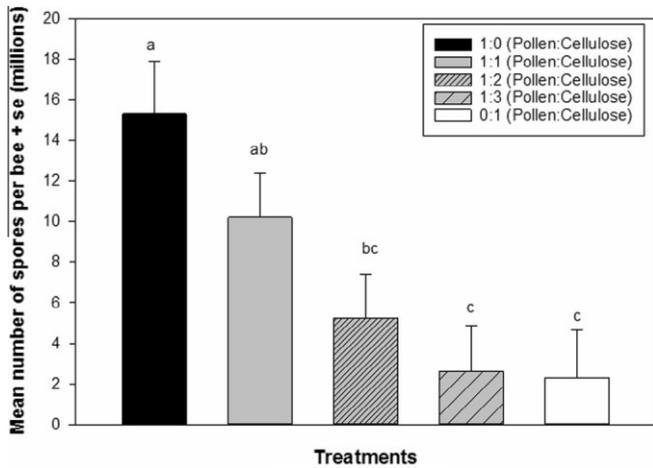


Fig. 1. Mean number of *Nosema ceranae* spores per bee (+se) fed different pollen concentrations and sampled 16 days after infection. Means with different letters indicate significant differences among the treatments ( $P < 0.0001$ ).

pollen:cellulose treatments. The lowest midgut proteolytic enzyme activity was observed in 0:1 and 1:3 pollen:cellulose treatments (Fig. 4).

#### 4. Discussion

This study provides clarity and new insights on how pollen quantity in the diet influences *N. ceranae* intensity, prevalence and adult survival in honey bees. Interestingly, here we found that bees in the treatments receiving higher pollen quantity had higher *N. ceranae* intensities, but also higher survival. Similar findings were reported by other studies with *N. apis* (Rinderer and Elliot, 1977) and *N. ceranae* (Basualdo et al., 2014; Zheng et al., 2014; Fleming et al., 2015), but to our knowledge this is the first comprehensive study to examine the effects of diet on *N. ceranae* spore intensities, prevalence and survival of infected bees while simultaneously observing changes in critical physiological parameters (hypopharyngeal gland protein content and midgut enzyme activity). Further, unlike similar studies mentioned above, here we used a different approach to formulate varying pollen diets using a non-nutritious cellulose. A significant advantage of this approach is that it facilitates a repeatable quantitative alteration in nutritional content. This approach also helps minimize any potential confounding

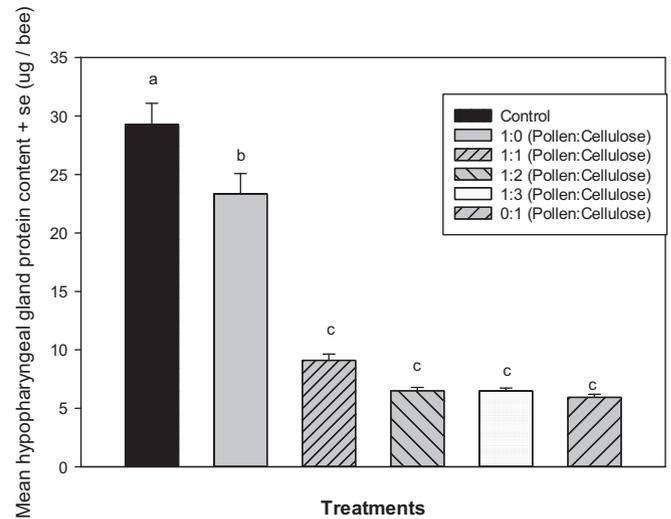


Fig. 3. Mean hypopharyngeal gland protein (HPG) quantities of bees (+se) fed different pollen concentrations. Different letters indicate significant differences among the treatments ( $P < 0.0001$ ).

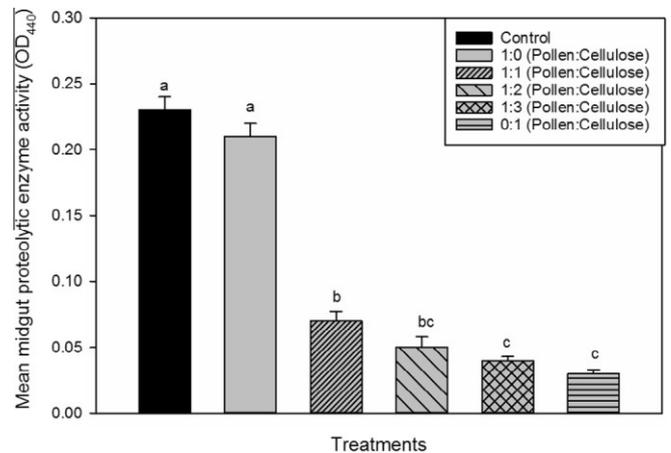


Fig. 4. Mean midgut proteolytic enzyme activities of bees (+se) fed different pollen concentrations. Different letters indicate significant differences among the treatments ( $P < 0.0001$ ).

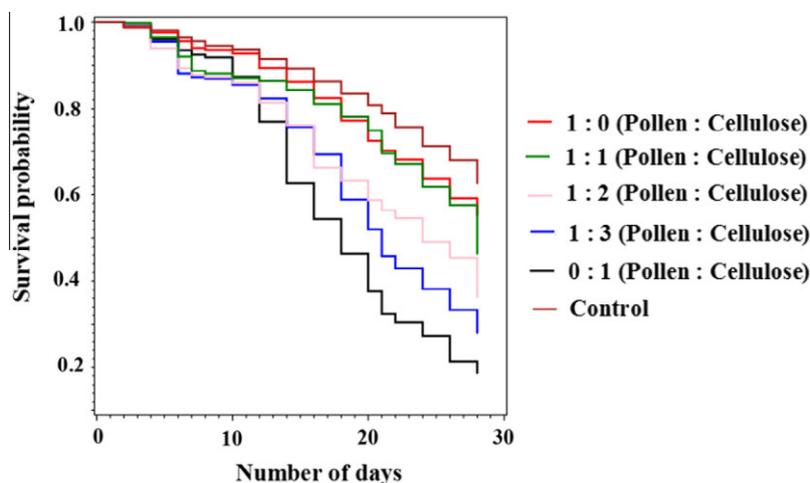


Fig. 2. Survival of *Nosema ceranae*-infected bees fed different concentrations of pollen. (Control group was not infected.)

effects resulting from total quantity of the diet consumed on *N. ceranae* development, as a few studies (Gontarski and Mebs, 1964; Rinderer and Elliot, 1977; Porrini et al., 2011) speculate that midgut expansion due to pollen ingestion results in higher surface area for *Nosema* infection, which in turn leads to greater spore production. In this study we assumed that cellulose mixed in the diets doesn't have any significant effect on consumption of sugar solution and water by bees, and also on their survival. This is a limitation of this study due to gap in knowledge regarding effects of cellulose on the above listed parameters. Future research using cellulose to manipulate diets should also focus on examining any potential effects of cellulose on consumption and survival.

Different pollen diets used in this study appear to have no significant effects on the prevalence of *N. ceranae*. Once ingested, spores germinate in response to cues in the gut lumen of the host (Weiss and Becnel, 2014). It appears that honey bee nutrition does not influence the cues or factors responsible for *N. ceranae* spore germination as spores were detected in all diet treatments regardless of the type of diet. However, our results suggest that the successful reproduction of the pathogen is largely dependent upon host nutrition.

We observed that increases in pollen quantities in the diet also increased the intensity of *Nosema* infection—observations similar to those made by Rinderer and Elliot (1977), Porrini et al. (2011), Basualdo et al. (2014), and Zheng et al. (2014). This phenomenon may be due to the fact that *N. ceranae* is highly dependent on host nutritional status for its development. It has been suggested that *N. apis* uses amino acids from their honey bee hosts (Wang and Moeller, 1970; Panek et al., 2014), and microsporidians including *N. ceranae* hijack ATP directly from the cytoplasm of their host (Weidner et al., 1999; Williams, 2009). Therefore, it is likely that an individual honey bee receiving a high pollen diet becomes an ideal host for greater proliferation of the parasite by providing an ideal nutritional environment. Weiss and Becnel (2014) suggest that microsporidia may appropriate other core nutrients from their host, which further supports the idea that *N. ceranae* might replicate faster in a host receiving, and therefore providing, better nutrition.

Even though *N. ceranae* spore intensities were higher in bees that received more pollen in their diet, the bees in these treatments had greater survival, which appears to be counterintuitive. However, certain nutritional factors appear to be contributing to honey bee survival, despite increased infection intensities. For instance, Alaux et al. (2010b) demonstrated that the presence of pollen in the diet increased fat body content, the site of immunoprotein synthesis. The results of Zheng et al. (2014) and Di Pasquale et al. (2013) suggest that the expression of vitellogenin, an immunoprotein involved in honey bee longevity (Amdam and Omholt, 2002), may contribute to the increased survival of *N. ceranae* parasitized bees. We further speculate that higher pollen availability might compensate for the energy and nutrients lost in bees with high *Nosema* intensity, leading to greater survival. These results are in agreement with the conclusions made by Eischen and Graham (2008), that nutrition influences survival during colony level *Nosema* infection.

Hypopharyngeal gland protein content in all the treatments that were inoculated with *N. ceranae* was significantly lower than that of the control, which was not inoculated with *Nosema* spores. These findings are similar to the results from studies with *N. apis* (Wang and Moeller, 1971; Malone and Gatehouse, 1998); and they reaffirm the assertion that *N. ceranae* disrupts protein metabolism in bees, in turn, affecting hypopharyngeal gland protein biosynthesis. The total midgut protease activity significantly decreased with the reduction of pollen in the diet. However, we did not observe any negative effects on midgut enzyme activity as a result of

*N. ceranae* infection. Malone and Gatehouse (1998) observed that proteolytic enzymes in honey bees are at highest levels during the first few days post-emergence and then decline quickly with age. Since the bees in our treatments were inoculated with *N. ceranae* spores 5 days after emergence, it may be possible that proteolytic enzymes were synthesized abundantly in the 1:0 pollen:cellulose treatment bees, similar to the bees in control cages, before the spores potentially caused damage to epithelial cells. Hence no significant differences were detected between the high pollen treatment and the control.

Since newly emerged adult bees were used in this experiment, the rate of pollen consumption was highest during the first week in all treatments, as that is the typical time of pollen consumption for young bees (Crailsheim et al., 1992). The amount of assigned diet consumed was also highest in bees fed 100% pollen, demonstrating the ability of the bees to recognize the pollen quality. Our results also show that bees in the control cages consumed similar volumes of sucrose syrup when compared to *Nosema* infected treatments. However, previous studies have demonstrated that sucrose syrup consumption was higher in *Nosema* infected bees when compared to controls (Mayack and Naug, 2009; Naug and Gibbs, 2009; Martín-Hernández et al., 2011). The difference between their results and ours may be attributed to low mortality in our control cages, causing the amount of syrup consumption to remain on par with *Nosema* infected treatments. Water consumption was highest in the 100% pollen treatments, possibly indicating the necessity of water for proper pollen digestion.

Protein feeding is a common practice among beekeepers, especially when there is pollen dearth or during late fall when colonies are rearing winter bees (diutinus bees). It seems logical to feed protein to honey bee colonies during pollen dearth to boost colony growth, but caution should be exercised while feeding protein as high protein diets have been shown to reduce longevity in honey bees (Pirk et al., 2010) and in ants (Dussutour and Simpson, 2012). The presence or absence of brood in the colonies might also impact supplemental feeding decisions, as it appears that broodless bees consume more carbohydrates (Altaye et al., 2010). Further, the timing of feeding honey bee colonies may also be critical, as the nutritional requirements of honey bees vary with age and behavioral role (Paoli et al., 2014).

As the primary goal of this study was to determine the effects of pollen quantity on *N. ceranae* infection, this study exclusively focused on pollen quantity manipulation. Future studies should examine the effects of pollen quantity and quality simultaneously to obtain greater insights. Further, there is also a need for studies focusing on understanding the factors affecting *N. ceranae* spore germination inside the midgut in order to mitigate infections. Since *N. ceranae* intensities are more severe in honey bees fed a higher quantity of pollen in their diet, it may also be important to understand the core nutrients that *N. ceranae* acquires from the host and allocates to spore proliferation. Though challenging, more field studies investigating the effects of different pollen sources, as well as protein supplements, will be instrumental in determining if the same effects of increased spore intensity are observed under natural conditions.

## 5. Conclusions

Here we demonstrate that higher pollen quantity increases *N. ceranae* intensity, but also enhances the survival or longevity of honey bees. Pollen quantity doesn't appear to influence the prevalence of *N. ceranae*. Information from this study could be potentially used by beekeepers to formulate appropriate protein feeding regimens for their colonies based on *N. ceranae* phenology in their respective regions.

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