UNIVERSITY OF CALIFORNIA SAN DIEGO

Intensity of *Nosema ceranae* infection and survival effects strongly depend on colony background

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by

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The thesis of Edmund Lau is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT OF THE THESIS

Intensity of *Nosema ceranae* infection and survival effects strongly depend on colony background

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The European honey bee, *Apis mellifera*, plays an important role in agriculture worldwide, but managed colonies have faced considerable losses in recent years, including a

41% winter loss of U.S. managed colonies in 2019. A widespread contributing factor is a microsporidian pathogen, *Nosema ceranae,* which has spread to colonies worldwide, developed a resistance to antibiotic treatment, and can alter the host's immune response and nutritional uptake. These obligate gut pathogens share their environment with a natural honey bee microbiome whose composition can affect pathogen resistance. We tested the effect of *N. ceranae* infection on this microbiome by feeding 5 d old adult bees (with developed natural microbiomes) with live *N. ceranae* spores (40,000 per bee) or a sterile 2.0 M sucrose solution. We caged and reared these bees in a controlled lab environment and tracked their mortality over 12 d, after which we dissected them, measured their infection levels (spore counts), and prepared samples for future microbiome analyses. Bees fed live spores had two-fold higher mortality by 12 d, as well as 36.5-fold more spores per bee as compared to controls. In addition, we found strong colony effects on infection, confirming that susceptibility to *N. ceranae* varies widely between colonies. By analyzing the microbiome of the most resistant bees, future studies can be performed to determine how these microbes affect resistance of honey bees to microsporidian infections.

Introduction

Animal pollination services contribute about 35% of global food crop production (Lonsdorf et al., 2011; Winfree et al., 2011), and 75% of global food crops are dependent on insect pollination in some capacity (Bommarco et al., 2013). Although wild insects, including many native bee species, make meaningful contributions as pollinators (Ollerton et al., 2012), commercial honey bees like the European honey bee, *Apis mellifera,* are important for the successful pollination of many of these crops (Sumner and Boriss, 2006). The monetary value that commercial *Apis mellifera* colonies provide as pollinators was estimated to be $12.3 to $16.4 billion annually in the U.S. (Losey and Vaughan, 2006). In California alone, western honey bee pollination of almonds accounts for an estimated $6.4 billion in 2015 (USDA, 2015).

The proportion of pollinator-dependent crops in agriculture has tripled over the past half century, driving the demand for commercial European honey bee pollinators (Aizen and Harder, 2009). However, fluctuations in colony management have been seen worldwide. Colony decline in Central Europe and some increase in the Mediterranean can possibly be explained by the variable decrease in beekeeping operations throughout Europe over the past few decades (Potts et al., 2010). However, the U.S. has seen a great decline in managed honey bee health in recent years. Winter colony losses range from 29% to 36% in 2006-2014 (Lee et al., 2015), around 40% in 2014-2016 (Seitz et al., 2015; Kulhanek et al., 2017), and 41% in 2018-2019 (Bruckner et al., 2019). Similar losses have been observed around the world (McMenamin and Genersch, 2015). Although beekeepers have managed to maintain a fairly stable global population of managed honey bee, this has only been accomplished at considerable effort and expense (Bruckner et al., 2019). Winter losses in the range of 30-40% are not normal and point to poor overall health

(Bruckner et al., 2019). Certain key factors have been identified as significantly contributing to this decline: migratory beekeeping, poor nutrition, infestation with *Varroa destructor*, pathogens like *Nosema ceranae*, and pesticides (Staveley et al., 2014; Goulson et al., 2015; Smith et al., 2013; Rennich et al., 2012). Multifactorial interactions, such as pesticide exposure increasing susceptibility to both *V. destructor* and *N. ceranae*, have proven problematic (Pettis et al., 2012; Goulson et al., 2015).

One particular pathogen, *Nosema ceranae*, infected 57% to 85% of honey bees sampled from 2009 to 2011 in the U.S. (Rennich et al., 2012). Runckel et al. (2011) tested for *Nosema* spp. in a migratory beekeeping operation and reported that nearly 100% of colonies were infected (75% with *Nosema ceranae* and 25% with *Nosema apis*) in August and September. *Nosema ceranae* now infects a number of *Apis* species such as *Apis mellifera*, *Apis florea*, and *Apis dorsata*, (Chaimanee et al., 2012), but this microsporidian originated from the Asian honey bee, *Apis cerana,* before transferring to other species (Vávra and Lukeš, 2013). Within *A. mellifera*, the recently invasive *N. ceranae* has overtaken the endemic pathogen, *Nosema apis*, in distribution and infectivity (Charbonneau et al., 2016; Martín-Hernández et al., 2012; Chen and Huang, 2010). In France, some colonies have been found to contain eight times more *N. ceranae* than *N. apis* (Chauzat et al., 2007), likely due to commercial trade in infected *A. mellifera* colonies (Klee et al., 2007). Our research therefore focused on *N. ceranae*.

Nosema apis and *N. ceranae* infects bee midgut epithelial cells (Gisder et al., 2011; 2010; Chen et al., 2009; 2008; Huang and Solter, 2013). After germinating for approximately seven days, these cells burst and release *Nosema* spores. The released spores then infect other cells or

are excreted fecally, where they infect other bees via oral-fecal transmission (Higes et al., 2007; Huang and Solter, 2013; Chen et al., 2008).

Nosema ceranae infection has multiple effects including suppressed immune response, digestive tissue degeneration, and premature mortality (Paris et al., 2018; Dussaubat et al., 2012; Martín‐Hernández et al., 2011). Infection can also modify pheromones essential for normal worker behavior, reduce brood rearing, and induce earlier foraging activity (Botías et al., 2013; Paris et al., 2018; Kralj and Fuchs, 2009). These effects reduce colony fitness (Higes et al., 2008), and *Nosema ceranae* can thus significantly decrease the survival of colonies in poor health, particularly in conjunction with other factors such as insecticide exposure (Aufauvre et al., 2012; Pettis et al., 2012; Wu et al., 2012).

In general, many of *Nosema's* effects seem to manifest in the bee gut. Infected bees exhibit modified behaviors such as increased sucrose consumption and lower tendencies to share food (Naug and Gibbs, 2009). Infected midgut cells also have reduced ability to absorb nutrients (Martín‐Hernández et al., 2011). Because *Nosema* exclusively infects the bee gut, it should have a strong effect on the gut microbiome (Rubanov et al., 2019; Huang and Solter, 2013; Zhang et al., 2019; Paris et al., 2020).

The microbiome of western honey bees worldwide has the same nine bacterial types (Raymann and Moran, 2018; Kwong and Moran, 2016) and takes about 5 d after emergence for their guts to be colonized (Martinson et al., 2012). These microbes contain genes that are involved in host immune response, metabolism, growth and development (Engel et al., 2012; Kwong et al., 2017; Raymann and Moran, 2018). Honey bee specific microbes and *Nosema* co-occur abundantly in the midgut and hindgut (Martinson et al., 2012; Bourgeois et al., 2012).

Some studies have begun to analyze these interactions. Pollen patties inoculated with honey bee gut bacterium, *Parasaccharibacter apium,* lowered *Nosema* levels in workers that were challenged with *Nosema* (Corby-Harris et al., 2016). Inoculation with *Bifidobacterium* and *Lactobacillus* strains isolated from the bee gut also reduced *Nosema* infection levels (Baffoni et al., 2015). However, some studies have shown that an imbalance of microbiota composition (dysbiosis), induced via bacterial inoculation, can increase mortality and susceptibility to parasites such as *Nosema* (Sommer and Bäckhed, 2013; Peterson and Round, 2014; Maes et al., 2016; Raymann and Moran, 2018; Schwarz et al., 2016). Therefore, understanding how *Nosema* infection alters the gut microbiome has implications for developing treatments to combat the effects of infection and dysbiosis.

Materials and Methods

Study site and colonies

We conducted our experiment on sixteen *Apis mellifera* colonies at the Biology Field Station (32°53'07.9"N 117°13'55.1"W) apiary at the University of California San Diego. All colonies were healthy, based upon standard inspection techniques (Dietemann et al., 2013).

General methods

Collection of newly-emerged bees

We took out the frame of a colony and located a large patch of capped worker brood. Using a brush, we carefully cleared away all the adult worker bees from the patch of capped brood. With the corner of a hive tool, we gently removed the caps off of worker broods to reveal the developing pupae, looking for pupae with deep purple eyes which signify that they are close to eclosion. After locating a good area, we placed a sterile wire cage (5x7.5x2.5 cm) around the region. We pushed the cage into the wax so that all sides are kept even and tight. We replaced the frame in its original slot in the colony, making sure to avoid scraping the cage off the side of other frames. We returned every 24 h to check for adult emergence inside the cages such as in Figure 1.

After emergence, we waited 5 d to collect the bees as honey bee workers need to develop their characteristic microbiome, acquired from food exchange and grooming with adult workers, 5 d after emergence (Martinson et al., 2012). After 5 d, we removed the frame and thoroughly brushed off all other bees. In a separate area, away from the colonies, we placed two sterile plastic cages (11.5x11.5x9 cm) on top of a table facing upward with their sliding door open. We

held the frame over the plastic cages with the wire cages closest to the opening. We removed the wire cage from the frame and quickly brushed the bees into the plastic cages. Once all the bees were emptied into the plastic cages, we closed the cages with the sliding door shown in Figure 2.

Figure 1. A wire cage inserted into the frame's wax covering newly emerged bees

Figure 2. A plastic cage with a closed door pierced with holes for gas exchange. This cage contains bees that emerged 5 d ago.

Spore Preparation and Counting

We prepared fresh spores that were less than 12 h old and maintained at room temperature throughout the purification process, which consisted of standard extraction and purification of spores from heavily infected bees (Eiri et al., 2015). We followed the standard procedure for measuring infection levels, using a hemocytometer to count the number of spores per bee (Eiri et al., 2015).

Feeding Newly-Emerged Bees with *Nosema*

After collecting the bees, we split them into two treatments: a control treatment (each bee fed 5 µl of pure 2.0 M sucrose solution) and an experimental treatment (each bee fed 40,000 spores per bee diluted in 5 µl of 2.0 M sucrose solution). To feed the bees, we placed them individually inside separate sterile vials and inserted a micropipette tip with 5 µl of the appropriate treatment into the vial lid (Fig. 3). We placed the vials in a tray with ultraviolet LED (SMD 3528, 240 lumens/m, 395–405 nm) strip lights, making sure to align the light with the pipette tip, to encourage feeding (Rubanov et al., 2019). If the bees did not consume all of the solution within 30-60 min, we then manually fed them the remaining solution to ensure that each bee received the same dose. After they finished feeding, we placed exactly 25 bees into a cage. Per cage, all bees received the same treatment. To sustain the bees during the experiment, we placed a 5 ml syringe filled with approximately 3 ml of 2.0 M sterile sucrose solution in each plastic cage and returned the plastic cages into the incubator.

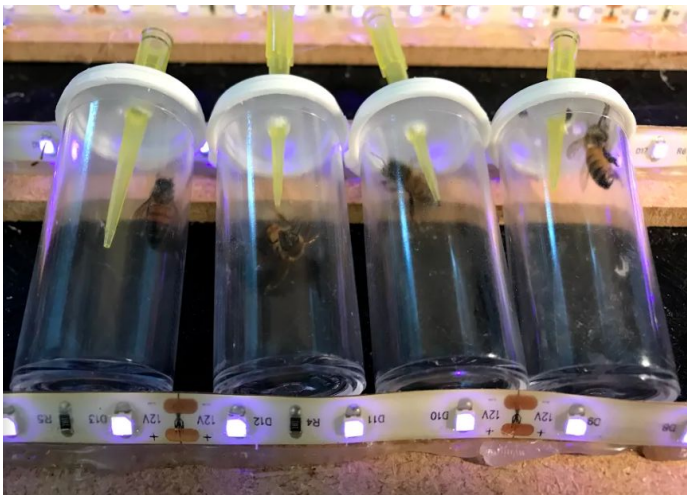

Figure 3. Bees are placed into separate vials with a pipette full of their treatment solution for feeding. Ultraviolet LED lights are placed at the tops of each vial to attract bees to the pipette tips with treatment solutions.

Gut Dissection and *Nosema* Extraction

We kept the caged bees in a dark incubator at 34°C and 70% relative humidity, standard conditions for maintaining caged adult worker bees in an incubator (Williams et al., 2013). Every 2 d, we refilled the sucrose syringe so that the bees could feed *ad libitum*, recorded mortality, and removed dead bees.

After allowing the spores to fully mature over 12 d (Fries et al., 2013), we chilled all remaining living bees on ice for 10-15 min and then dissected them. For each bee, we quickly dissected out the midgut and rectum because the midgut is where *Nosema* is predominantly found (Bourgeois et al., 2012) while the rectum contains the majority of the gut microbiota (Martinson et al., 2012). We placed the gut and rectum into a microcentrifuge tube filled with 100 µl of bee gut extraction buffer on ice, and then homogenized it with a Kontes motorized pestle for 30 s.

Each liter of bee gut extraction buffer (pH 7.4), contained 1.45 mM NaCl, 0.02 g Peptone, and 500 µl Tween 20. The buffer was autoclaved to sterilize it.

We then transferred 30 µl of the homogenized gut solution into a separate microcentrifuge tube for microbiome analysis. To preserve part of the sample for future culturing, we transferred another 30 µl of the original gut solution to a third tube containing 30 µl of 30% reagent grade glycerol and vortexed it.

We sterilized all of our dissection instruments between each bee dissection by spraying them with 100% ethanol. All samples were kept on ice at all times and were stored at -70°C to prevent microbiome degradation.

Experiment 1: Effects of treatment on survival and infection levels

We wanted to measure the survival and infection levels of bees fed live *Nosema* spores as compared to bees fed a sterile sucrose solution, using a lab environment. In previous experiments, bees were returned to their colonies after feeding them their appropriate treatment, but this yielded low levels of infection in the control bees due to expected trophallaxis between infected and control bees (Rubanov et al., 2019). We therefore wanted to run a more controlled experiment in a lab environment in which we could isolate the control and experimental bees in separate cages. For this experiment, we followed all of the general methods listed above.

Experiment 2: Selection experiment to collect microbiomes of the most resistant bees

In experiment 2, we wanted to determine the microbiome composition of bees that were the least infected despite being fed live *Nosema* spores. We determined which bees were the

least infected from those tested in experiment 1. Infection was measured with hemocytometer spore counts (see above). After counting the spores of all the infected bees, we sent preserved samples of the least infected gut material to the McFrederick lab in University of California, Riverside to perform a future microbiome analysis of the most resistant microbiomes.

Statistics

To determine the effects of treatment on spore levels, we used Analysis of Variance (ANOVA) with log-transformed spore counts (after inspecting model residuals) as our response variable and used colony identity, treatment, and the interaction colony x treatment as fixed effects. Cage name was a random effect nested within colony. Colony identity was a fixed effect because we explicitly wished to test the hypothesis that colonies varied. Although these data are counts, the measurement per bee ranged from 0 to over 41 million in increments of 5,000 and therefore closely approximated a continuous variable suitable for ANOVA. To correct all-pairwise comparisons, we used Tukey Honestly Significant Difference (HSD) tests.

To determine the effects of treatment upon survival, we used a Proportional Hazards Survival model with censoring and colony, treatment, and the interaction colony x treatment as fixed effects. We report model results as Effect Likelihood Ratio tests (L-R chi-square tests). We used JMP Pro v14.3.0 and reported a mean±1 standard error.

To test the effects of treatment and average mortality per cage after 12 days on average spore counts per surviving bee per cage, we ran a Mixed Model (REML algorithm) with colony as a random effect.

Results

Experiment 1a: Effects of treatment on survival

Overall, there was a significant effect of treatment on bee survival. Across all 15 colonies, 41.5% of bees fed *Nosema* spores died before 12 d and only 21.7% of control bees died before 12 d. In our Proportional Hazards fit model, there were no significant effects of treatment (L-R Chi-square=2.90×10^{-6}, 1 *df*, P=0.9986), but significant effects of colony (L-R Chi-square=122.54, 14 *df*, P<0.0001), and the interaction colony x treatment (L-R Chi-square=31.90, 14 *df*, P=0.004). For seven colonies, mortality was significantly greater for experimental bees than control bees (L-R Chi-square≥4.13, 1 *df*, $P \leq 0.04$). For the remaining colonies, there was no significant difference in mortality between treatments (L-R Chi-square≤3.50, 1 *df*, $P \geq 0.06$).

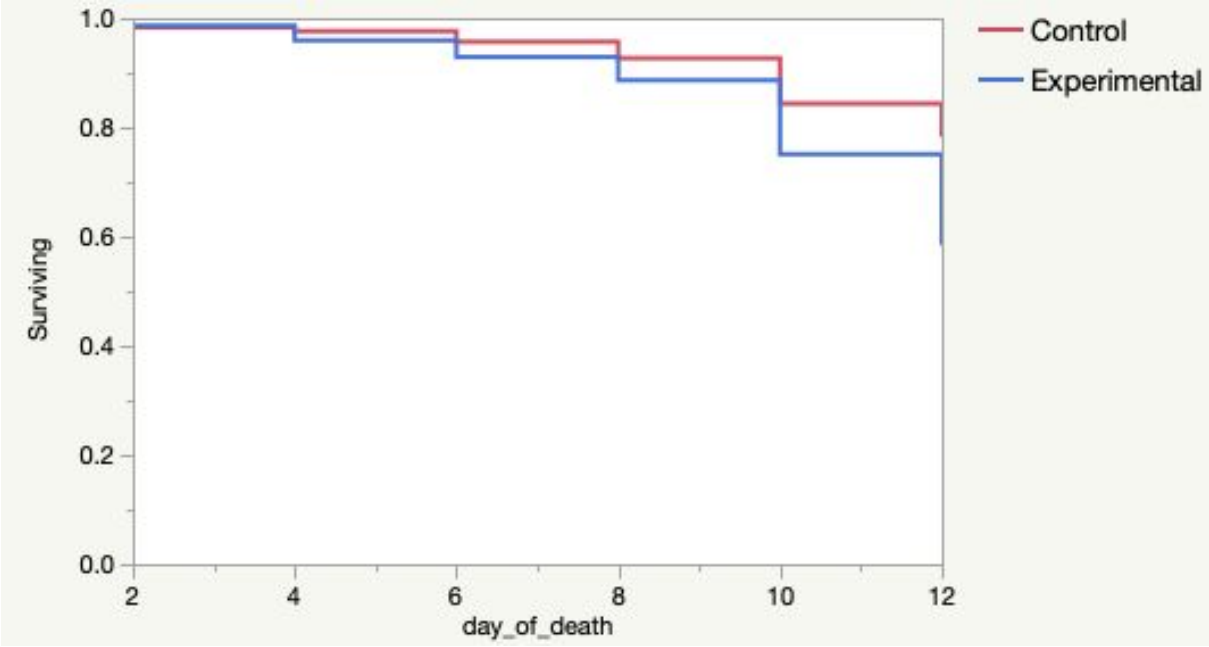

Figure 4. Survival plot of bees consuming pure sterile sucrose solution (control) or the experiment treatment (live *Nosema* spores).

Experiment 1b: Effects of spore treatment on infection levels

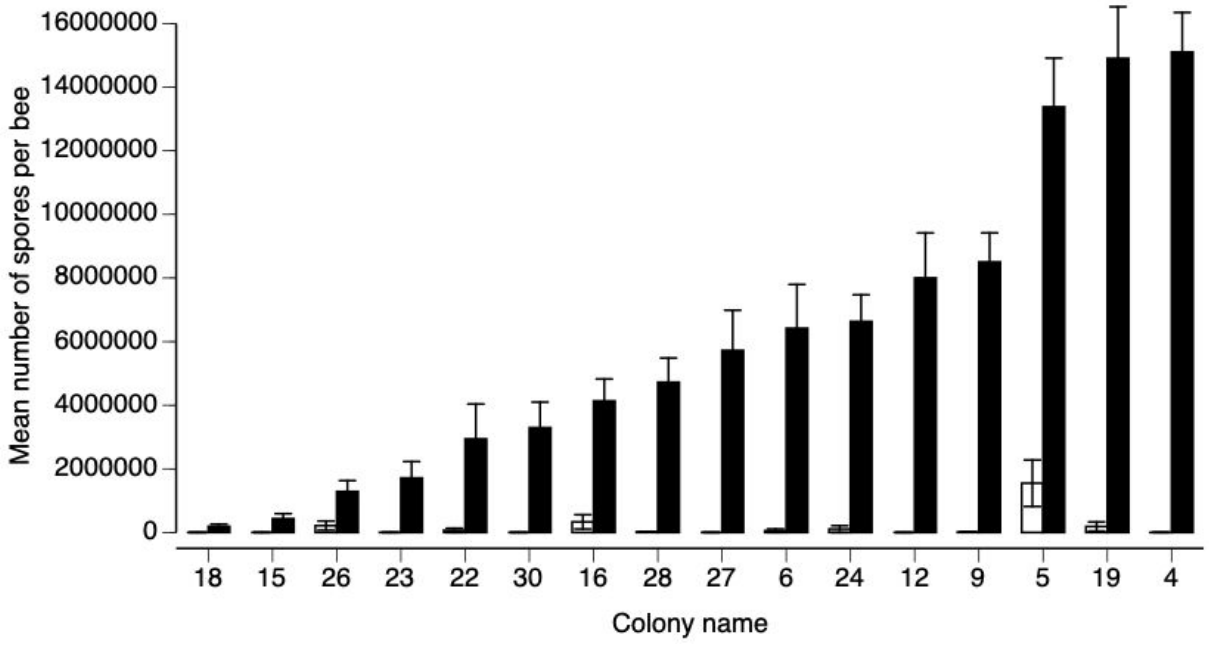

Figure 5. Effect of treatment on mean bee spore counts, organized by increasing severity of infection in different colonies. All experimental bees (black bars) were fed 40,000 live spores per bee. Control bees (open bars) received no spores. Means and standard deviations are shown.

Bees varied in the severity of the infection that they developed, according to their colony background (Fig. 4). The maximum spore count, after 12 days, was 41,400,000. Among the bees fed live spores (n=543), 8.5% were completely uninfected (zero spore counts). Among the control bees fed sterile sucrose solution and no spores (n=533), 87.6% were uninfected. Our overall model accounted for 78% of variance in spore count. There were significant effects of treatment ($F_{1,38}$=387.04, P<0.0001), colony ($F_{15,38}$=2.27, P=0.02), but no significant effect of the interaction treatment x colony ($F_{15,23}$=1.70, P=0.12). There was a significant effect of cage (Wald P-value=0.006), which accounted for 21% of model variance. The Tukey HSD test (P<0.05) revealed that all control bees were significantly less infected than experimental bees. Visual

inspection of the data did not show any natural bins into which colonies could be assigned: the degree to which bees from these colonies could become infected was fairly continuous, although colonies 5, 19, and 4 were perhaps somewhat more susceptible, as a group, than other colonies (Fig. 4)

We tested for the possibility that mortality and treatment could predict spore counts. As expected, treatment significantly predicted average spore count per bee per cage ($F_{1,29}$=58.10, P<0.0001). However, there was no significant effect of average mortality on spore counts ($F_{1,36}$=0.88, P=0.36). The interaction average mortality x treatment ($F_{1,29}$=3.06, P=0.09) was not significant. Colony accounted for 25% of model variance,

Experiment 2: Effects of spore treatment on microbiome composition

In experiment 1, we found significant variation in susceptibility of bees to infection according to their colony background. Therefore, we selected the least infected bees of each colony for a future microbiome analysis and culturing.

Table 1. Number of trials performed with each colony; each trial had one cage per treatment.

Colony	Trial #	# of bees/cage
4	1	25
4	2	25
5	1	25
5	2	25
6	1	25
6	2	25
9	1	25
9	2	25
9	3	25
12	1	25
12	2	25
15	1	20
16	1	25
16	2	25
18	1	25
19	1	25
19	2	25
22	1	25
23	1	25
24	1	25
24	2	20
26	1	25
27	1	25
28	1	25
30	1	25

Discussion

Feeding bees live *N. ceranae* spores resulted in significant increases in bee gut spore levels and decreased bee survival, as expected. Interestingly, there were significant differences in infection levels between different colonies, suggesting that colony genetic background, microbiome composition, or both contribute to *Nosema* resistance and susceptibility. Because we examined mortality over a fairly short time period and had relatively low mortality overall (Fig. 4), there was no significant relationship between overall cage mortality after 12 days and bee spore counts, although treatment strongly and very significantly predicted spore counts.

Multiple studies have shown that *N. ceranae* infection harms *Apis mellifera* so it is not surprising that infection can greatly reduce worker lifespan (Paris et al., 2018). On average, 12 days post inoculation (dpi), our bees fed *Nosema* spores had a mortality of 39%, significantly higher than the 19% mortality in our control bees. These results are roughly similar to those reported in other studies. Goblirsch et al. (2013) found that a 50% mortality rate occurred in bees inoculated with 10,000 spores at 16 dpi, as compared to 25 dpi for control bees which is an average of 9 d longer. Milbrath et al. (2015) stated that feeding bees 30,000 spores each resulted in a median survival time of 20 dpi whereas the median survival time of control bees was 27 dpi. Higes et al. (2007) inoculated bees with 125,000 spores each and found that, by 7 dpi, 100% of the bees had died, whereas the control bees' mortality was only 6% at this point in time. Martín-Hernández et al. (2011) compared the mortality of bees fed 0, 1,000, 10,000, 50,000, and 100,000 spores and measured respective mortalities of 4, 11, 20, 67, and 93 percent mortality at 7 dpi. Both Higes et al. (2007) and Martín-Hernández et al. (2011) used similar methods, including CO_2 anesthetization during inoculation. However, Milbrath et al. (2013) showed that

this technique can affect bee survival which can account for their higher mortality rates than those we measured at 12 dpi.

Our treatment of bees differs from other studies in multiple ways, which may account for some of the differences that we report. Milbrath et al. (2015) and Goblirsch et al. (2013) inoculated their bees within 1 day post eclosion (dpe) while Higes et al. (2007) and Martín-Hernández et al. (2011) caged their bees in an incubator within 1 dpe and inoculated them 5 dpe. However, this may cause longevity stress by impairing natural microbiome development. Newly emerged bees are sterile and only acquire their full microbiome from adult colony workers after 5 d of food exchange and contact with adult workers (Martinson et al. 2012). Subsequently, Powell et al. (2014) showed that bees removed from the colony before microbial colonization can remain completely sterile of gut bacteria throughout adulthood. Lastly, we only fed our caged bees a sterile sucrose solution. All of the other studies cited above provided caged bees with non-sterile pollen and in some cases, non-sterile dietary supplements such as Promotor L, a commercial mixture of amino acids and vitamins (Higes et al., 2007; Martín-Hernández et al., 2011). We did not provide any protein supplements, in part to ensure that our hemocytometer gut content counts could be made without obscuring digested pollen or other material. Thus, our higher mortality could arise from lack of protein nutrition. Relevantly, honey bees infected with *N. ceranae* have increased longevity when given diverse and high quality pollen (Jack et al., 2016; Basualdo et al., 2014; Di Pasquale et al., 2013).

We found that bees fed with live spores were significantly more infected than control bees, as shown in multiple other experiments. On average, bees that were fed 40,000 live spores, had infection levels of 7.7 million spores per bee at 12 dpi and 91.5% were infected (defined as

having at least one spore). Smart and Sheppard (2012) sampled bees from naturally infected colonies at various life stages and found that bees 0-3 dpe were completely uninfected, while only 0.4% of bees 8-11 dpe were infected with 3.35 million spores. Infection levels increase with bee age and infection period, potentially accounting for our higher mean infection levels. Older bees, particularly foragers, are most susceptible and most intensely infected by *Nosema* (Mayack and Naug, 2009; Higes et al., 2008). Smart and Sheppard (2012) found that 8.3% of 22-25 dpe were infected, with an average of 2.38 million spores per infected bee. The bees that we treated were approximately 17 dpe when we tested for spore counts. In general, there is considerable variation in infection levels between experiments, perhaps because of differences in *Nosema* purification and feeding techniques, bee ages, and colony backgrounds. Huang et al. (2015) fed bees 5 dpe with 100,000 spores each and reported an average infection level of approximately 50,000 spores 10 dpi. Forsgren and Fries (2010) found that inoculation with 10,000 spores yielded an average of 20 million spores per bee 12 dpi. Our highest infection level was 41.4 million spores per bee. By comparison, Milbrath et al. (2015) reported *N. ceranae* infection levels of over 30 million spores per bee after 15 days of infection and, in our case, found a bee with a mixed *Nosema* spp. infection of over 182 million spores.

Our results showing that bees from some colonies were more readily and highly infected were perhaps the most interesting and are confirmed in other studies. Rubanov et al. (2019) also found that colonies varied significantly in infection levels, even though the same number of bees fed identical spore numbers were returned to each colony. In this study, at 21 dpi, bees fed live spores in high infection colonies were significantly more infected as compared to control bees from the same colonies. In low infection colonies, infection levels were not significantly

different between control and experimental treatments. After analyzing the microbiome of the bees, they determined that high infection bees had greater amounts of two *Gilliamella* ESVs (Exact Sequence Variants) as compared to low infection bees. It would be interesting to test the effects of inoculating bees with a probiotic consisting of typical *A. mellifera* gut microbes with little to no *Gilliamella* ESVs present.

For the future, determining the microbiome of bees from our selection experiment will be revealing. We hypothesize that the microbiome of bees that are less infected, despite receiving the same dose of live, fresh *Nosema* spores, will be different from the microbiome of bees that are heavily infected. In addition, culturing the gut contents of bees that show resistance to *Nosema* infection to potentially isolate bacteria associated with resistance would be interesting, paving the way for the development of bee-based natural probiotics.

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