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Effects of larval *Nosema ceranae* infection on adult honey bee (*Apis mellifera*)
morphology and physiology

A Thesis submitted in partial satisfaction of the requirements for the degree Master of

Science

in

Biology

by

Lee BenVau

Committee in charge:

Professor James Nieh, Chair
Professor David Holway
Professor Joshua Kohn

2014

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University of California, San Diego

2014

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ABSTRACT OF THE THESIS

Effects of larval *Nosema ceranae* infection on adult honey bee (*Apis mellifera*)
morphology and physiology

by

Lee BenVau

Master of Science in Biology

University of California, San Diego, 2014

Professor James Nieh, Chair

Nosema ceranae is an important honey bee pathogen that has been implicated in CCD. Until recently, it was thought to be exclusively a disease of adult bees, but recent findings indicate that it can also infect larvae. Thus, little is known about the effects of

larval *Nosema* infection. Vitellogenin (Vg) is a key protein necessary for egg-laying, production of brood food, proper immune function, and promotion of longevity through the reduction of oxidative stress. In worker bees, Vg increases in bees of nursing age, at approximately 7 days of age. We therefore investigated the effects of larval *Nosema* infection on hemolymph Vg and total protein concentration. Larvae infected by a high dose of *Nosema ceranae* spores developed into adults with 2 fold higher Vg titers. However, they also had 1.3 fold lower total hemolymph protein titers. Thus, *Nosema* infection resulted in a corresponding 1.7 fold increase in percentage Vg compared to control. Infected bees also had 1.1 fold fewer barbs than did control bees and, we found an inverse relationship between Vg and the number of barbs per lancet. Overall, adult infected bees were not significantly different from controls. Our results indicate that larval *N. ceranae* infection has a greater impact than does adult infection on adult honey bees. Overall, *Nosema ceranae* accelerates aging and causes infected bees to resemble older workers or queens.

Introduction

Honey bees (*Apis mellifera*) are important pollinators whose services are valued at more than \$15 billion per year in the United States. However, domesticated honey bee colonies have been steadily declining in the US since the 1940's (Pettis & Delaplane 2010). Since 2006 beekeepers have reported unusually high colony losses, up to 30-90% (vanEngelsdorp et al. 2012), and some European countries have also reported similar losses (Higes et al. 2010; Genersch et al. 2010). These losses have been attributed to colony collapse disorder (CCD) in which a colony suddenly loses its workers and dies as a result.. It is currently believed that CCD is caused by a combination of factors such as pesticides, parasites, and pathogens that act synergistically to decrease honey bee health (Neumann & Carreck 2010).

Concurrent with the spread of CCD, the microsporidian *Nosema ceranae* expanded from its original host, the Asian honey bee (*A. cerana*), to also infect the European honey bee, *A. mellifera* (Higes et al. 2006; Huang et al. 2007; Cox-Foster et al. 2007; Higes et al. 2008). Nosemosis, the disease caused by *Nosema apis*, is one of the most common diseases among adult honey bees, though it generally does not lead directly to colony death (Bailey 1981; Matheson 1993). In general, *N. ceranae* infection decreases bee health by weakening immune systems and causing energetic stress (Mayack & Naug 2009; Alaux et al. 2011). However, *Nosema ceranae* is more pathogenic than *N. apis* (Paxton et al. 2007) and has been associated with CCD in Spain (Higes et al. 2008; Martín-Hernández et al. 2009; Higes et al. 2009). For example, a metagenomic analysis of bees that died during the 2006-2007 CCD crisis showed that

nearly all bees from CCD-positive colonies had four pathogens: two viruses and two species of Microsporidia, *N. apis* and *N. ceranae* (Dainat et al. 2012).

While the effects of *Nosema ceranae* infection manifest themselves in all honey bee castes (Mayack & Naug 2009; Alaux et al. 2011; Traver & Fell 2011), it is thought that *Nosema* only infects adult honey bees (Hassanein 1951). However, larvae can also be infected in a laboratory setting (Eiri et al. 2014), but little is known about the effects of larval *N. ceranae* infection. Studying the effects of larval infection is important because a variety of larval diseases can affect development and thus adult morphology. A well known example is deformed wing virus that causes crippled wings and bloated abdomens in adults that developed from infected larvae (de Miranda & Genersch 2010). However, no studies that have investigated the effects of *N. ceranae* on honey bee morphology because this pathogen was not believed to infect larval honey bees (Hassanein 1951; Smart & Sheppard 2011).

Nosema infection may also alter normal bee age polyethism, the progression of tasks from nurse to forager that a bee engages in as it ages. Bees in normal hives can transition to foraging at 14-65 days old (Free 1965). Early evidence suggested that *N. ceranae* infection could prematurely accelerate age polyethism, causing infected nurse bees to adopt the behaviors of forager bees at an earlier age than normal (Wang & Moeller 1970a). Recently, Goblirsch et al. (2013) showed that nurse-aged bees infected with *N. ceranae* were 2 fold more likely to engage in precocious foraging as non-infected in controls. This behavioral response may be a colony behavioral immunity response (Evans & Spivak 2010) because foragers have a higher probability of dying outside the nest than nurse bees. The colony may limit the spread of infection if nurse

bees refrain from feeding larvae and tend to die outside the nest. In fact, honey bees treated with drugs or otherwise impaired tend to leave the colony, a potential example of altruistic self-removal (Rueppell et al. 2010).

Acceleration of age polyethism should alter multiple physiological traits that are normally associated with bee aging. For example, vitellogenin (Vg), a 180-kDa glycolipoprotein that is a precursor to the major yolk protein vitellin, regulates important aspects of the honey bee life cycle including age polyethism, the division of labor critical to colony function (Hartfelder & Engels 1998; Amdam & Omholt 2002). *Nosema ceranae* infection of adults can decrease Vg levels (Antúnez et al. 2009), giving them levels more similar to those found in older bees (i.e., foragers).

Vitellogenin is found in females of all oviparous species. In insects, it is synthesized in fat body cells and released into hemolymph (Hagedorn & Kunkel 1979; Wheeler & Kawooya 1990). Vitellogenin is necessary for egg-laying in queens and is also used by nurse bees to produce the protein-rich glandular secretion known as brood food. Vitellogenin levels are low in newly eclosed workers and reach a peak at 5-10 days (Engels 1974; Engels & Fahrenhorst 1974) when nurse bees use the protein to synthesize royal jelly to feed larvae and the queen (Amdam et al. 2003). As the nurse bees age, their Vg stores decrease and they transition to the far riskier job of foraging in their final weeks of life.

Vitellogenin also plays a key role in regulating longevity (Amdam et al. 2004), promoting longevity in workers and queens by reducing oxidative stress (Seehuus et al. 2006; Corona et al. 2007). Suppressed Vg expression will reduce worker longevity (Nelson et al. 2007; Remolina et al. 2007). Recently, Goblirsch et al. (2013) showed

that nurse-aged bees infected with *N. ceranae* had decreased gene expression levels of Vg as compared to controls. *Nosema ceranae* infection may therefore contribute to poor bee health and longevity by decreasing Vg levels. Viewed in terms of age polyethism, these bees also appear age-accelerated in that their Vg levels were more similar to those of older bees.

However, nothing is known about how *N. ceranae* infection contracted by larvae alters subsequent adult Vg levels. Our study therefore examines how *N. ceranae* infection on larvae affects adult Vg and total protein levels. We compared the effects of larval infection with those of adult infection. We hypothesized that larval *Nosema* infection would result in young bees with elevated Vg levels. In addition *N. ceranae* infection causes energetic stress that can induce adult bees to consume more food (Mayack & Naug 2009; Alaux et al. 2011). It is therefore possible that our infected larvae, which we reared *in vitro* on a diet that included royal jelly, would exhibit more queen-like traits, such as a queen-like morphology, as a result of consuming more royal jelly (Kamakura 2011). We therefore examined the effects of larval infection on an indicator of queen-like morphology, the number of barbs on an adult stinger.

Methods

Adult infection experiment

We conducted three trials with bees from three different healthy colonies that were free of *Nosema* infection (determined by randomly collecting foragers and examining their midguts for spores) and kept at the UCSD Biological Field Station. For each trial, we obtained comb with capped brood that we incubated at 33 C and 70%

humidity. We collected the newly emerged bees (60, 90, and 150 bees for trials 1, 2, and 3 respectively). Within a trial, the newly emerged bees were equally separated into *Nosema* treatment or control among multiple plastic cages. Each cage (9 x 11.5 x 11.5 cm) held only one treatment group. Control bees were fed 2.0 M sucrose, water, and bee bread (pollen mixed with 2.0 M sucrose solution) *ad libitum*. *Nosema* treated bees were given 2.0 M sucrose solution containing 10,000 spores per 40 μ L of solution, bee bread, and water *ad libitum*. Sucrose solution was contained in syringes that bees could readily access and that were refilled when low. Cages were kept queen-right with half of a queen pheromone stick in each cage (PseudoQueen, Contech Inc.). Each day sucrose solutions were weighed to determine how much the bees had consumed. Clean tissues placed in the bottom of the cage were regularly discarded and replaced when soiled. Any dead bees in the cages were discarded and the mortality recorded. After 7 days, the surviving bees were frozen and prepared for hemolymph extraction following the methods described below. Seven days is within the 5-10 day window of peak Vg in hemolymph (Engels 1974; Engels & Fahrenhorst 1974) and is also the length of time that Antunez et al. (2009) used for their infected bees. While Vg in normal one-day old bees is typically too low to detect (Engels 1974; Engels & Fahrenhorst 1974), we nonetheless wanted to measure adult Vg levels upon emergence to see if *N. ceranae* infection would alter this.

Larval infection experiment

We obtained *N. ceranae* spores from infected *A. cerana* workers in Thailand and fed them to *A. mellifera* workers in La Jolla, California to create a constantly renewed fresh stock for our experiments. Spore-producing bees were not fed pollen, only sucrose solution to ensure that gut contents consisted mainly of spores. To obtain spores, we dissected out the midguts of adult honey bees, homogenized them in sterile double-distilled water, and filtered them through Fisherbrand P8 filter paper with 20-25 μm pores. We collected the filtrate in microcentrifuge tubes that we centrifuged at 10,000 RPM for 15 min. We subsequently removed the supernatant and resuspended the pellet in double-distilled water (ddH₂O). We measured spore concentrations under a microscope with a hemocytometer (Cantwell 1970). This procedure resulted in fairly pure spore preparations. We made appropriate dilutions with ddH₂O to obtain the necessary treatment doses. We confirmed that our spores were *N. ceranae* by sequencing them (primer pairs NoscRNAPol-F2 and NoscRNAPol-R2 from Gisder & Genersch 2013) and comparing our sequences with GenBank data.

Bees from five different healthy colonies that were free of *Nosema* infection were raised *in vitro* based upon methods of Aupinel et al. (2005) and as described in Eiri et al. (2014). Day 4 larvae were treated with 10,000 *N. ceranae* spores, 40,000 spores, or controls (10 μL of fluid added only at day 4, the control consisting of sterile ddH₂O). Bees were frozen at -70°C upon emergence or 7-9 days after emergence.

Determining infection levels

To determine if our larval *Nosema* treatments actually infected bees, we counted spores (see method above) in the midguts of bees from the same trials that we used to obtain bees infected as larvae. Our bees were generated as part of another project testing if honey bee larvae can be infected by *N. ceranae* (Eiri et al. 2014), and thus a part of these data have been published. However, we use this data only to demonstrate that our larvae were infected with *Nosema*. There is no other overlap between this publication and Eiri et al. (2014), which does not examine the effects of infection on hemolymph proteins. For bees infected as adults, we counted the spores from bees with intact midguts.

Vitellogenin quantification

Bees were stored at -70 °C prior to use. Bees were thawed for 10 min at room temperature and their mouthparts were glued shut with cyanoacrylate adhesive (Goodale & Nieh 2012). We then used dissecting scissors to sever the legs close to the body and placed the bee in a microcentrifuge tube with a membrane-less insert (Costar Spin-X #9301) that allowed hemolymph to freely flow through. The bees were then centrifuged for 1 minute at 1000 rpm in an Eppendorf 5415 D centrifuge (modified methods of Mayack & Naug 2010). Bees were prepared in small subgroups of typically five individuals to minimize the time between severing the legs and centrifugation in an attempt to reduce hemolymph clotting, which could affect extraction. Pure hemolymph is clear immediately after extraction. We carefully inspected bees after centrifuging and did not use hemolymph samples that were contaminated by ruptured abdomens (cloudy

or yellow in color) or bees in which the hemolymph extraction did not yield at least 1 μL . Typically, we obtained yields of 1 to 3 μL hemolymph from all treatment groups. Hemolymph samples were then frozen (-70°C) for later purification or immediately purified with the SDS-PAGE Sample Prep Kit (Pierce Biotechnology, Rockford, IL, USA # 89888) according to the manufacturer's recommendations. The purification yielded 50 μL of protein solution: we used 25 μL to measure Vg levels and 12.5 μL to assay total protein levels. We used a larger fraction of our total purification to more accurately measure the Vg content, which is a fraction of the total protein.

There are several methods of quantifying proteins, and SDS-PAGE separation coupled with the BCA assay is standard technique. SDS-PAGE is widely used to separate honey bee Vg from other hemolymph proteins (Wheeler & Kawooya 1990; Lin et al. 1999; Pinto et al. 2000; Barchuk et al. 2002; Guidugli et al. 2005; Amdam et al. 2005; Seehuus et al. 2006; Corona et al. 2007; Gaetschenberger et al. 2012). The BCA assay is used to quantify Vg or other hemolymph proteins after separation with SDS-PAGE in honey bees and other insects (Wheeler & Kawooya 1990; Hartfelder & Engels 1998; Lewis et al. 2001). Antúnez et al. (2009) and Goblirsch et al. (2013) used quantitative real-time PCR as another way to measure Vg, but SDS-PAGE and the BCA assay allow us to directly measure the amount of protein in the hemolymph.

In order to isolate and quantify Vg, we used sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE (Laemmli 1970) and the bicinchoninic acid (BCA) assay (Smith et al. 1985). Hemolymph samples were run on 7% acrylamide gels with a 4% stacking gel at 200 V at room temperature until the protein bands were

sufficiently resolved, approximately 1 hour (based upon methods of Amdam et al. 2006). The gels were run on a Mini-PROTEAN Tetra Cell (Bio-Rad #165-8000), and we used a Thermo Scientific Spectra Multicolor High Range Protein Ladder (#26625) that included a 180 kDa protein marker to correspond with honey bee Vg, a 180 kDa protein (Wheeler & Kawooya 1990; Lin et al. 1999). Gel bands corresponding to 180 kDa (Vg) were excised into 120 μ L elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5, Thermo Scientific), homogenized with a motorized Kontes pellet pestle for 1 minute, and eluted for 24 hours. The samples were then centrifuged for 10 minutes at 10,000 rpm and 100 μ L of the supernatant was collected for measuring protein content.

To measure total hemolymph protein, we followed the same procedure, but did not run our samples through the SDS-PAGE gel because we did not need to isolate the Vg. We placed 12.5 μ L of the purified hemolymph in 87.5 μ L of the elution buffer for a total volume of 100 μ L (matching the Vg samples) and ran this mixture through the BCA assay. We followed the manufacturer recommended protocol for the BCA assay (Thermo Scientific Kit # 23225) and used a spectrophotometer (Pharmacia Biotech Ultrospec 2000, model # 80-2106-00) to visualize protein concentrations.

Sting morphology

Queens and workers differ in morphology. For example, queens do not leave their sting inside the victim, and thus queens have fewer barbs per sting lancet (2-5 barbs/lancet) compared to workers (8-11 bars/lancet) (Weaver 1957). We used this

measure of “queenliness” because the number of barbs per sting is easily counted. We used forceps to pull out the two lancets of the sting in an adult bee, placed one lancet on a slide, covered it with a drop of water and a coverslip, and viewed it under 100x magnification (Zeiss Axioskop compound microscope).

Measuring infection levels

We measured the infection levels of adult bees in our adult exposure experiment. We carefully dissected out the midgut of each bee, and applied the method of Cantwell (1970) to extract spores and measure the number of spores per bee with a hemocytometer in a compound microscope (Zeiss Axioskop), making two independent measures of each sample and recording the average spore count (Cantwell 1970).

Statistics

Analysis of hemolymph proteins, including Vg, as well as all morphological measurements were done through ANVOA and tested for significance with *post-hoc* Tukey HSD analysis (JMP v10.0 statistical software). All statistical tests used $\alpha = 0.05$ to establish significance. Total protein data were log transformed and percentage Vg data were arcsine square root transformed (Zar 1984). For the analysis of treatment effects on Vg titers >100 ug Vg/ uL hemolymph in the bees infected as adults, we log transformed Vg titers. Following transformations, data met normal assumptions as determined through inspection of the residuals. To determine if our treatments resulted in infected adults, we used non-parametric Wilcoxon/Kruskal-Wallis tests because the data were not normally distributed.

Results

In total, we analyzed the hemolymph proteins, including Vg, and the morphologies of 391 bees from eight different colonies. Each *in vivo* adult control bee consumed an average of 22.5 ± 17.7 μL of 2.0 M sucrose solution per day. Each *in vivo* adult *N. ceranae* infected bee consumed an average of 21.3 ± 19.6 μL of 2.0 M sucrose solution containing 10,000 spores per 40 μL every day. *In vivo* treated bees consumed the same amount of sucrose as did controls ($F_{1,124} = 0.20$, $P = 0.65$). In the trials from which we obtained our larvae, *Nosema* spores given to larvae resulted in significantly infected prepupae (10K vs control: $z = 5.54$, $P < 0.0001$; 40K vs control: $z = 3.68$ $P = 0.0002$) and adults (10K vs control: $z = 5.77$ $P < 0.0001$; 40K vs control: $z = 4.23$, $P < 0.0001$). In the trials in which we infected adult bees, adults were significantly more infected than were controls (Wilcoxon/Kruskal-Wallis test, Chi-square = 25.79, $df = 1$, $P < 0.0001$).

Effect on Vg titers

Larval treatment

There is a significant effect of treatment on the Vg titers of bees treated as larvae ($F_{5,126} = 4.12$, $P = 0.0017$, $n = 136$ bees). The 1-day old 40K-treated bees have a significantly 2 fold higher Vg titer than the 1-day old control bees (Tukey HSD test, Fig. 1A). There are no significant differences between any other groups. At 7 days of age, control and infected bees had equal Vg titers. Thus, 1-day old adults infected as

larvae with a 40K dose had an average Vg titer that was similar to those found in 7-day old controls, which would normally have far higher titers.

Adult treatment

In bees, infected as adults, there is no significant overall effect of *Nosema* treatment on Vg titers in 7-day old bees ($F_{1,85} = 1.21$, $P = 0.27$, $n = 87$ bees, Fig. 1B). However, there is a bimodal distribution in the Vg titers of the *Nosema* treated bees such that a natural gap occurs at 100 ug Vg /uL hemolymph (Fig. 1B). We therefore excluded Vg titers below this value and tested for a treatment effect on this more restricted range of Vg titers. In this range of Vg titers, there is a significant effect of treatment ($F_{1,37} = 4.97$, $P = 0.03$, $n = 39$) such that control bees had significantly 1.3 fold higher Vg titers than did infected adult bees.

Effect on total hemolymph protein titer

Larval treatment

There is a significant effect of treatment on the total hemolymph protein titers of bees treated as larvae and measured as adults on day 7 after eclosion ($F_{5,39} = 4.45$, $P = 0.0026$, $n = 45$ bees). In 1-day old bees, there is no significant effect of the larval treatment. However, 7-day old bees given the highest *Nosema* dose (40K) have a significantly 1.3 fold smaller total hemolymph protein titer compared to any other treatment group, including the 7-day old controls (Tukey HSD test, Fig. 2A). Thus, larval infection by *N. ceranae* led to significantly decreased levels of total hemolymph protein 7 days after eclosion.

Adult treatment

In bees infected as adults, there is no significant effect of treatment on total hemolymph protein titers in 7-day old adults ($F_{1,82} = 1.26$, $P = 0.27$, $n = 90$ bees, Fig. 2B).

Effect on percentage Vg titers

Larval treatment

We then determined the effect of treatment on the percentage of the total protein titer that consisted of Vg. There is a significant effect of treatment ($F_{5,39} = 6.33$, $P = 0.0002$, $n = 45$ bees). The 7-day old 40K treatment bees have a significantly 1.7 fold higher percentage of Vg compared to 7-day control bees and 2.7 fold higher compared to the 1-day 40K bees (Tukey HSD test). Bees give the same treatment (the highest *Nosema* dose as larvae) therefore had proportionally more Vg in their hemolymph as 7-day old adults (Fig. 3A), even though their overall hemolymph protein titers had significantly decreased by this age (Fig. 2A).

Adult treatment

There is no significant effect of treatment on 7-day old adults infected as adults ($F_{1,83} = 0.53$, $P = 0.47$, $n = 87$).

Effect on queen-like morphology

For these data, we only looked at the effect on larval bees because the stings are already fully developed in newly emerged adult bees. There is a significant effect of treatment ($F_{5,87} = 4.08$, $P = 0.0023$, $n = 116$) such that *Nosema*-treated bees tended to be more queen-like and had slightly fewer barbs per lancet compared to controls (0.95 fold and 0.90 fold fewer barbs in both *Nosema* treatments as measured in 1-day and 7-day old bees respectively). Specifically, in 1-day old bees, 40K-treated bees had significantly fewer barbs compared to 1-day old control bees (Tukey HSD test, Fig. 4A). Similarly, the 7-day old 10K bees had significantly fewer barbs compared to the 7-day old controls (Tukey HSD test). However, we would expect both 10K and 40K treated groups to have fewer barbs compared to controls, regardless of the adult bee age. Variation between the treatment groups may account for this result.

Furthermore, there is a significant correlation such that higher Vg levels resulted in significantly fewer barbs per stinger ($F_{1,85} = 10.69$, $P = 0.002$, $R^2 = 0.11$). Thus, larval *N. ceranae* infection causes the developed adults to become slightly more queen-like with fewer barbs on their stings. Moreover, as expected, bees with a higher Vg titer had a more queen-like stinger morphology.

Discussion

No previous studies have examined the effect of larval honey bee *N. ceranae* infection upon Vg levels. Our results show that *N. ceranae* has a greater impact when it infects larvae rather than adults in that larvae infected with *N. ceranae* had increased Vg titers. The 1-day 40k bees had a 2-fold increase in Vg compared to controls and to all

other treatments, including 7-day old bees. Thus, at this early age, they had unexpectedly high levels of Vg that are typically found only in older bees. However, this Vg increase did not persist for long and by 7 days of age, had disappeared. Overall, *Nosema* infection decreased total hemolymph protein titers (7-day 40k bees had a 1.3-fold decrease in total protein as compared to control). Because of this decrease in overall protein levels, the percentage Vg levels of 7-day 40k bees were 1.7 fold higher compared to control and 2.7-fold higher compared to 1-day 40k bees. Bees infected as adults did not differ from uninfected bees in any of these measures, with one exception. Adult-treated bees with $> 100 \mu\text{g Vg}/\mu\text{L}$ hemolymph had significantly less Vg titers than controls. However, this is a weak effect that disappears when the entire range of Vg titers is considered. Thus, *Nosema* treatment had a greater effect when given to larvae than when given to adults. The 7-day old adults treated as larvae can be most directly compared to the 7-day old adults treated as 1-day old adults. In this comparison, *Nosema* treatment resulted in adults with decreased total protein titers and increased percentage Vg titers when given to larvae, but not when given to adults.

Finally, bees infected as larvae had a more queen-like morphology; they developed fewer barbs on their stinger (1-day 40k bees had 1.1 fold fewer barbs than control bees). Thus, *N. ceranae* has the greatest impact on larval infected individuals and causes them to develop into adults that resemble older workers or queens.

Vitellogenin titers

Amdam et al. (2005) showed that naturally reared 2-day and 8-day old honey bees (*A. mellifera*) had Vg titers of approximately $5 \mu\text{g}/\mu\text{l}$ and $45 \mu\text{g}/\mu\text{l}$, respectively. In

our *in vitro* reared control bees, the 1-day old bees had higher Vg titers (at least 30 $\mu\text{g}/\mu\text{l}$) while our 7-day old control bees were quite similar to the naturally reared 8-day old bees (40-50 $\mu\text{g}/\mu\text{l}$). The elevated levels of Vg that we found in our *in vitro* reared 1-day old bees may arise from the *in vitro* rearing, which provides larvae with a diet rich in royal jelly that could stimulate Vg production. However, once bees were adults, they were fed only sucrose and pollen, and, after 7 days, exhibited more typical Vg titers. Despite the elevated levels of Vg in control 1-day old bees, the levels in the corresponding 40K larval treatment bees were significantly 2-fold higher (50 $\mu\text{g}/\mu\text{l}$). Based upon this Vg level, our 1-day old bees were thus more similar to naturally reared 8-day old bees.

Our goal in infecting bees as adults was to provide a comparison group with the bees infected as larvae and also assayed at 7 days of adult age. In both larval- and adult-infected groups assayed at 7 days of adult age, there is no significant effect of *Nosema* treatment. This finding for adult-infected bees is similar to that reported by Goblirsch et al. (2013), who showed that there is no overall significant effect of *N. ceranae* infection on Vg gene expression levels.

However, Antunez et al. (2009) showed that there is a significant decrease in Vg gene expression in 14-day old adults infected with *N. ceranae* at 7 days of age. At 14 days of age, Vg levels are typically low (Engels 1974) because bees are transitioning away from nursing, and these results fit with the idea that *Nosema* infection induces a premature shift towards foraging behavior (Goblirsch et al. 2013). We measured Vg in 7-day old adults (infected on the day of emergence or as larvae) because this age corresponds to the peak of Vg production in naturally reared workers (Engels 1974;

Engels & Fahrenhorst 1974). We did not see significant effects of adult infection on overall Vg or total protein titers. However, this may not be surprising since bees infected upon adult emergence have highly variable levels of infection which do not become pronounced until they reach foraging age (Smart & Sheppard 2011).

We did observe a bimodal distribution in the Vg titers of adults (Fig. 2B) and therefore used a natural break in the data at 100 $\mu\text{g Vg}/\mu\text{L}$ to analyze a more restricted subset of Vg titers. In this analysis, the infected adults had significantly reduced Vg titers compared to controls. Thus, there is no overall effect of infection on adult Vg titers, but an effect is found in adults with a higher titer of Vg. In these adults, the infected bees appeared more like foragers with reduced levels of Vg. In analyzing these data, we tried a variety of non-parametric tests, including a Kolmogorov-Smirnov test, but found no significant effect. It is possible that the Vg titer bump observed (Fig. 2B) in the *Nosema*-treatment group came from adults that were more heavily infected (infection level hypothesis) and we therefore counted the spores in the midguts of bees that we obtained Vg from. However, in the hemolymph extraction process, the midgut sometimes ruptured and thus spore counts of these bees were not possible. Although we were careful not to use hemolymph from bees in which the hemolymph was contaminated by leaking gut contents (easily visible as cloudiness or yellow color in a normally clear sample), the number of bees from which hemolymph and spores could be counted were too few to test this infection level hypothesis.

Percentage Vitellogenin

As with Vg titers, the percentage of hemolymph protein that is Vg decreases with age and corresponding tasks. Nurse bees (between 1-12 days old) typically have Vg levels of 32-40% and this decreases to 15-26% Vg in foragers, older bees (Fluri et al. 1982). However, %Vg begins at negligible levels in 1-day old bees before peaking at about 7 days of age (Engels 1974; Engels & Fahrenhorst 1974). The %Vg in all of our 1-day old bees was similarly low, <10%.

Previous studies show that in naturally reared bees about 7-days old, approximately 28% of the protein in their hemolymph is Vg (Engels 1974; Engels & Fahrenhorst 1974). Our naturally reared 7-day old control adults had roughly similar levels (mean of 19%). In addition, our *in vitro* reared bees infected as larvae had similar levels once they were 7-day old adults (23% and 28% for the 10K and 40K treatment groups respectively). However, the corresponding control group (*in vitro* reared bees assayed as 7-day old adults) had a significantly lower percentage of vitellogenin (10%). It is unclear why %Vg levels are so low for this group, though *in vitro* and subsequent cage rearing may contribute to this difference. Nonetheless, larval *Nosema* treatment did elevate infection levels compared to controls in the 7-day old adults (Fig. 3A).

Total protein titers

Total hemolymph protein levels slightly increase from 1-day to 7-day old bees (Rutz et al. 1976; Fluri et al. 1982), so the result that 7-day 40k bees had significantly less total protein compared to 1-day old 40k bees is unexpected. This suggests that the effects of *N. ceranae* infection are more pronounced in older individuals. It is possible

that *Nosema* infection affects bee nutrition. If fewer nutrients can be absorbed by the infected midguts of bees, then a lower total hemolymph protein titer could result. It has been shown that infection results in lower protein levels and consequently reduced hypopharyngeal glands (Wang & Moeller 1970b; 1971; Malone & Gatehouse 1998).

Lass and Crailsheim (1996) showed that caged bees have significantly lower protein synthesis rates than bees of the same age in a normal colony. In a colony setting, protein synthesis decreased only slightly with age while in caged bees the effect was far more dramatic. Additionally, Page et al. (1992) showed that some young workers will become precocious foragers in groups that lack older bees. These observations could help to explain why all of our *in vitro* treatments, including controls, had consistently lower protein levels than would be expected for their age and appeared physiologically more similar to foragers rather than nurse bees. Nonetheless, in the 7-day bees infected as larvae, total protein levels were still lower, following the general trend of infected bees being “time shifted” forwards and exhibiting the protein levels of much older bees.

Summary

These results provide additional insight into a virulent and widespread pathogen that affects a globally important pollinator. Adults that develop from larvae exposed to *N. ceranae* suffer greater harm compared to bees infected upon adult emergence. Interestingly, infected larvae suffer, as adults, from decreased total hemolymph protein levels and temporarily elevated Vg levels that are indicative of accelerated age polyethism. These results agree with recent findings that bees infected with *N. ceranae* as adults have temporally fast-forwarded; that is, they exhibit higher juvenile hormone

levels titers that characterize older bees and precociously forage (Goblirsch et al. 2013).

Whether such behavior is an adaptive response to infection is unclear, but this is an intriguing possibility (Rueppell et al. 2010). Future studies focusing on the results of larval infection in natural colonies or which examine the age-related tasks that bees perform will shed more light upon this phenomenon of accelerated age polyethism in honey bees

Figures

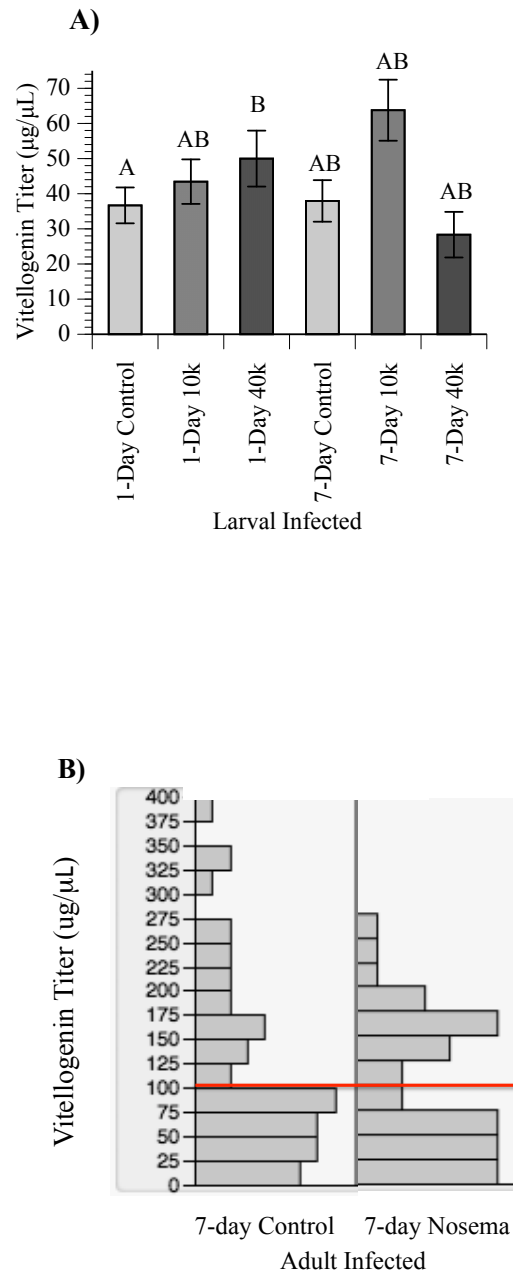


Figure 1: Vg titers in (A) larval and (B) adult infected bees. Different letters indicate significant differences. Error bars indicate the standard error of the mean.

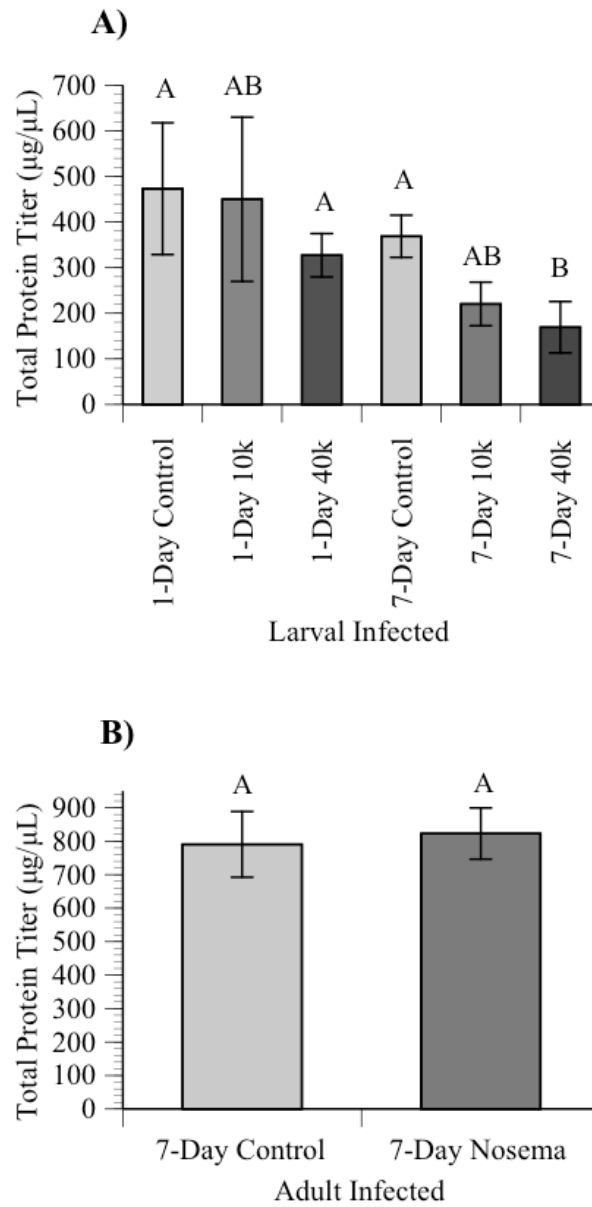


Figure 2: Total hemolymph protein titers in (A) larval and (B) adult infected bees. Different letters indicate significant differences. Error bars indicate the standard error of the mean.

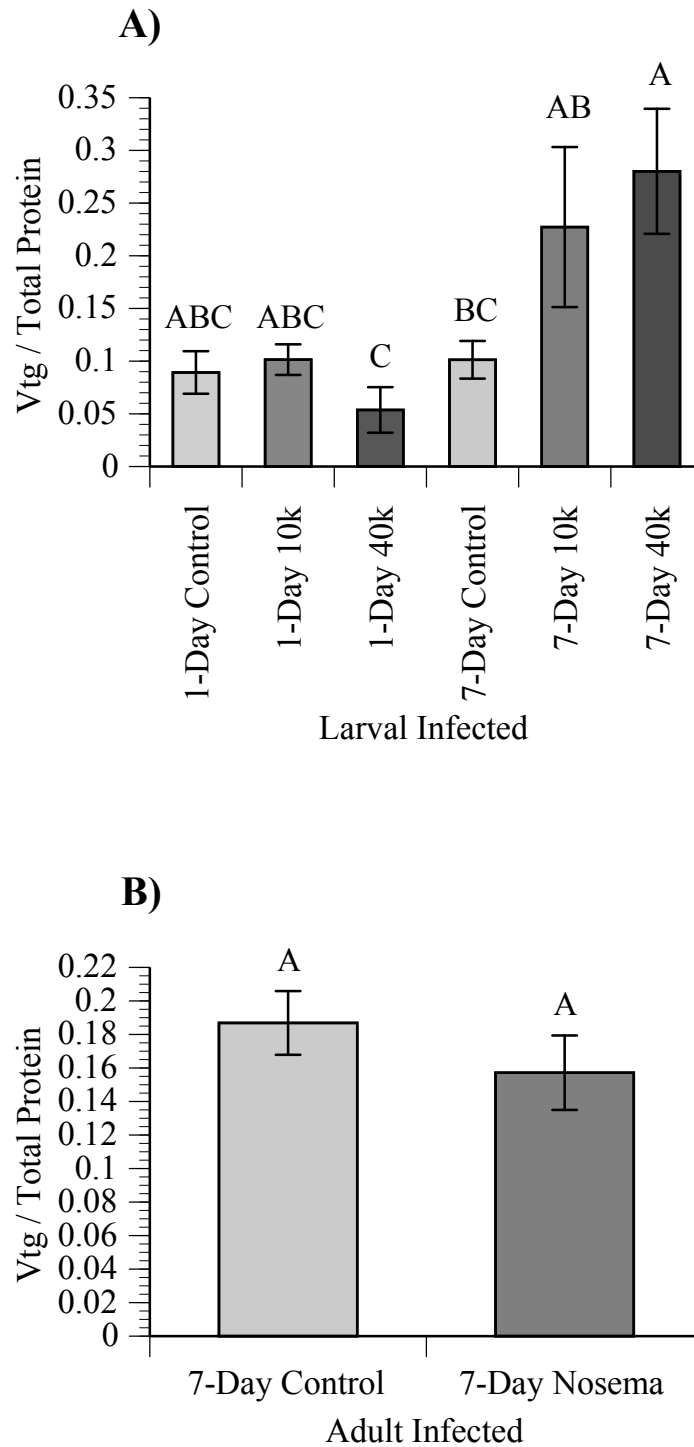


Figure 3: Proportional Vg titers in (A) larval and (B) adult infected bees. Different letters indicate significant differences. Error bars indicate the standard error of the mean.

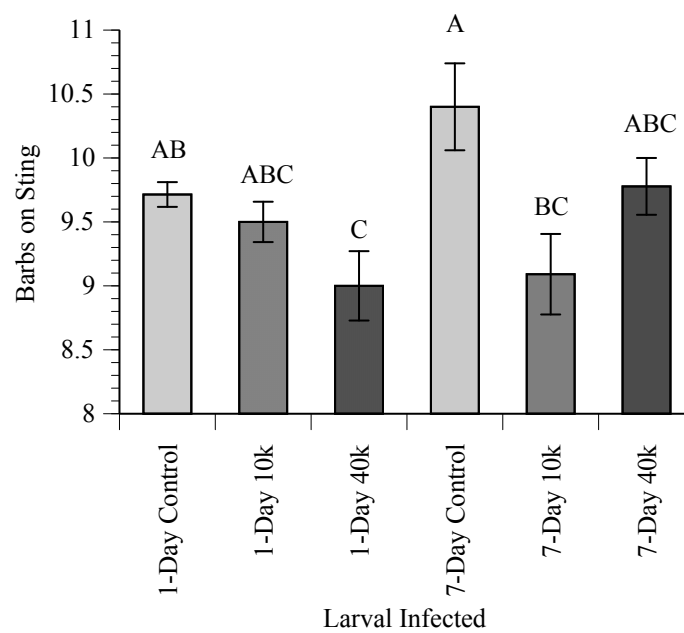


Figure 4: Number of barbs in larval infected bees. Different letters indicate significant differences. Error bars indicate the standard error of the mean.

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