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Autoclaved *Nosema ceranae* spores immune prime adult *Apis mellifera* against future infection

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

in

Biology

by

Andrey Rubanov

Committee in charge:

Professor James Nieh, Chair

Professor Joshua Kohn

Professor David Holway

2017

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ABSTRACT OF THE THESIS

Autoclaved *Nosema ceranae* spores immune prime adult *Apis mellifera* against future infection

by

Andrey Rubanov

Master of Science in Biology

University of California, San Diego, 2017

Professor James Nieh, Chair

ABSTRACT:

Apis mellifera, the Western honey bee, is an essential pollinator in a multitude of worldwide ecosystems, and occupies a prominent niche in the agricultural industry. Microsporidia like *Nosema ceranae* are a serious threat to colony health; we demonstrated that immune priming (IP) of newly emerged *A. mellifera* workers using autoclaved *N. ceranae* spores can activate an immune response, resulting in lower infection levels after subsequent challenge with live *N. ceranae* spores. Upon death, bees that were immune primed and challenged were observed to have significantly lower (34%) infection levels compared to control bees that were not immune primed. Immune primed bees also demonstrated a

decreased rate of infection. However, the benefit of IP was partially offset by tradeoff in longevity. IP bees survived slightly (1 d) but significantly less than did control bees that were not primed or challenged. To determine the mechanisms involved, we analyzed immune gene expression levels of four Toll genes involved in fighting *N. ceranae* infection. We did not sobtain clear results due to high variation in gene expression. However, IP significantly increased expression of *apidaecin*, *hymenoptaecin*, and *defensin-1*, and this may account for a decreased level and rate of infection in IP bees that were subsequently challenged with live spores.

INTRODUCTION:

The Western honey bee, *Apis mellifera* is a pollinator of high importance to agricultural prosperity (Klein et al., 2007). In the United States alone, commercial honey bee pollination of staple crops like almonds, apples, cotton, oranges, strawberries, and more yields upwards of $14 billion annually (Calderone, 2012). However, recent trends show a decline in managed honey bee populations partly attributed to the microsporidian pathogen *Nosema ceranae*, exposure to agricultural pesticides, environmental variation, and the synergistic effects between these factors (Goulson et al., 2015).

Microsporidia (of the phylum Microspora) are unicellular, eukaryotic, spore-forming parasites that use an evaginable polar filament to infiltrate host cells, injecting the host with sporoplasm to propagate (Weiss and Becnel, 2014; Vávra and Lukeš, 2013). Microsporidia lack typical eukaryotic organelles like mitochondria, facilitating a high reliance on direct importation of host ATP (Keeling and Fast, 2002) and nutritional metabolites as evidenced by an amplified number of ATP transporters and transport proteins in a relatively simplified genome (Vávra and Lukeš, 2013).

Although initially believed to be solely a parasite of the Asian honey bee *Apis cerana*, since being detected outside of Asia in 2006 (Higes et al., 2006), *Nosema ceranae* has been observed on a global scale (Chauzat et al., 2007; Cox-Foster et al., 2007; Klee et al., 2007; Martin-Hernandez et al., 2007; Chen et al., 2008) and is associated with poor health in the western honey bee *Apis mellifera* (Higes et al., 2008). Spread via fecal-oral or oral-oral transmission (Smith, 2012), *Nosema* spores germinate in midgut epithelial cells, replicate, and eventually rupture host epithelial cell membranes (Fries et al.,1996). In addition to the continuous degeneration of epithelial cells by *Nosema*, its inhibition of the Wnt pathway.

prevents gut tissue renewal (Dussaubat et al., 2012), culminating in malnutrition and subsequent death (Martín-Hernández et al., 2011).

The antibiotic fumagillin is the primary treatment option for *N. ceranae* but increasing resistance by *Nosema* (Williams et al., 2011; Huang et al., 2013), toxicity to mammals (van den Heever et al., 2014), residual presence in honey (van den Heever et al., 2015), and ability to even exacerbate *N. ceranae* infection (Huang et al., 2013) has researchers searching for alternative treatments. In addition, agricultural antibiotic use can exacerbate antibiotic resistance in human commensal bacteria (Smith et al., 2002). A new treatment that uses natural honey bee immunity is therefore desirable.

Despite the lack of an adaptive immune system (Evans et al., 2006), invertebrates have other forms of immunity, such as physical, chemical, and behavioral defenses (Nouvian et al., 2016). Immune priming is defined as exposure of low level doses or non-pathogenic elements of disease-causing agents, causing increase in defense to subsequent exposure (Best et al., 2012), and is possible due to the presence of a variety of inducible cellular and molecular mechanisms (Evans et al., 2006; Evans and Pettis, 2005). Invertebrates have been repeatedly demonstrated to be able to resist future pathogenic infection by prior inoculation with immune-activating factors: Mealworm beetles primed with non-pathogenic *Escherichia coli* lipopolysaccharides demonstrated higher haemolymph antibacterial activity and reduced death rates when challenged with the entomopathogenic fungus *Metarhizium anisopliae* (Moret and Siva-Jothy, 2003). *Bombus terrestris* inoculated with survivable doses of pathogenic bacteria showed resistance to subsequent challenge with high doses up to 22 days post-priming (Sadd and Schmid-Hempel, 2006).

Immunity can also be passed, to a limited degree, from one generation to another: such trans-generational immune priming has been demonstrated in bumble bees (Sadd et al., 2005) and honey bees (López et al., 2014), and may occur via the transfer of pathogen protein fragments by the protein vitellogenin (Salmela et al., 2015). In honey bees, preliminary research suggests that immune priming may be effective against *Nosema* infection. *Apis mellifera* larvae inoculated with heat-killed (autoclaved) *N. ceranae* and then challenged with pathogenic *N. ceranae* upon emergence demonstrated a 97% reduction in infection level and a 71% reduction in infection rate (Endler et al. in prep).

In insects, antimicrobial peptides (AMPs) play a key role in immune defense and immune priming. Immune primed *Drosophila* activated their antimicrobial peptide (AMP) Toll pathway (Christofi and Apidianakis, 2013). The Toll pathway is also involved in honey bee immune defense against *Nosema*. *Apis mellifera* upregulated five Toll pathway genes when infected by *N. ceranae* (Schwarz and Evans, 2013), and *A. mellifera* drones with natural resistance to *N. ceranae* displayed upregulation of six Toll AMPs. (Huang et al., 2012). Similarly, immune primed *A. mellifera* larvae upregulated two Toll pathway genes (Endler et al. in prep).

Prior research on honey bee immune priming focused on the immune priming of bee larvae. We wished to test the hypothesis that immune priming newly emerged adults would also be effective by lowering infection levels after subsequent infection. Such immune priming would be a more practical way to deal with *Nosema* infection because it is easier to feed adult bees than larvae, and would also offer insight, if effective, into the honey bee immune system. We also hypothesized that high levels of immune priming would incur heavy metabolic costs, resulting in a reduced lifespan (Moret and Schmid-Hempel, 2000).

Finally, we hypothesized that Toll immune genes known to be involved in defense against *Nosema* infection would be upregulated in immune primed bees. We therefore measured spore loads to quantify infection levels, adult longevity, and immune gene expression levels.

MATERIALS & METHODS:

Obtaining bees for immune priming

Apis mellifera ligustica (Spinola, 1806) workers were collected from 13 colonies housed at UCSD's Biology Field Station apiary from September 2015 to April 2017. We collected comb frames with at least 200 capped brood cells, removed all adult bees, and transferred them into a nucleus (nuc) box that was placed into incubators at 33°C and 60-80% relative humidity. Frames were kept in incubators for no longer than 5 days and checked daily for newly emerged worker bees, which were used for the experiments.

Maintaining and harvesting *N. ceranae* stock

To generate spore stock, we placed 25 newly emerged worker bees into a cage equipped with a syringe containing 5 ml of 2.0 M sucrose solution mixed with one million *N. ceranae* spores (40,000 per bee). Bees were fed solely 2.0 M sucrose *ad libitum* after the initial 5mL, and were given 10-12 days to develop heavy infections (Fries et al., 2013). Spores were harvested within 12 h of each new trial and stored at 4°C to ensure viability (Fries et al., 2013). Spores were extracted by dissecting out the midgut of infected bees. Three midguts were placed per 100 µl DD H_2O in a 1.5 ml Eppendorf tube, ground with Kimble polypropylene pestles, diluted to 1 ml, vacuum filtered through a Buchner funnel lined with Fisherbrand P8 Filter paper, and concentrated by centrifuging for 15 minutes at 10,000 rpm. The supernatant was discarded, and the precipitates were compiled and re-suspended in 500 µl DD H_2O(modified from Webster et al. 2004). A hemocytometer containing 0.1 µl of re-suspended solution was observed at 400x total magnification under a bright-field light microscope to determine spore concentrations (Fries et al., 2013). Heat-killed spore

solutions were prepared by autoclaving spores for 30 min at 121°C (Fenoy et al., 2009), then subsequently recounted to verify spore numbers.

Immune priming newly-emerged workers

One hundred newly emerged workers were removed from the nuc box and starved in a large cage for 4 h, then separated into individual sterile plastic vials. Fifty bees were each fed 7 μL of 2.0 M sucrose, the rest 7 μl of 2.0 M sucrose containing 40,000 autoclaved *N. ceranae* spores. This treatment was dispensed via a 100 μl pipette tip inserted into a hole in the plastic vial lid. Newly emerged bees can be reluctant to feed. To facilitate faster feeding, bee-feeding trays were designed modeled after Thomas Rinderer's mass-feeding method (Rinderer, 1976), which takes advantage of natural bee phototaxis (Scheiner et al., 2014). We placed strips of ultraviolet (395-405 nm) light emitting diodes along the tops of each vial. This UV light, shining through the pipette tips, attracted bees and increased the likelihood of ingestion. Thirty minutes after complete ingestion of treatment (based upon careful inspection of the pipette tips), 25 bees per treatment were transferred into separate cages: "0-0", "IP-0", "0-40", or "IP-40". 0-0 bees were never exposed to autoclaved or pathogenic spores, IP-0 bees received only autoclaved spores upon emergence, 0-40 bees were solely challenged with pathogenic spores on their seventh day, and IP-40 bees were primed upon emergence and challenged seven days post inoculation (dpi) with autoclaved spores. Bees treated with 40,000 autoclaved spores were transferred, at random, to either the IP-0 or IP-40 treatment cage. Bees given solely sucrose were grouped in the 0-0 or 0-40 cages. Cages were provided with 2.0 M sucrose via syringe *ad libitum*. Cages were cleaned daily as necessary. Bees were not isolated in vials for longer than six hours.

Challenging adult workers with live *N. ceranae* spores

Seven days post-inoculation with autoclaved spores, bees were starved for no more than two hours in their cages by removing their supply of sucrose, then separated into individual sterile plastic vials. Bees in the 0-0 and IP-0 treatments were given solely 7 μl of sterile 2.0 M sucrose with no *Nosema* spores. The IP-40 and 0-40 treatments were given 7 μl of 2.0 M sucrose mixed with 40,000 *N. ceranae* spores (freshly harvested within 12 h of feeding). Feeding trays were utilized as described above. Thirty minutes after all bees had completely consumed the treatments, the bees were returned to their respective cages and fed sterile 2.0 M sucrose (without *Nosema* spores) *ad libitum*.

Gathering and quantifying data post-mortem

Cages were checked each 24 h and the death of all bees was recorded. Dead bees were removed and their midguts were dissected out and spores extracted (see above) into 100 μl of double-distilled H_2O. Samples were stored at 0°C until they were spore counted to determine total quantity of *N. ceranae* spores in the midgut. For each bee, we counted spores twice and used the average of these two counts.

Gene expression measurements

Samples were analyzed for four AMP Toll immune genes shown to be upregulated in bees infected by *N. ceranae: abaecin*, *defensin-1*, *apidaecin*, and *hymenoptaecin* (Chaimanee et al., 2012). Two of these genes, *abaecin* and *defensin-1*, were significantly upregulated in larvae that were immune primed (Endler et al. in prep). We used two reference genes: actin (Chaimanee

et al., 2012) and GAPDH (Scharlaken et al., 2008). From the trials described above, we removed two bees per treatment and immediately froze each bee in liquid nitrogen at three different time points: pre-priming at 0 dpi, pre-challenge at 7 dpi, and post-challenge at 17 dpi. We severed the frozen abdomens of these bees and stored each individually in 300 µl of Invitrogen RNAlater-ICE at -70°C. For RNA extraction, abdomens were thawed on ice and blotted dry of RNAlater-ICE. The midgut was extracted and immersed in 300 µl of Trizol reagent (Invitrogen, Carlsbad, CA, USA) per manufacturer protocol. RNA concentration and quality was determined by measuring absorption ratio at 260 nm over 280 nm with a Nanodrop 2000 spectrophotometer. We added 1 µg of RNA to RNase and DNase free water (12 µl mixture volume) to which we added 2 µl of gDNA wipeout buffer, followed by incubation at 42°C for 2 min.

We converted RNA to cDNA using a QuantiTect Reverse Transcription (RT) Kit (Qiagen, Venlo, Netherland): 4 µl RT buffer, 1 µl Reverse Transcriptase, and 1 µl RT primer mix was added to each 14 µl RNA preparation mix, incubated at 42°C for 30 minutes, then 95°C for 3 minutes, and stored at -70°C. qPCR was performed by adding 30 µl DNase and RNase free water to the 20 µl cDNA mix, then adding 2 µl into a well containing 7.5 µl QuantiTect SYBR Green PCR Master Mix (Qiagen), 1.9 µl each of the forward and reverse primers (at 50 nM), and 6.2 µl DNase and RNase free water. After mixing, qPCR was performed using an Applied Biosystems® 7500 Real-Time PCR System. After an initial phase of 50°C for 2 minutes, followed by 95°C for 10 minutes, 40 cycles were performed at 95°C for 15 seconds and ended at 60°C for 60 seconds. A plate reading was made at each cycle. Melting curves were recorded: 95°C (15 seconds) to 60°C (15 seconds) and back to

95°C (15 seconds). Equal numbers of treated and control bees were run on a 96-well plate with all 6 genes and each gene per sample was run in triplicate.

We used the $2^{-\Delta\Delta}C_T$ method (Livak and Schmittgen, 2001) to calculate the fold change in the expression level of each immune gene relative to the mean expression level of the two references genes (Actin and GAPHD), as shown in equation 1 below.

[Eq. 1] $$2^{-\left(\text{CT of immune gene} - \left(\frac{\text{CT of actin reference gene} + \text{CT of GAPHD reference gene}}{2}\right)\right)}$$

Freezing trials

Two trials were run in typical fashion involving immune priming and challenge as described above, except they were challenged at different timepoints and frozen to end the trial. Trial A was challenged at 9 dpi and frozen at 16 dpi, Trial B was challenged at 10 dpi and frozen at 20 dpi. These trials were included to monitor the rate of *N. ceranae* infection; we wanted to quantify the average infection level at certain timepoints.

Statistics

To analyze the effect of treatment on spore counts, we used non-parametric Steel-Dwass tests to conduct all pairwise comparisons, corrected for Type I error. To determine if colony (fixed effect) had an effect upon spore counts, we used an Analysis of Variance (ANOVA) Standard Least Squares Fit given that colonies vary in resistance to *Nosema* infection. For our survival analyses, we ran a Proportional Hazards Log-rank fit with colony as a fixed effect. Finally, we used linear regression to determine the effect of time on infection levels. For our analyses, we used JMP Pro v13 software. We report mean±1 standard deviation.

RESULTS

We used a total of 1486 bees and 9 colonies over all experiments.

Immune priming reduced infection in subsequently challenged bees

There was no significant difference in spore count levels in treatments that never received live *N. ceranae* spores (0-0 and IP-0). Bees in both treatments were essentially un-infected and not significantly different from each other (Steel-Dwass Z=0.06, p=1.0). Bees challenged with 40,000 live spores (0-40) were significantly more infected than 0-0 bees (Steel-Dwass Z=18.51, p<0.0001) and IP-0 bees (Steel-Dwass Z=-18.35, p<0.0001, Fig. 1). Bees that were immune primed prior to challenge (IP-40) were significantly less infected (34% less infected) compared to bees challenged without priming (Steel-Dwass Z=-3.34, p<0.005). Bees that were challenged without immune priming developed infection 65.45% (252/385) of the time, while IP-40 bees became infected only 58.77% (201/342) of the time.

As expected, bees that lived longer were more heavily infected: 0-40 bees ($F_{1,415}$=100.60, p<0.0001) (Fig. 3A) and IP-40 bees ($F_{1,367}$=71.26, p<0.0001, Fig. 3B). Twenty days after emergence, 0-40 bees were, on average, 1.4-fold more infected compared to IP-40 bees. There was small, but significant variation among how colonies responded to infection: colonies accounted for 4.4% of variance in spore count ($F_{3,1596}$=76.26, p<0.0001). By running a Fit Least Squares test we observed that the infection rate in the IP-40 treatment was retarded compared to the 0-40 treatment; there was a significant effect due to treatment ($F_{1,772}$=6.08, p=0.01), days alive ($F_{1,751}$=131.9, p<0.0001), and the interaction of treatment and days alive ($F_{1,779}$=7.22, p=0.007).

Treatment and Colony had significant effect on survival

A Proportional Hazards Fit survival analysis that included colony as a fixed effect showed a significant effect of colony (Log-Rank Chi-square=377.21, 8 df, $p<0.0001$) and treatment (L-R Chi-square=22.39, 3 df, $p<0.001$) (Fig. 2A), as expected. Bees that received both autoclaved spores and live spores, or solely autoclaved spores (IP-40, 0-40, and IP-0) had lower survival than 0-0 bees (Fig. 2B, 2C, & 2D). As expected, the 0-0 bees lived significantly longer (mean 0.79 days) than did 0-40 bees (Chi-square=5.40, 1 df, $p=0.020$, Fig. 2C) and mean 1.82 days longer than IP-40 bees (Chi-square=11.67, 1 df, $p=0.0006$) (Fig. 2B). Bees that were primed but not challenged (IP-0) lived significantly less (mean 0.97 days) than 0-0 bees (Chi-square=5.01, 1 df, $p=0.025$) (Fig. 2D), demonstrating the cost of immune activation even without exposure to pathogen. Despite having lower infection levels, bees that were primed and challenged (IP-40) did not live significantly longer than 0-40 bees (Chi-square=2.01, 1 df, $p=0.16$) (Fig. 2E), suggesting that immune priming does impose a longevity cost.

Gene expression of four Toll AMPs

The difference between Gene expression levels for *abaecin*, *apidaecin*, *hymenoptaecin*, and *defensin-1* were analyzed by Tukey's HSD test as a factor of age and treatment. For *hymenoptaecin* and *defensin-1* we observed a significant expression increase in the IP-0 treatment between Day 8 and Day 18. We noticed a similar trend for *apidaecin*, but the increase in expression was not significant. It appears that *apidaecin*, *hymenoptaecin*, and *defensin-1* were upregulated as bees grew older and began to prepare for foraging duties, and treatments that received pathogenic spores experienced suppression of expression due to

infection. For *abaecin* we observed the highest levels of expression on Day 8, seven dpi with autoclaved spores. On Day 18, *abaecin* was the only gene of the four that did not have strikingly higher expression levels in the IP-0 treatment.

DISCUSSION

Preventing or lowering *N. ceranae* infection in *A. mellifera* is important for maintaining colony health and ensuring the ability of this species to provide necessary agricultural pollination services. Immune priming (IP) newly-emerged *A. mellifera* workers with autoclaved *N. ceranae* spores resulted in significantly lower infection levels when these bees were subsequently challenged with live *Nosema* spores. IP reduced mean spore count by 34% and the proportion of infected bees (defined as bees with at least one spore) by 6.68% (Fig. 1). Prior research suggested that IP of larvae resulted in lower adult infection levels (Endler et al., in prep). Here, we demonstrated that similar results can be achieved by IP newly-emerged adults, an approach that should be simpler to implement. However, we observed a longevity tradeoff. IP bees, even without being challenged, had lower survival compared to 0-0 control bees (Fig. 2B & 2D). This finding suggests that the activation of immune pathways incurs a metabolic cost.

Without some costs, one would expect bees to always upregulate their immune system. Selective upregulation only in the presence of infection or pathogens demonstrates there are fitness costs, consisting of the energetic investment an organism makes to prepare or defend itself. Such tradeoffs have been demonstrated in a variety of insects, including honey bees and bumble bees. For example, bumble bees increase consumption to offset the metabolic costs of immune responses (Tyler et al., 2006). Moret and Schmid-Hempel (2000) showed that inducing bumble bee immune responses by challenging them with non-pathogenic lipopolysaccharides (LPS) and micro-latex beads resulted in 50-70% reduction of survival. Immune responses can also have other costs arising from the diversion of metabolic resources. For example, injection of non-pathogenic LPS impaired learning of

rewarding flower colors by *B. terrestris*, perhaps because resources were diverted from the brain (Alghamdi et al., 2008). Experimental bees took a significantly longer time to learn the color of rewarding flowers and demonstrated a lower probability of choosing the most rewarding flower during initial visits (Alghamdi et al., 2008). *Apis mellifera* injected with LPS also had an impaired ability to associate odors with sugar reward (Mallon et al., 2003). Immune responses and associative learning both utilize intracellular messenger molecules like amines, and Mallon et al. (2003) hypothesize the observed impaired learning ability is due to a lack of available messenger signals necessary for both pathways. In addition, exposure to natural antimicrobial peptides found in propolis, an alternative to innate immune defenses, led to downregulation of immune genes in honey bees (Simone et al., 2009).

Our immune gene expression results, based upon the small sample size of six bees per age and treatment, and the high variation measured, require additional data. However, the results are suggestive. We propose the following explanation, which will need to be tested with further data. IP-0 bees activated three Toll immune genes known to be related to defense against *Nosema* infection: *apidaecin*, *hymenoptaecin*, and *defensin-1*. However, the costs of fighting *Nosema* infection result in an eventual downregulation of these genes measured at day 18, 10 days after bees were challenged. Even though bees typically upregulate these genes as they approach forager age (Bull et al., 2012), immunosuppression due to *N. ceranae* infection has been observed (Badaoui et al., 2017; Antúnez et al., 2009). This downregulation applies to all bees infected with live spores and explains why the IP-40 and 0-40 bees have essentially the same levels of gene expression. In addition, we note that we reduced infection levels, on average, by 34%, not 100%. It is perhaps not surprising that bees infected with

100% and 66% of average spore levels would show somewhat similar immune impairments. More conclusive results will require a larger sample size for each time point and treatment.

There was no significant difference between the survival of IP-40 and 0-40 bees. This suggests that the costs of being infected and activating immune defenses (IP-40) and the costs of simply being infected (0-40) are similar with respect to survival (Fig. 2E). In addition, IP-0 and 0-40 bees all had significantly lower survival than 0-0 control bees (Fig. 2). As expected, infection levels rose as bees lived longer (Fig. 3). However, IP reduced the severity of this rise: 20-day old 0-40 bees showed a 1.4-fold increase in mean spore count compared to IP-40 bees.

IP therefore has costs that should be weighed against its potential benefits. The survival reduction due to immune priming alone is a mean 1.03 days decrease in longevity. This is likely a fairly small cost given that workers live, on average, 35-45 days (Winston, 1991). The benefits of reducing the spread of *Nosema* infection within a colony and protecting the queen from becoming infected with *Nosema* may outweigh the decreased worker survival costs. In addition, *Nosema* infection can reduce colony foraging, by reducing homing ability (Wolf et al., 2014), thereby reducing food intake. A colony depends upon foraging, and *Nosema* can disrupt colony division of labor by inducing early foraging (Goblirsch et al., 2013). Future studies that test our four treatments with whole colonies and assess fitness by measuring brood production, number of workers, colony weight, and colony honey stores would be informative.

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FIGURES

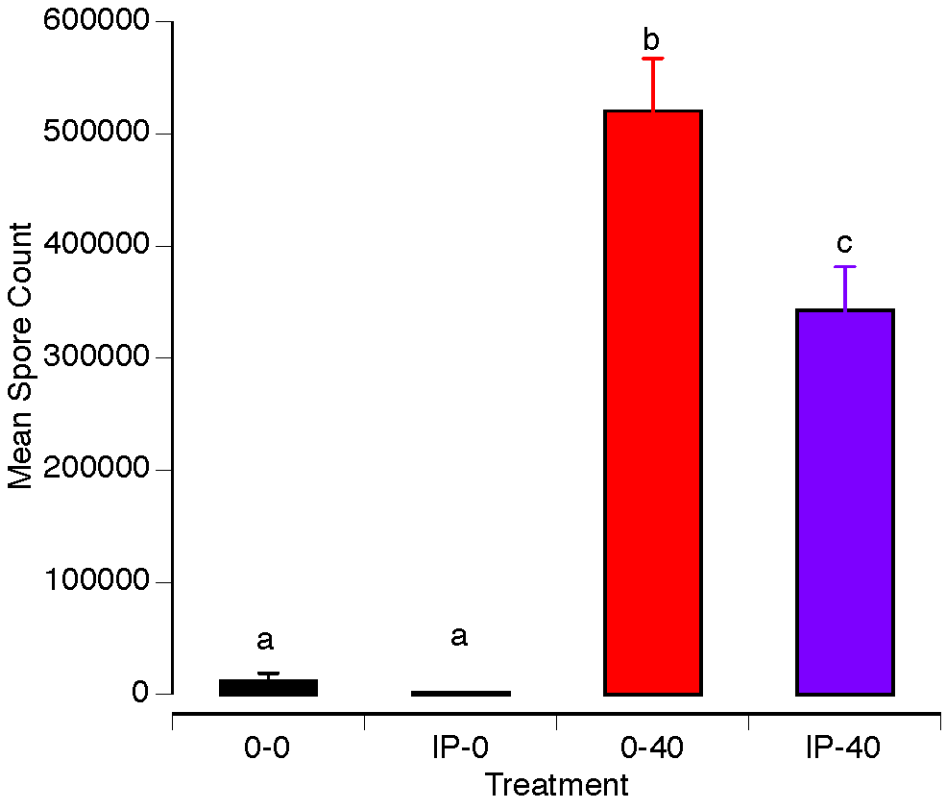

Figure 1. Treatment effects on mean spore count (infection levels) upon bee death. Different letters indicate significant differences (Steel-Dwass tests, $P<0.05$). Means and standard errors shown. The color coding shown here is also applied to other applicable figures.

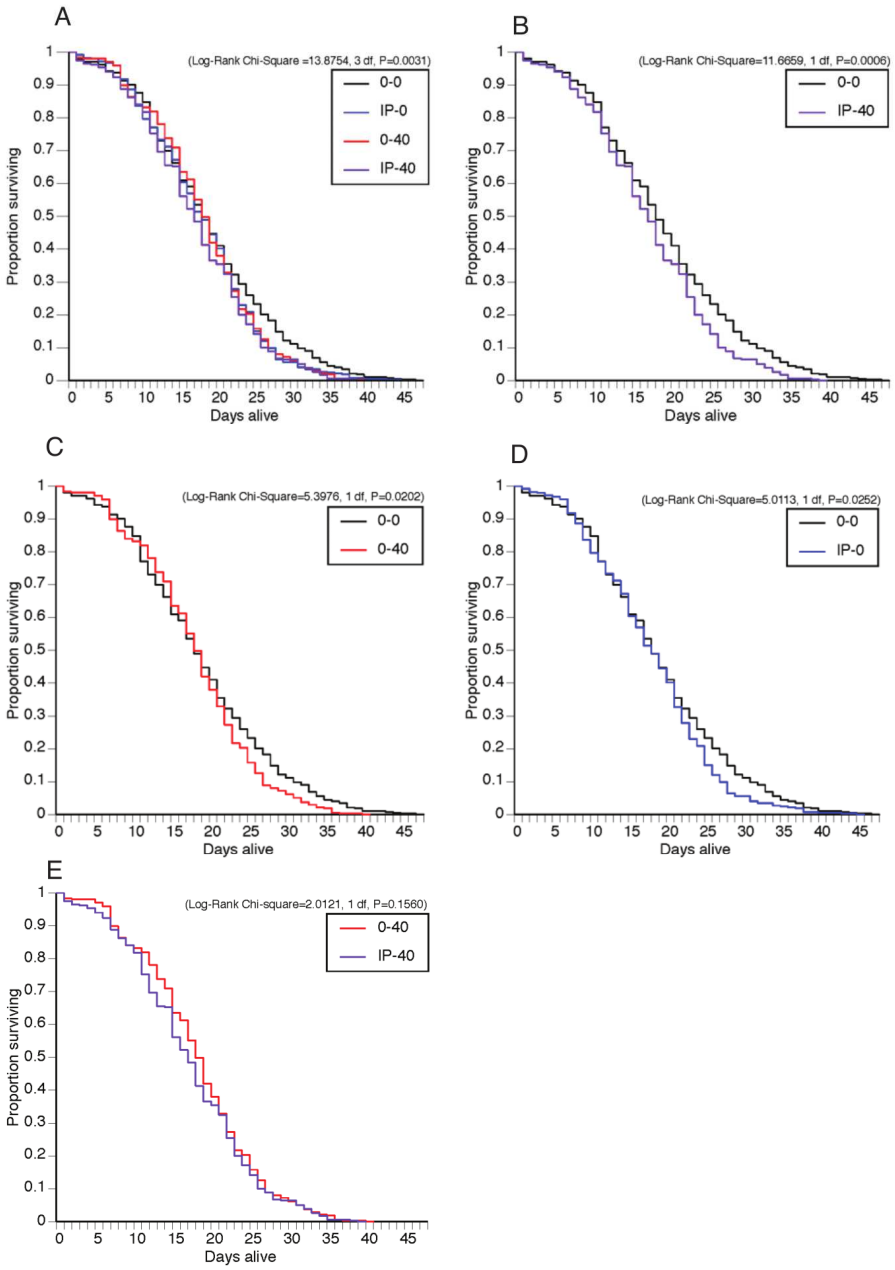

Figure 2. Effects of different treatments on bee survival. A) Overall survival analysis comparing all four treatments, showing a significant effect. B) Immune priming followed by exposure to pathogenic spores resulted in a significantly shorter lifespan compared to 0-0 bees. C) Exposure to solely pathogenic spores reduced lifespan significantly compared to 0-0 bees. D) Immune priming was enough to significantly reduce lifespan compared to 0-0 bees. E) Immune priming and challenge by pathogenic spores did not significantly alter lifespan compared to bees exposed to solely pathogenic spores.

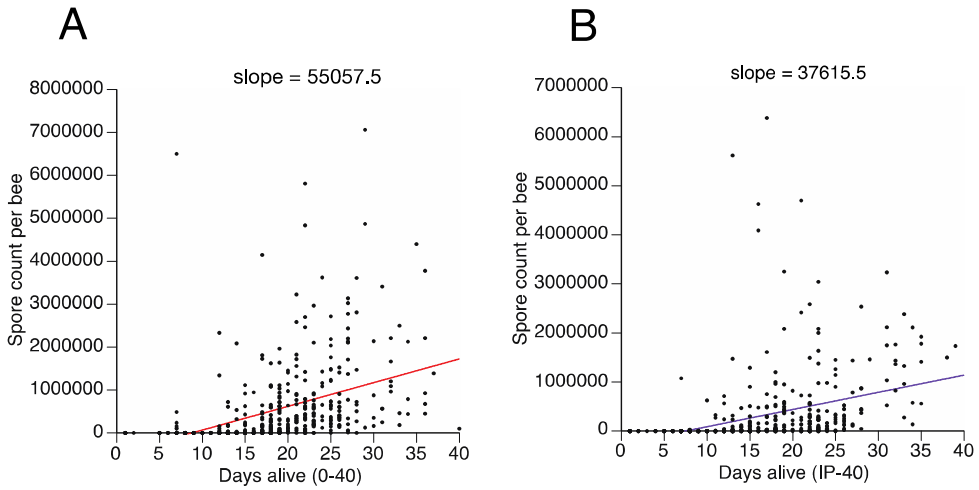

Figure 3. Bivariate fit of Spore Count by Days Alive. **A**) Bees challenged with live *N. ceranae* spores (0-40) developed heavier infections the longer they lived. **B**) Bees immune primed and challenged (IP-40) developed infections at a slower rate than solely challenged bees. These plots only examine 0-40 and IP-40 treatment groups because the other groups never were fed live spores and were essentially uninfected.

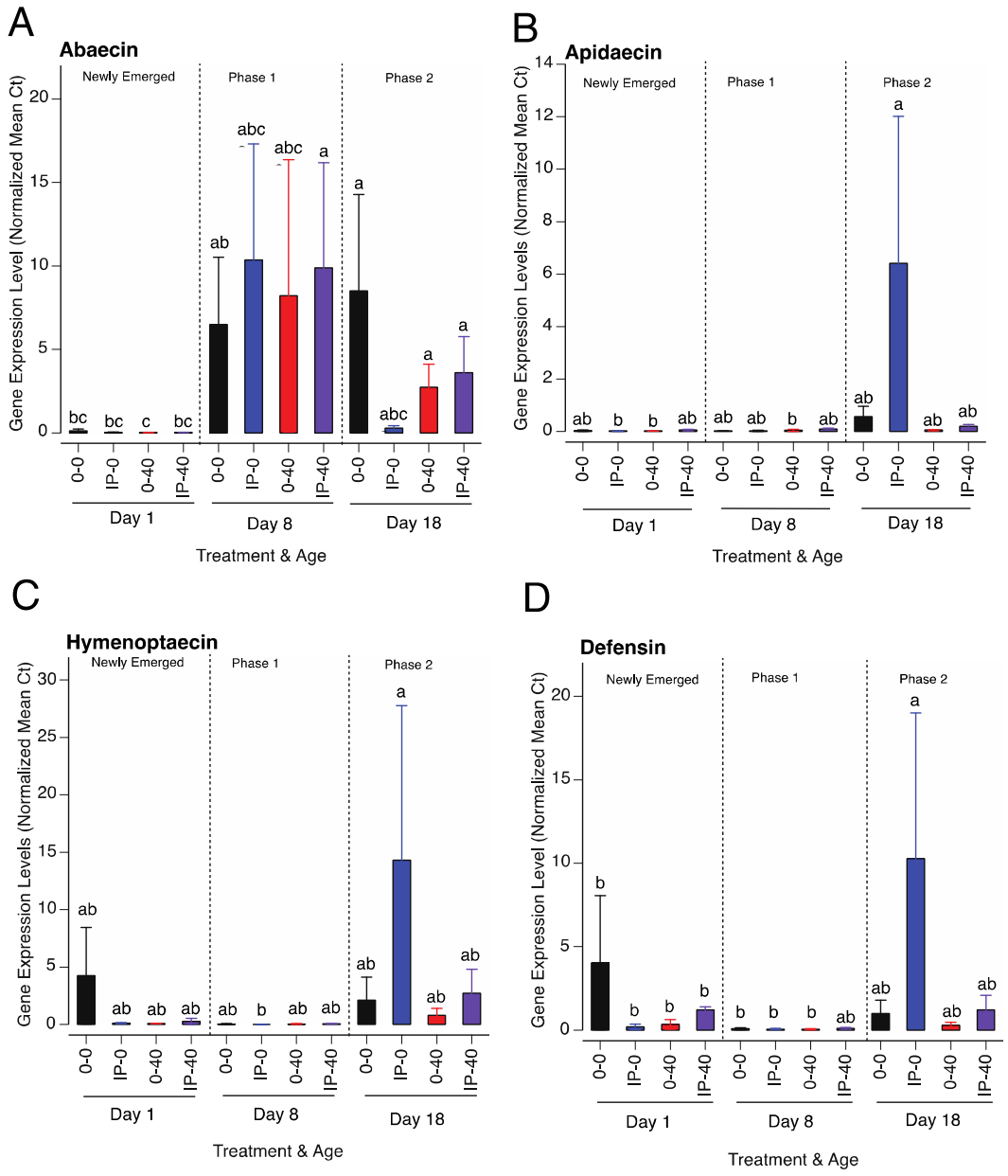

Figure 4. Effects of treatments and bee age upon Toll immune gene expression. Genes were chosen based upon preliminary results and prior research demonstrating a correlation between these genes and immune defense against *N. ceranae*. The reference genes were actin and GAPDH. Treatment names indicate the overall treatment groups, but treatments were applied at two time points. The ages are therefore divided into three time groups. *Newly emerged* bees had not yet received any treatment. *Immune primed* bees had all been immune primed but none of these bees had been challenged yet. *Challenged* bees reflect the effects of being fed live *Nosema* spores at the end of day 8. Different letters indicate significant differences between all age groups and treatments. We conducted a single Tukey HSD test per gene. Means and standard errors are shown.