Immune Priming in *A. Mellifera*

by

R. Starling Krentz

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Director of Thesis: Dr. Michael Brewer

Major Department: Biology

Trans-generational immune priming (TgIP) is the transfer of maternal immune experience to progeny, producing offspring pathogen resistance and ultimately survival from infections. In colony-forming insects like the honey bee *Apis mellifera*, TgIP would yield a form of lasting immunity benefiting subsequent generations. TgIP has been demonstrated in multiple social insects, but the efficacy and longevity of this immune protection is yet to be fully understood. To induce “priming” we inoculated honeybee queens with *Paenibacillus larvae* (Pl), a spore-forming bacterium causing American Foulbrood, a brood disease that once plagued beekeepers worldwide. Following inoculation, offspring of “primed” queens were fed a diet containing *P. larvae* spores and mortality rates were measured to assess TGIP. Our data reflects a dramatic reduction in larval mortality in *A. mellifera* colonies with “primed” queens, and demonstrates the efficacy of this protection at multiple timepoints.

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R. Starling Krentz

APPROVED BY:

DIRECTOR OF
THESIS: _____

Michael Brewer, Ph.D.

COMMITTEE MEMBER: _____

Erin Field, Ph.D.

COMMITTEE MEMBER: _____

Chris Balakrishnan, Ph.D.

CHAIR OF THE

DEPARTMENT OF BIOLOGY: _____

Jeff Mckinnon, Ph.D.

DEAN OF THE

GRADUATE SCHOOL: _____

Paul J. Gemperline, PhD

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Introduction

The evolutionary relationship between a parasite and its host is often described as an arms race, signifying rapid adaption and counter-adaption, resulting in co-evolution of the host and its parasite (Dawkins and Krebs 1979). The “red queen” hypothesis is another representation of this race (Keith Clay 1996; Pearson 2001), and can drive positive selection in immunologically important genes in many taxa including insects (Schmid-Hempel, 2013). A trend found numerous times in recent studies is the fact that immune proteins of various plants and animals evolve more rapidly than corresponding non-immune proteins (Harpur and Zayed 2013; Viljakainen et al. 2007). This accelerated evolution of immune mechanisms, sometimes significantly faster than the neutral rate, is assumed to be a consequence of the host-parasite arms race described above and is a distinct sign of adaptive evolution (Obbard et al. 2006; Sackton et al. 2007).

Invertebrates are traditionally considered to lack a form of Immune memory and immune specificity (Hoffmann and Reichhart 2002). Immune specificity, or the ability to respond to one type of pathogen without coinciding cross-reactivity against other pathogens, was previously considered unique to the vertebrate immune system (Kurtz and Franz 2003). Vertebrates have both an acquired and innate immune defense, and homologies with invertebrates are only found for the latter (Little, Hultmark, and Read 2005)(Tzou, De Gregorio, and Lemaitre 2002). The adaptive immune system implements responses primarily by T and B lymphocytes, while innate immune responses are executed by macrophages, neutrophils, basophils, natural killer cells, and various proteins (Klein 1989; Medzhitov 2002). The survival benefit gained through

evolving towards an adaptive immune system led to the evolution of alternative methods for lymphocytes to generate diverse antigen receptors for use in distinguishing and resisting pathogens (Cooper et al. 2006)

Although the innate immune system lacks the anticipatory mechanisms found in the adaptive immune system, it is capable of mounting a defense against a variety of threats including malignant or cancerous cells, viruses already present in a cell, and multi-cellular parasites (De Gregorio et al. 2002). In the model system *Drosophila melanogaster*, innate immune responses are mediated primarily by the Toll and Imd pathways, which regulate antimicrobial peptide (AMP)-encoding genes(De Gregorio et al. 2002; Hoffmann and Reichhart 2002). The Toll pathway is thought to function primarily for resistance to Gram-positive bacterial and fungal infections, whereas the Imd pathway is for defense against Gram-negative bacterial infections (Hoffmann and Reichhart 2002; Tzou, De Gregorio, and Lemaitre 2002). Mammals utilize TLR/IL-1 and TNF-R signaling pathways, homologous to the Toll and Imd pathways found in invertebrates (Hoffmann and Reichhart 2002; Tzou, De Gregorio, and Lemaitre 2002).

Eusocial insects and their parasites are exceptional cases within the host-parasite-interaction complex. Colony-forming, social insects like ants, termites and social bees are expected to be very susceptible to parasites, since colonies produce a high frequency of physical interaction within a constant nest environment, simplifying the spread of the parasite within the colony (Cornman et al. 2012; Verlag 1995). In addition to the individual immune system, social insects are equipped with an additional level of immune defense provided by their unique lifestyle (Cremer et al. 2007). Group strategies and specific behavioral patterns such as mutual grooming, resin collection,

and the removal of diseased and deceased individuals by honeybees have been shown to have huge impacts on the success of colonies to defend and recover from pathogens and parasites (Simone, Evans, and Spivak 2009; Spivak and Reuter 2001). Rothenbuhler first described such hygienic behavior in honeybee colonies and showed that resistant inbred lines removed brood killed by the American foulbrood (caused by *Bacillus larvae*) completely, whereas susceptible lines left remains of infected brood within the nest **(**Rothenbuhler, 1964). Another mechanism to improve colony resistance to parasites found in many social insects is polyandry, or multiple mating by queens. This taxonomically widespread behavior promotes obvious risks and drawbacks to the individual female (predation, energy costs, etc.) as well as the entire colony (reducing intracolony relatedness and thereby lowering potential fitness gains of altruistic workers), yet was shown to reduce parasitic and pathogenic infestation by yielding colonies with higher diversity (Baer and Schmid-Hempel 1999; Tarpy 2002).

In recent years there has been growing evidence for immune specificity and memory in invertebrates, described in many species including Drosophila (Pham et al. 2007), along with many others(Roth et al. 2009; Sadd and Schmid-Hempel 2006; Tidbury, Pedersen, and Boots 2011). This phenomenon is referred to as "**priming**", or the mediated increase in defense to a subsequent exposure following an initial exposure, relative to control individuals. When combined with specificity invertebrates can attain the immunological application of specific immune priming, functionally comparable but mechanically different to vertebrate immune memory (Schmid-Hempel 2005).

Invertebrate priming has challenged previous thinking towards antigen-specific responses in invertebrates. Recent studies have demonstrated the transfer of maternal immune experience to progeny and may therefore provide offspring resistance and ultimately survival from infections (Grindstaff, Brodie, and Ketterson 2003; López et al. 2014; Pham et al. 2007; Ben M Sadd et al. 2005). This capability has been described as trans-generational immune priming (TgIP), and is demonstrated in both vertebrates (Beemelmanns and Roth 2016; Grindstaff, Brodie, and Ketterson 2003) and invertebrates (Freitak, Heckel, and Vogel 2009; Little, Hultmark, and Read 2005; Tidbury, Pedersen, and Boots 2011). Trans-generational immune priming is particularly beneficial for invertebrates where generations and environments overlap, such as social insects. In a 2005 study by Sadd et al. bumble bee species *B. terrestris* queens were exposed to a bacterial-based immune challenge prior to colony founding. Consequently, daughter workers displayed significantly increased levels of antibacterial activity in relation to the corresponding challenge. Salmela et. al (2015) discovered that immune-priming signals are mediated via the egg-yolk protein vitellogenin, the carrier of immune elicitors. Trans-generationally primed individuals possess an upregulation of antimicrobial peptides, providing an instant immune defense against subsequent exposure (Barribeau et al. 2016). Given that the spectrum of pathogens in which a queen is exposed to over her lifetime would be similar to that of her offspring, TgIP would be a critical adaptive advantage to ensure colony protection from future exposure to pathogens (Sadd et al. 2005).

Evans et al. (2006) found that social insects such as the honeybee *Apis mellifera* possesses only about one-third as many genes within gene families associated with

insect immunity compared to the solitary *Drosophila* or *Anopheles* . Social defenses were suspected to contribute to an effective protection against diseases and were hypothesized to be the reason for the observed reduction in immune flexibility in bees (Evans et al. 2006). A similar study conducted by Gerardo and colleagues on the pea aphid *Acyrthosiphon pisum* showed that this insect species is missing many of genes from the IMD pathway which were thought to be relevant for the recognition, signaling and killing of microbes, and Gram-negative bacteria. Gerardo and colleagues considered the aphid life style, such as their association with microbial symbionts, could facilitate survival without strong immune protection. (Gerardo et al. 2010).

Bees have been declining, leading to widespread concern because of their importance for pollination in both wild and agricultural systems (Cameron et al. 2011; Cox-Foster et al. 2007; Kim et al. 2007; Vanengelsdorp et al. 2009). Parasites, broadly construed to include both micro- and macroparasites, have been implicate in bee declines (Cameron et al. 2011; Verlag 1995; Sumpter and Martin 2004). Our reliance on both managed and wild pollinators for a large part of our diet has engendered rare public interest in the conservation of insects. Bumblebees represent important wild pollinators in both natural and agricultural systems (Goulson, Lye, and Darvill 2008; Velthuis et al. 2006), which are supplemented by managed pollinators like honeybees (Gallai et al. 2009).

Chapter 2: Immune Priming in *A. Mellifera*

Background:

Trans-generational immune priming is the transfer of maternal immune experience to progeny(López et al. 2014). Trans-generational immune priming was initially thought only to exist in vertebrates, whom possess an adaptive immune system featuring antibodies (Cooper et al. 2006). Insects lack antibodies and were thought to rely on innate defenses, but recent literature shows significant degrees of immune specificity in insect pathogen defense, along with vertical transmission of immune experience to progeny (Barribeau et al. 2016; Sadd et al. 2005; Salmela et. al 2015; Tidbury et. al 2011). Freitak et al. (2009) found that feeding non-pathogenic bacteria to female cabbage loopers *Trichoplusia ni* during larval stage resulted in increased immune response in progeny, demonstrating that nonpathogenic bacteria in diet can trigger systemic immune responses (Freitak, Heckel, and Vogel 2009). Roth et al. showed using the red flour beetle *Tribolium castaneum* that parental exposure to the Gram-positive bacterium*, Bacillus thurngiensis*, could elicit strain-specific TGIP (Roth et al. 2009). Also, Lopez et al. (2014) showed that injecting *A. mellifera* queens with dead *Paenibacillus larvae* (American Foulbrood) leads to higher resistance against this pathogen in progeny (López et al. 2014). These findings demonstrate that immune priming can be mediated by mechanisms other than antibodies, and have created a central dilemma in insect immunological physiology.

The western honey bee *Apis mellifera* is a social insect, forming colonies of up to 50,000 individuals or more, residing in a minimal space that offers near-perfect conditions for the transmission of pathogens and parasites (Cornman et al. 2012;

vanEngelsdorp et al. 2009). Colonies of the *A. mellifera* are in increasing demand for commercial crop pollination (Gallai et al. 2009). Further frustrating an increasing demand, beekeepers in North America and Europe have suffered severe losses due to Colony Collapse Disorder (CCD) within the past fifteen years (vanEngelsdorp et al. 2009). CCD is defined simply as a sudden decline in available adult worker bees, with various proposed causes such as disease, pesticides, and environmental changes leading to nutrient deficiencies (Underwood and Vanengelsdorp 2007). One proposed cause is American Foulbrood (AFB), attributable to the spore-forming bacterium *Paenibacillus larvae* (Fünfhaus, Poppinga, and Genersch 2013). *P. larvae* spores can be transferred to honey bee larvae up to three days old, but larvae are most susceptible from birth to 24 hours old (Genersch 2010). The spores infect larvae, and adult honey bees (tolerant to infection) serve as vectors within and between colonies delivering spores to the brood while nursing. Larvae die within the first week of infection, depending on the spore load and strain of AFB (Genersch 2010; López et al. 2014).

A. mellifera maternal immune experience via TGIP triggers differentiation of prohemocytes (the invertebrate equivalent to stem cells) to hemocytes, to prepare progeny for a prevailing pathogen exposure (López et al. 2014). Maternally primed honey bee larvae possess increased levels of immune competent cells, providing the ability to react more rapidly to infection and resulting in reduced mortality rates. *A. mellifera* workers are short lived, and are thought to rely on a system of social immunity (Cremer et al. 2007) to compensate for a lack of immune genes (Evans et al. 2006). If honey bee queens (avg. lifespan: 2-6 years) can retain even a portion of this immune

response, and transfer it to their worker offspring, the queen as a single individual could positively impact the immunological status of the entire colony.

To further outline the effects of TgIP, we conducted a series of *P. larvae* dose experiments to determine the virulence of three strains of *P. larvae.* Following the dose experiments we then decided to further explore TgIP by immune-challenging queens with heat-killed vegetative cells of *P. larvae*, to instigate an immune response. We then compared the mortality rates of *A. mellifera* larvae exposed to *P. larvae* to those prior to queen challenge, to outline the level of protection provided by TgIP in *A. mellifera*. To explore the length of protection, we conducted mortality experiments 48 hours after priming, and again two and three weeks after priming (see methods). Understanding the extent of protection, the level of protection in response to other pathogens, and if this is a practical option for protection against AFB would be of great benefit to entomologists, commercial queen breeders, and invertebrate immunologists alike. In 2014 Lopez et. al demonstrated trans-generational immune priming in *A. mellifera*, and here we seek to outline the duration and efficacy of this priming effect (López et al. 2014). We hypothesize that we will be able to prime *A. mellifera* queens against AFB using heat-killed vegetative cells of *P. larvae,* resulting in a reduced mortality of ~30% (López et al. 2014). We also project that this priming effect will last for multiple seasons, if our colonies successfully overwinter.

Methods:

Colony Establishment

The *A. mellifera* colonies used for this experiment will be kept and maintained by the Tarpy Lab at N.C. State University. Initially we established a “cell builder” colony to raise the queens for this project. This is accomplished by removing the standing queen from a healthy colony and replacing her with approximately 90 uncapped queen cups containing larvae grafted from the same colony. Within the following week the queen cups will be capped by the workers of this queen-less cell builder colony, and each capped cell is transferred to a small mating nucleus colony containing healthy workers. The queens will then emerge from their cells, perform mating flights, and lay eggs within the next two weeks (Aupinel et al. 2005). In 2017, we experienced a 50% mortality rate when raising queens, with some death due to queen supersedure.

Once the queen has successfully started to lay eggs colonies are fed a 50/50 sugar water diet for healthy worker and brood population growth, as needed. In addition to feeding colonies are upgraded to 10-frame Langstroth hives, which is their final location. We produced forty colonies for AFB experimentation, thirty of which were sourced from the same queen, and ten contained queens raised from local colonies at the N.C. State University Apiary, at random.

Spore Preparation

For all AFB experimentation, *P. larvae* spores were provided by Adam Groth of the Miller lab at N.C. State University. Spores were isolated from a hive located in North Wilkesboro, NC. The comb sample was heated to 80°C for 10 mins in phosphate buffered saline (PBS) and then allowed to cool to room temperature before plating on

2xYeast-Tryptone plates with additional glucose (final concentration of 0.4%) and thiamine-HCL (Final concentration 1ug/mL). The enrichments were then incubated at 30°C until colonies formed (Walls and Chuyate 2000). The colonies were confirmed to be P. larvae through specific primers and universal 16s primers (Govan and Allsopp 1999). Typing of *P. larvae* spores in their lab gave rise to three populations of NW6, a known and local strain of AFB. To obtain spores, a few colonies of *P. larvae* were used to inoculate Columbia sheep blood agar slants and incubated at 35.0°C for 12 days. Next, the liquid supernatant was collected, heated for three repetitions of 10 min at 85°C to eliminate vegetative forms. Using both spore serial dilutions and a hemocytometer, spore concentrations were determined for all three preparations. The spore suspensions were stored at 4°C and used throughout all experiments and all three preparations were screened for viability by the Miller lab.

48-hour/10-Day Dose Experiments

Prior to any study of TgIP, the virulence level of the three spore preparations was assessed by using larvae from two different colonies. The purpose of the dose experiment is to discover a dosage for all three preparations of NW6 that results in ~50% larvae mortality, where any positive or negative effect from the priming of queens can be evaluated. The appropriate doses for the following experiments was determined by exposing larvae to various AFB concentrations. Initially a 48-hour dose experiment was conducted, with observations occurring every hour. Following this experiment daily observations were conducted, and we found that mortality from *P. larvae* in *A. mellifera* occurs between 5-8 days following exposure. We prepared larval diets (Aupinel et al. 2005) with AFB spore loads of 0, 1, 5, 10, and 20 spores/ul and then pipetted the diet

into 96-well plates. Larvae for the dose experiments are sourced from three different colonies (10 per colony, per dose) to capture variation among colonies (see Appendix). Larvae were stored in an incubator at 35.0°C and checked for mortality every 24 hours for 10 days. After three days the larvae were fed an additional 50 ul diet, following standard in vitro larval rearing protocol (Aupinel et al. 2005).

Pre-priming Experiments

Using the dose determined from the dose experiments above we conducted mortality experiments to test baseline resistance to *P. larvae*. For the AFB priming experiment, we grafted 20 larvae (10 control, 10 exposed) from each colony into 96-well plates. Larvae designated for infection will receive a 50 ul diet containing AFB spores, while the control larval diet will include water in the place of spores. Larvae will be stored in an incubator at 35°C and checked for mortality every 24 hours for 10 days. After 3-5 days the larvae are fed an additional 50 ul diet, just as the dose experiment. In addition to the 20 larvae for the initial AFB pre-priming experiments, 15 larvae were grafted from each colony and designated for RNA analysis. Five larvae were grafted into a noninfectious diet and immediately flash-frozen in liquid nitrogen. Five of these designated brood received an infected diet, while the remaining five received a standard diet and serve as controls. After 48 hours both the infected and control larvae are flash-frozen (resulting in time 0, 48 hr exposed and 48 hr control samples).

Queen Challenge

For queen inoculation, we injected 10 *A. mellifera* queens with 2 ul of heat-inactivated *P. larvae* vegetative cells at 1 x 10^8 cells/mL and another 10 queens with 2 ul of ringer solution to serve as controls(Ben M. Sadd and Schmid-Hempel 2007).

Queens are chilled on ice for 5–10 minutes to ensure sedation and injected between the fifth and sixth abdominal tergites, using a pulled capillary with a respirator. After regaining consciousness, the queens will be returned to their colonies to allow for the buildup of an immune response. Queens will be momentarily monitored to prevent rejection, but we expect some mortality due to handling. With an abnormal amount of supersedures taking place over the past two years, it is common for something as simple as a broken antennae to cause rejection.

Post-priming Experiments

Post-priming experiments began 48 hours following queen innoculation to allow time for an immune response. In addition, another round pf post-priming experiments will begin 14 days after the queens have been primed, and again at 21 days. Testing for AFB protection after experimental manipulation followed the methods used for the pre-priming experiments with 20 larvae (10 controls, 10 exposed) from each colony grafted into 96-well plates with exposure as described above. In addition to the 20 larvae for the initial AFB post-priming experiments, 15 larvae are grafted from each colony and designated for RNA analysis. Five larvae are grafted into a noninfectious diet and immediately flash-frozen. Five of these Fifteen designated brood received an infected diet, while five received water and serve as controls. After 48 hours both the infected and control larvae will be flash-frozen (resulting in time 0, 48 hr exposed and 48 hr control samples).

Results:

Dose Experiments

From these experiments, we selected a suitable spore dose of 1 spore/ul of diet, which produces mortality rates of around 40%, 50%, and 40% for spore preps 1 (Figure 1), 2 (Figure 2), and 3 (Figure 3) respectively. Using a wide range of spore concentrations from three preparations, it was determined that *A. mellifera* larvae raised *in vitro* with a diet of 50 *P. larvae* spores/ul (1 spore/ul) from spore preparation 3, produced a mortality rate of 50-60% (Appendix tables 8-10).

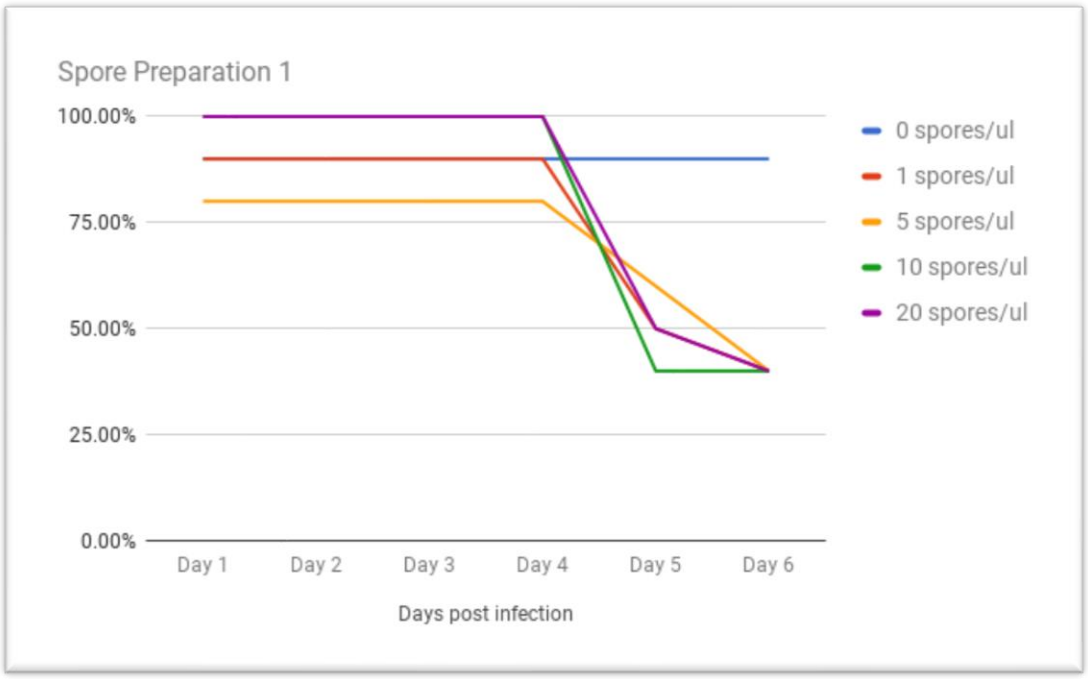

Figure 1: Mortality experiment for spore preparation 1. (n=30, per dose)

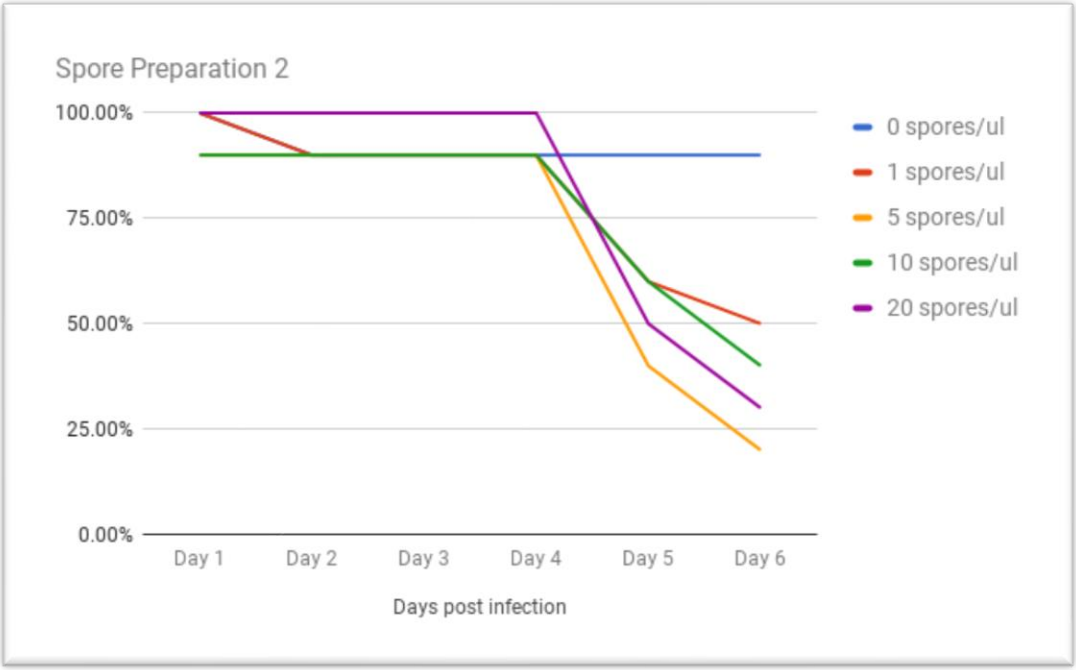

Figure 2: Mortality experiment for spore preparation 2. (n=30, per dose)

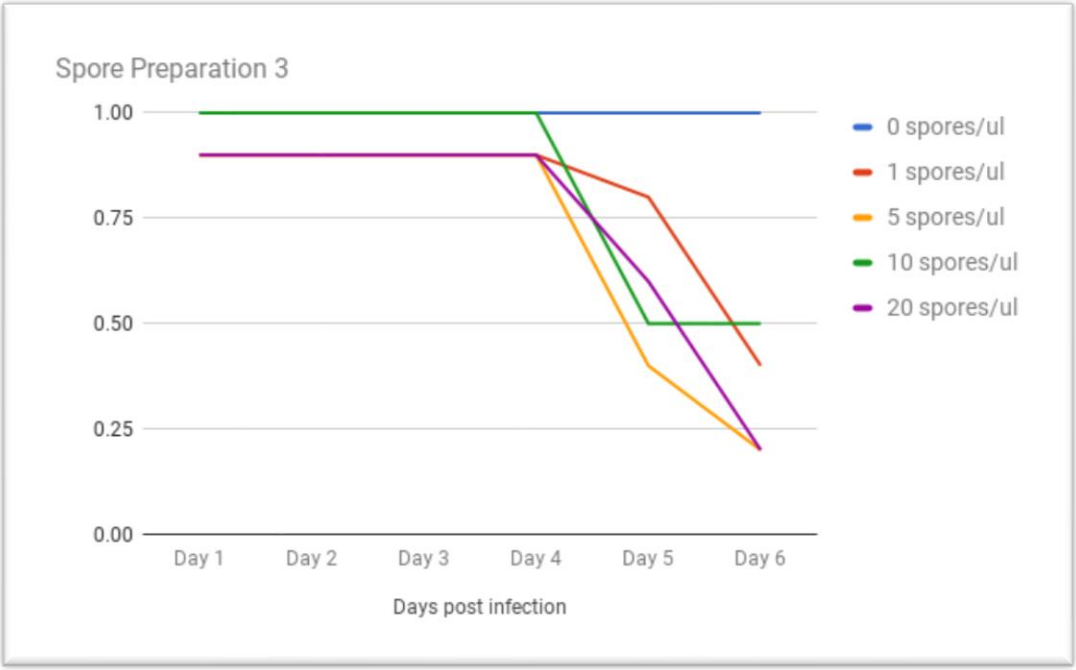

Figure 3: Mortality experiment for spore preparation 3. (n=30, per dose)

From this experiment, we can conclude that spore loads higher that 1 spore/ul are unnecessary, and provide only a slight increase in virulence. This supports current AFB literature, although our spore concentration represents the lower limit of dosage

(López et al. 2014; Peng et al. 1996). We will use spore preparation 3, and store preparations 1 and 2 for future experimentation. Although the three preparations were sourced from the same colony of *P. larvae*, maintaining virulence is often an issue with *P. larvae* spores(López et al. 2014). Spores stored between seasons are often ineffective for later experiments, which created many delays for this project. Future experimentation may require the preparation of new spores, and of course new dose experiments.

Queen Challenge

Of the twenty queens injected, three died from injection immediately after. Of these seventeen that survived, one died within the first 48 hours post injection. She was more than likely rejected by her colony. Sixteen queens were available for the 48-hour post-priming experiments. Only twelve survived until the two-week post-priming experiments, and their larvae provided the data that we used to show the priming effect. Of that twelve, another three colonies are no longer available for larval grafting at three weeks post-priming.

Eleven of the twenty colonies have been declared “dead” or “unusable” for several reasons. Many colonies replaced their queen (using her previously laid eggs), completing a “supersedure” with both queens momentarily present. Supersedures can be caused by a few factors, but here it was likely due to poor handling of the queens. Many of her reproductive organs are in her abdomen, so it is likely that the injections caused the majority of these supersedures. As mentioned above, a broken antennae or a change in pheromone profile can cause a colony to reject the queen, which can result from general beekeeping practices.

Food becomes scarce during the end of the season, and feeding the hives encourages “robbing” from other colonies. This can cause weaker hives to starve in the absence of resources. Grafting causes general stress but more importantly it creates generational gaps in the colonies, due to larval mortality from removing the grafting frame from the colony. Under stress, even the strongest colonies will swarm, leaving us with a virgin queen that is irrelevant for our experiments. The nine colonies that have survived will hopefully overwinter and be available for the spring, for future priming experimentation.

Post-priming

The data for the post-priming experiments can be found below (Figures 4-6). Figure 4 (below) is a mean comparison of naïve larvae between the primed and unprimed colonies. Primed (green) and unprimed (blue) larvae have no difference in mortality when not given AFB (X^2 =.061, df= 1, p=0.085). Any mortality beyond day two is likely the result of AFB.

Overall Comparisons[a]

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.061	1	.805

Test of equality of survival distributions for the different levels of Pretreat.

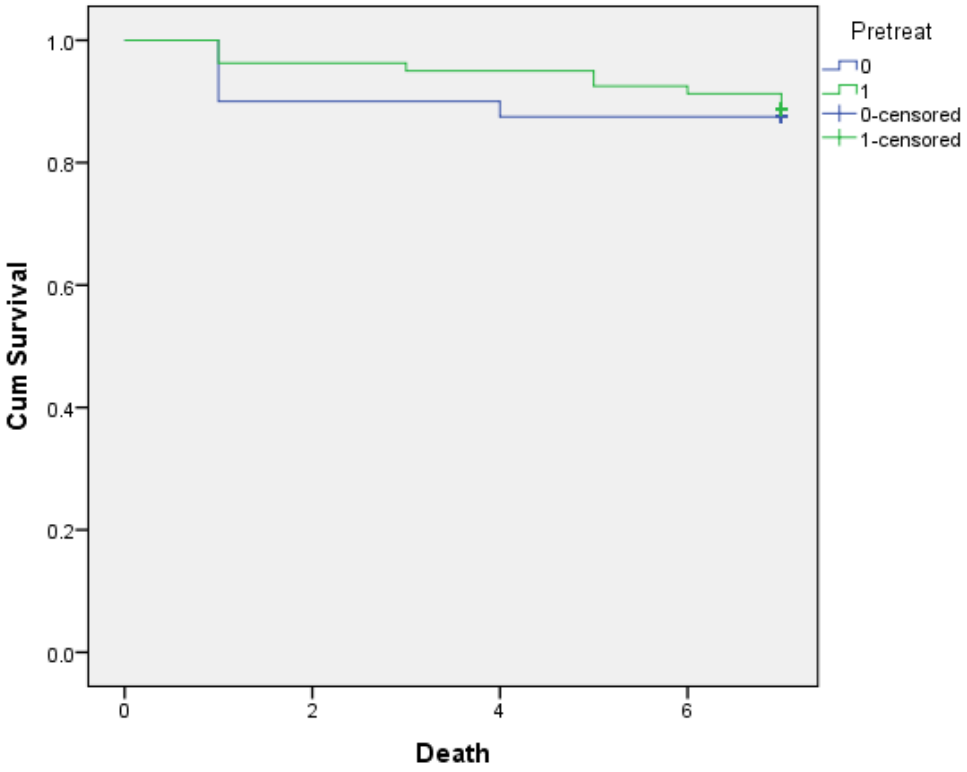

Figure 4: Comparison of unexposed (control) larvae between primed (green) and unprimed (blue) colonies.

Figure 5 (below) is a comparison of exposed larvae between the primed and unprimed colonies. Exposed larvae from unprimed queens (blue) produce an expected survival of about 40%, while exposed larvae from primed queens (green) have a survival rate of 75% (X^2 =18.957, df= 1, p=0.000).

Overall Comparisons[a]

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	18.957	1	.000

Test of equality of survival distributions for the different levels of Pretreat.

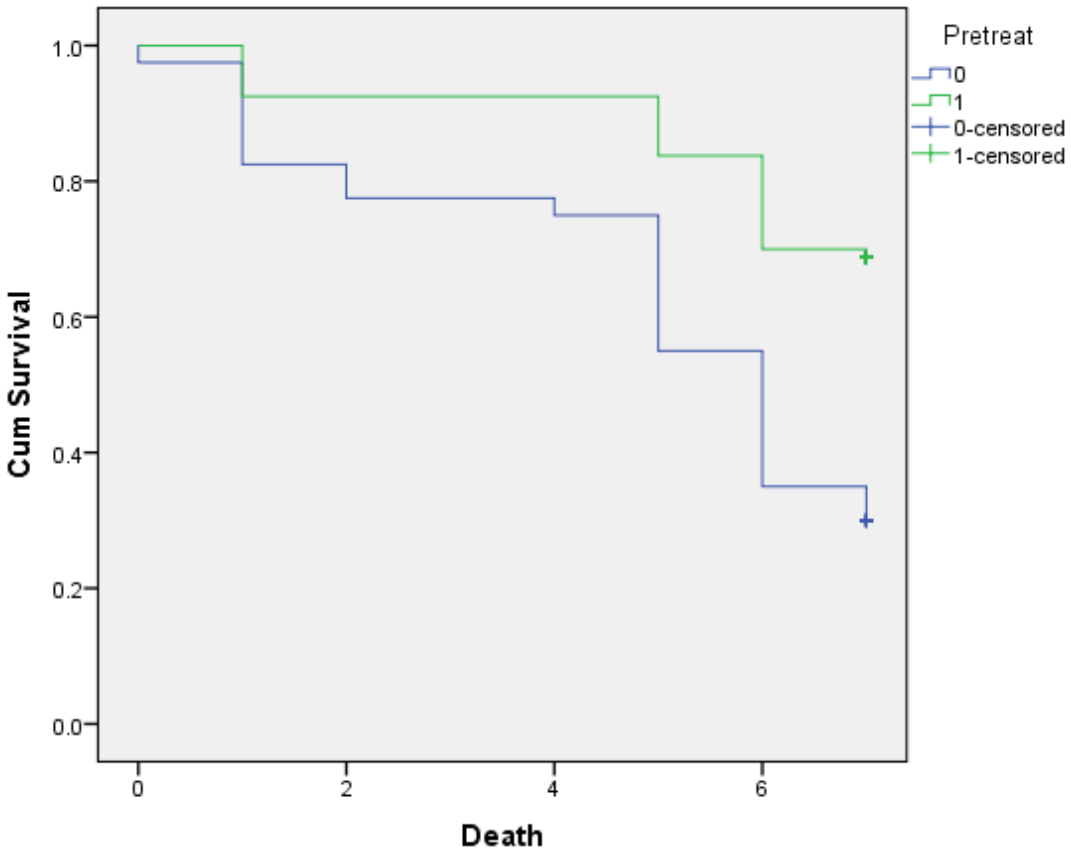

Figure 5: Comparison of exposed (control) larvae between primed (green) and unprimed (blue) colonies.

Figure 6 (below) compares exposed larvae between primed and unprimed queens (as in Figure 5), but shows the survival curve for each colony. Primed colonies

are colored in red, while unprimed are in blue. Although there is colony variation of mortality towards AFB, the priming of queens produces an obvious effect. A breakdown of daily mortality is provided in the appendix (Table 4).

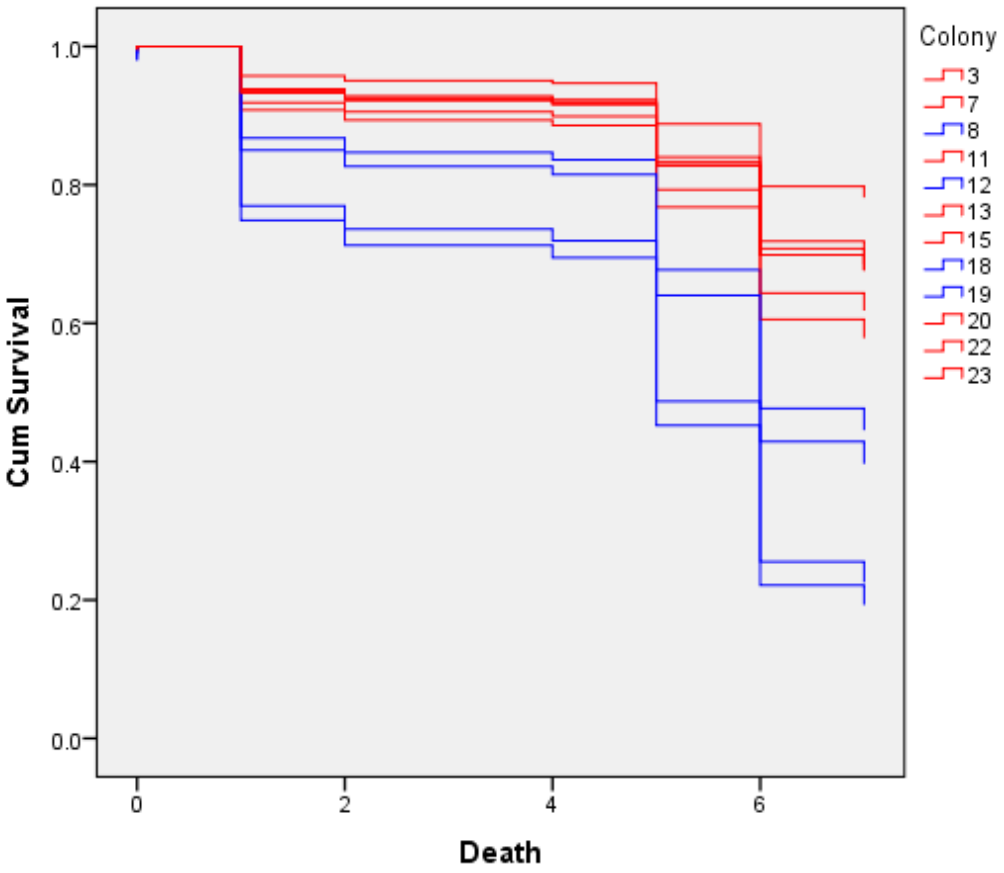

Figure 6: Post-priming experiment data for exposed larvae of Primed (red) vs. Unprimed (blue) colonies.

Discussion:

Our goal for this project was to confirm the existence of TGIP and outline the length and degree of protection. This project follows a similar approach as that of Lopez et. al, with the addition of mortality experiments at multiple time points, from multiple colonies (López et al. 2014). We demonstrate reduced mortality in worker offspring of *A. mellifera* when their mother queen received a bacterial-based immune challenge of *P. larvae* prior to colony founding, confirming the findings in recent literature (Barribeau et al. 2016; López et al. 2014; Moret 2006; Sadd et al. 2005). The efficacy of TGIP is still yet to be fully understood, but here we report a 40% reduction in larval mortality due to the priming effect. We are currently conducting experiments with increased sample sizes to fully outline the statistical significance of TGIP. In addition to our mortality experiments, we have stored hundreds of larvae (flash-frozen at various time intervals) for future analyses. We hope that these samples will shed light on the genetic mechanisms of TGIP.

This project creates new questions towards TGIP and host-parasite interactions, such as “fitness costs” associated with TGIP. A high maternal investment in TGIP could be beneficial if it does not negatively affect the queen's reproductive fitness, and of course if the infection is consistently present in the colony. Maintaining and using immune defenses can be costly for organisms (Siva-Jothy, Moret, and Rolff 2005). In the bumble-bee *Bombus terrestris*, the stimulation of the females with a bacterial pathogen increased susceptibility to a parasite distinctly unrelated to the maternal challenge (B. M. Sadd and Schmid-Hempel 2009). In the mealworm beetle *Tenebrio molitor*, a maternal immune challenge elevates the haemocyte load of adult offspring

with the tradeoff of a prolonged developmental time (Zanchi et al. 2011). As *A. mellifera* queens create and transmit effectors and/or elicitors of immunity to their offspring, we expect this transmission to be costly for them, along with the typical costs of immune activation. Thus, queens may possess mechanisms to somehow estimate the magnitude of a current infection and act accordingly (Zanchi et al. 2011). With situations such as CCD, a high investment in immunity might be inevitable to prevent the loss of the entire colony. From our dose experiments we saw similar levels of mortality between preps, confirming the efficacy of NW6. In our post-priming experiments however, we discovered disparity between colonies, which should be equally apparent through RNA analysis.

Although paternal immune priming is still of interest, it is believed that the transfer of immune experience to progeny is likely done so via the eggs (Ben M. Sadd and Schmid-Hempel 2007; Salmela, Amdam, and Freitak 2015). Salmela et. al (2015) discovered that TGIP in *A. mellifera* is mediated via the egg-yolk protein Vitellogenin, which is found in many oviparous insects. Vg-mediated TGIP can allow for effective and specific immune priming in insects, but this mechanism does not rule out that other mechanisms also participate in TGIP. With our larval samples that we stored for future analysis, we hope to complete an in-depth study of the genetic expression and epigenetic factors that might be involved in this transfer of immunity from primed queens to offspring.

When compared with *Anopheles* or *Drosophila*, *A. mellifera* possess only one-third of genes involved in immunity in their genome (Evans et al. 2006). Regardless, *A. mellifera* retain the four known pathways (mentioned above) implicated in immunity in

invertebrates (Evans et al. 2006). Honey bee colonies compensate for this lack of genes via a system of social immunity (Cremer et al. 2007; Evans et al. 2006). With the proposed fitness costs previously discussed, this would suggest that a short-lived (3-4 months) worker honeybee is not expected to display a complex immune response but the long-lived honeybee queens (2-6 years) could positively influence the immunological status of the whole colony.

In a human context, *A. mellifera* TGIP experimentation could be applied towards understanding vaccination “costs” both at the individual and population level. With large colony sizes confined to small nesting cavities, honey bees will continue to serve as a relevant immunological model for the study of various pathogen transmission dynamics (Comman et al. 2012). Given that honey bees can be primed against bacterial diseases that contribute to CCD such as AFB (Cox-Foster et al. 2007; vanEngelsdorp et al. 2009), it is incredibly important to explore the limitations of TgIP (López et al. 2014). This project explores multiple facets of TgIP, which produces agricultural applications. Ideally commercial beekeepers will soon be able to prime *A. mellifera* queens against multiple pathogens to meet the immunological needs of their colonies. Given that common treatment methods for AFB require the total disposal of honey bee colonies (including equipment), TGIP provides a less economically taxing solution.

Bibliography:

Aupinel, Pierrick et al. 2005. “Improvement of Artificial Feeding in a Standard in Vitro Method for Rearing Apis Mellifera Larvae.” *Bulletin of Insectology* 58(2): 107–11.

Baer, Boris, and Paul Schmid-Hempel. 1999. “Experimental Variation in Polyandry Affects Parasite Loads and Fitness in a Bumble-Bee.” *Nature* 397(6715): 151–54. http://dx.doi.org/10.1038/16451.

Barribeau, Seth M. et al. 2016. “Royal Decree: Gene Expression in Trans-Generationally Immune Primed Bumblebee Workers Mimics a Primary Immune Response” ed. Sebastian D. Fugmann. *PLOS ONE* 11(7): e0159635. http://dx.plos.org/10.1371/journal.pone.0159635 (December 6, 2016).

Beemelmanns, Anne, and Olivia Roth. 2016. “Bacteria-Type-Specific Biparental Immune Priming in the Pipefish Syngnathus Typhle.” *Ecology and Evolution* 6(18): 6735–57.

Cameron, Sidney A, Jeffrey D Lozier, James P Strange, and Jonathan B Koch. 2011. “Patterns of Widespread Decline in North American Bumble Bees.”

Cooper, Max D et al. 2006. “The Evolution of Adaptive Immune Systems.” *Cell* 124(4): 815–22. http://www.ncbi.nlm.nih.gov/pubmed/16497590 (December 20, 2016).

Cornman, R. Scott et al. 2012. “Pathogen Webs in Collapsing Honey Bee Colonies” ed. Sarah K. Highlander. *PLoS ONE* 7(8): e43562. http://www.ncbi.nlm.nih.gov/pubmed/22927991 (December 6, 2016).

Cox-Foster, Diana L et al. 2007. “A Metagenomic Survey of Microbes in Honey Bee Colony Collapse Disorder.” *Science* 318(5848): 283 LP-287. http://science.sciencemag.org/content/318/5848/283.abstract.

Cremer, Sylvia et al. 2007. “Social Immunity.” *Current biology : CB* 17(16): R693-702.

http://www.ncbi.nlm.nih.gov/pubmed/17714663 (December 21, 2016).

Dawkins, R, and J R Krebs. 1979. “Arms Races between and within Species.” *Proceedings of the Royal Society of London. Series B. Biological Sciences* 205(1161): 489 LP-511. http://rspb.royalsocietypublishing.org/content/205/1161/489.abstract.

Evans, J D et al. 2006. “Immune Pathways and Defence Mechanisms in Honey Bees Apis Mellifera.” *Insect Mol Biol.* 15.

Freitak, Dalial, David G. Heckel, and Heiko Vogel. 2009. “Dietary-Dependent Trans-Generational Immune Priming in an Insect Herbivore.” *Proceedings of the Royal Society of London B: Biological Sciences* 276(1667).

Fünfhaus, Anne, Lena Poppinga, and Elke Genersch. 2013. “Identification and Characterization of Two Novel Toxins Expressed by the Lethal Honey Bee Pathogen *P Aenibacillus Larvae,* the Causative Agent of American Foulbrood.” *Environmental Microbiology* 15(11): n/a-n/a. http://doi.wiley.com/10.1111/1462-2920.12229 (December 21, 2016).

Gallai, Nicola, Jean-Michel Salles, Josef Settele, and Bernard E. Vaissière. 2009. “Economic Valuation of the Vulnerability of World Agriculture Confronted with Pollinator Decline.” *Ecological Economics* 68(3): 810–21.

Genersch, Elke. 2010. “American Foulbrood in Honeybees and Its Causative Agent, Paenibacillus Larvae.” *Journal of Invertebrate Pathology* 103: S10–19. http://www.ncbi.nlm.nih.gov/pubmed/19909971 (December 6, 2016).

Gerardo, Nicole M et al. 2010. “Immunity and Other Defenses in Pea Aphids, Acyrthosiphon Pisum.” *Genome Biology* 11(2): R21–R21.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2872881/.

Goulson, D, G C Lye, and B Darvill. 2008. “Decline and Conservation of Bumble Bees.” : 191–210.

Govan, V A, and M H Allsopp. 1999. “A PCR Detection Method for Rapid Identification of Paenibacillus Larvae.” 65(5): 2243–45.

De Gregorio, Ennio et al. 2002. “The Toll and Imd Pathways Are the Major Regulators of the Immune Response in Drosophila.” *The EMBO journal* 21(11): 2568–79. http://www.ncbi.nlm.nih.gov/pubmed/12032070 (December 6, 2016).

Grindstaff, Jennifer L, Edmund D Brodie, and Ellen D Ketterson. 2003. “Immune Function across Generations: Integrating Mechanism and Evolutionary Process in Maternal Antibody Transmission.” *Proceedings of the Royal Society of London. Series B: Biological Sciences* 270(1531): 2309 LP-2319. http://rspb.royalsocietypublishing.org/content/270/1531/2309.abstract.

Harpur, Brock A, and Amro Zayed. 2013. “Accelerated Evolution of Innate Immunity Proteins in Social Insects: Adaptive Evolution or Relaxed Constraint?” *Molecular Biology and Evolution* 30(7): 1665–74. http://dx.doi.org/10.1093/molbev/mst061.

Hernández López, Javier, Wolfgang Schuehly, Karl Crailsheim, and Ulrike Riessberger-Gallé. 2014. “Trans-Generational Immune Priming in Honeybees.” *Proceedings of the Royal Society of London B: Biological Sciences* 281(1785).

Hoffmann, Jules A., and Jean-Marc Reichhart. 2002. “Drosophila Innate Immunity: An Evolutionary Perspective.” *Nature Immunology* 3(2): 121–26. http://www.ncbi.nlm.nih.gov/pubmed/11812988 (December 6, 2016).

Keith Clay, Paula X. Kover. 1996. “THe Red Queen Hypothesis and Plant/Pathogen

Interactions.” *Annual Review of Phytopathology*.

Kim, L K et al. 2007. “Down-Regulation of NF-kappaB Target Genes by the AP-1 and STAT Complex during the Innate Immune Response in Drosophila.” *PLoS Biol* 5. http://dx.doi.org/10.1371/journal.pbio.0050238.

Klein, J. 1989. “Are Invertebrates Capable of Anticipatory Immune Responses?” *Scandinavian Journal of Immunology* 29(5): 499–505. http://doi.wiley.com/10.1111/j.1365-3083.1989.tb01152.x (December 6, 2016).

Kurtz, Joachim, and Karoline Franz. 2003. “Innate Defence: Evidence for Memory in Invertebrate Immunity.” *Nature* 425(6953): 37–38. http://www.ncbi.nlm.nih.gov/pubmed/12955131 (December 6, 2016).

Little, Tom J, Dan Hultmark, and Andrew F Read. 2005. “Invertebrate Immunity and the Limits of Mechanistic Immunology.” *Nature Immunology* 6(7): 651–54. http://www.ncbi.nlm.nih.gov/pubmed/15970937 (December 6, 2016).

López, Javier, Wolfgang Schuehly, Karl Crailsheim, and Ulrike Riessberger-Gallé. 2014. “Trans-Generational Immune Priming in Honeybees.” *Proceedings of the Royal Society of London B: Biological Sciences* 281(1785).

Medzhitov, Ruslan, and Charles A Janeway Jr. 2002. “Decoding the Patterns of Self and Nonself by the Innate Immune System.” 296(April): 298–301.

Moret, Yannick. 2006. “"Trans-Generational Immune Priming": Specific Enhancement of the Antimicrobial Immune Response in the Mealworm Beetle, Tenebrio Molitor.” *Proceedings. Biological sciences* 273(1592): 1399–1405. http://www.ncbi.nlm.nih.gov/pubmed/16777729 (December 21, 2016).

Obbard, Darren J, Francis M Jiggins, Daniel L Halligan, and Tom J Little. 2006. “Report

Natural Selection Drives Extremely Rapid Evolution in Antiviral RNAi Genes." 1: 580–85.

Parasites, P Schmid-hempel, and Springer Verlag. 1995. "Parasites and Social Insects To Cite This Version :" 26(3): 255–71.

Pearson, Paul N. 2001. "Red Queen Hypothesis." In *eLS*, John Wiley & Sons, Ltd. http://dx.doi.org/10.1038/npg.els.0001667.

Peng, Christine Ying-Shin et al. 1996. "Laboratory and Field Studies on the Effects of the Antibiotic Tylosin on Honey BeeApis melliferaL. (Hymenoptera: Apidae) Development and Prevention of American Foulbrood Disease." *Journal of Invertebrate Pathology* 67(1): 65–71. http://www.sciencedirect.com/science/article/pii/S0022201196900105.

Pham, Linh N., Marc S. Dionne, Mimi Shirasu-Hiza, and David S. Schneider. 2007. "A Specific Primed Immune Response in Drosophila Is Dependent on Phagocytes." *PLoS Pathogens* 3(3): e26. http://www.ncbi.nlm.nih.gov/pubmed/17352533 (December 6, 2016).

Roth, Olivia, Ben M Sadd, Paul Schmid-Hempel, and Joachim Kurtz. 2009. "Strain-Specific Priming of Resistance in the Red Flour Beetle, Tribolium Castaneum." *Proceedings of the Royal Society of London B: Biological Sciences* 276(1654).

Sackton, Timothy B et al. 2007. "Dynamic Evolution of the Innate Immune System in Drosophila." *Nat Genet* 39(12): 1461–68. http://dx.doi.org/10.1038/ng.2007.60.

Sadd, B. M., and P. Schmid-Hempel. 2009. "A Distinct Infection Cost Associated with Trans-Generational Priming of Antibacterial Immunity in Bumble-Bees." *Biology Letters* 5(6): 798–801.

http://rsbl.royalsocietypublishing.org/cgi/doi/10.1098/rsbl.2009.0458.

Sadd, Ben M., and Paul Schmid-Hempel. 2006. “Insect Immunity Shows Specificity in Protection upon Secondary Pathogen Exposure.” *Current Biology* 16(12): 1206–10. http://www.ncbi.nlm.nih.gov/pubmed/16782011 (December 6, 2016).

———. 2007. “Facultative but Persistent Trans-Generational Immunity via the Mother’s Eggs in Bumblebees.” *Current Biology* 17(24): R1046–47. http://www.ncbi.nlm.nih.gov/pubmed/18088585 (December 6, 2016).

Sadd, Ben M, Yvonne Kleinlogel, Regula Schmid-Hempel, and Paul Schmid-Hempel. 2005. “Trans-Generational Immune Priming in a Social Insect.” *Biology Letters* 1(4).

Salmela, Heli, Gro V Amdam, and Dalial Freitak. 2015. “Transfer of Immunity from Mother to Offspring Is Mediated via Egg-Yolk Protein Vitellogenin.” *PLOS Pathogens* 11(7): e1005015. https://doi.org/10.1371/journal.ppat.1005015.

Schmid-Hempel, Paul. 2005. “Natural Insect Host-Parasite Systems Show Immune Priming and Specificity: Puzzles to Be Solved.” *BioEssays* 27(10): 1026–34. http://doi.wiley.com/10.1002/bies.20282 (December 6, 2016).

———. 2013. *Evolutionary Parasitology: The Integrated Study of Infections, Immunology, Ecology, and Genetics*. Oxford: Oxford University Press. http://www.oxfordscholarship.com/view/10.1093/acprof:oso/9780199229482.001.0001/acprof-9780199229482.

Simone, Michael, Jay D Evans, and Marla Spivak. 2009. “RESIN COLLECTION AND SOCIAL IMMUNITY IN HONEY BEES.” *Evolution* 63(11): 3016–22. http://dx.doi.org/10.1111/j.1558-5646.2009.00772.x.

Siva-Jothy, M T, Y Moret, and J Rolff. 2005. “Insect Immunity: An Evolutionary Ecology

Perspective.” *Adv Insect Physiol* 32. http://dx.doi.org/10.1016/S0065-2806(05)32001-7.

Spivak, Marla, and Gary S Reuter. 2001. “Resistance to American Foulbrood Disease by Honey Bee Colonies Apis Mellifera Bred for Hygienic Behavior.” *Apidologie* 32(6): 555–65. https://doi.org/10.1051/apido:2001103.

Sumpter, D. J. T., and S. J. Martin. 2004. “The Dynamics of Virus Epidemics in *Varroa* -Infested Honey Bee Colonies.” *Journal of Animal Ecology* 73(1): 51–63. http://doi.wiley.com/10.1111/j.1365-2656.2004.00776.x (December 21, 2016).

Tarpy, David R. 2002. “Genetic Diversity within Honeybee Colonies Prevents Severe Infections and Promotes Colony Growth.” (November): 99–103.

Tidbury, Hannah J., Amy B. Pedersen, and Mike Boots. 2011. “Within and Transgenerational Immune Priming in an Insect to a DNA Virus.” *Proceedings of the Royal Society of London B: Biological Sciences* 278(1707).

Tzou, Phoebe, Ennio De Gregorio, and Bruno Lemaitre. 2002. “How Drosophila Combats Microbial Infection: A Model to Study Innate Immunity and Host-Pathogen Interactions.” *Current opinion in microbiology* 5(1): 102–10. http://www.ncbi.nlm.nih.gov/pubmed/11834378 (December 20, 2016).

Underwood, Robyn M, and Dennis Vanengelsdorp. “Colony Collapse Disorder: Have We Seen This Before?”

vanEngelsdorp, Dennis et al. 2009. “Colony Collapse Disorder: A Descriptive Study” ed. Justin Brown. *PLoS ONE* 4(8): e6481. http://www.ncbi.nlm.nih.gov/pubmed/19649264 (December 6, 2016).

Vanengelsdorp, Dennis et al. 2009. “Colony Collapse Disorder: A Descriptive Study.”

PloS one 4(8): e6481. http://www.ncbi.nlm.nih.gov/pubmed/19649264 (December 6, 2016).

Velthuis, Hayo H W, Adriaan Van Doorn, Hayo H W Velthuis, and Adriaan Van Doorn. 2006. “A Century of Advances in Bumblebee Domestication and the Economic and Environmental Aspects of Its Commercialization for Pollination To Cite This Version :”

Viljakainen, Lumi et al. 2007. “Rapid Evolution of Immune Proteins in Social Insects.” (Hoffmann 2003).

Walls, Isabel, and R Chuyate. 2000. 83 Journal of AOAC International *Isolation of Alicyclobacillus Acidoterrestris from Fruit Juices*.

Zanchi, Caroline et al. 2011. “Differential Expression and Costs between Maternally and Paternally Derived Immune Priming for Offspring in an Insect.” *Journal of Animal Ecology* 80(6): 1174–83. http://dx.doi.org/10.1111/j.1365-2656.2011.01872.x.

Appendix:

Dose Experiment

Spore Preparation 1						
spores/ul	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
0	90.00%	90.00%	90.00%	90.00%	90.00%	90.00%
1	90.00%	90.00%	90.00%	90.00%	50%	40%
5	80.00%	80.00%	80.00%	80.00%	60.00%	40.00%
10	100.00%	100.00%	100.00%	100.00%	40.00%	40.00%
20	100.00%	100.00%	100.00%	100.00%	50%	40.00%

Table 1: Dose Experiment Spore preparation 1 . (n=30, per dose)

Spore Preparation 2						
spores/ul	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
0	100.00%	90.00%	90.00%	90.00%	90.00%	90.00%
1	100.00%	90.00%	90.00%	90.00%	60%	50%
5	90.00%	90.00%	90.00%	90.00%	40.00%	20.00%
10	90.00%	90.00%	90.00%	90.00%	60.00%	40%
20	100.00%	100.00%	100.00%	100.00%	50%	30.00%

Table 2: Dose Experiment Spore preparation 2 (n=30, per dose)

Spore Preparation 3						
spores/ul	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
0	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%
1	90%	90%	90%	90%	80%	40%
5	90%	90%	90%	90%	40.00%	20.00%
10	100.00%	100.00%	100.00%	100.00%	50.00%	50.00%
20	90%	90%	90%	90%	60%	20.00%

Table 3: Dose Experiment Spore preparation 3 (n=30, per dose)

Mortality Experiments

Post-Priming (2 weeks)				
Days after Death	Survival %			
	Unprimed-exposed	Unprimed-control	Primed-exposed	Primed-control
D1	0.85	0.9	0.925	0.9625
D2	0.8	0.9	0.925	0.9625
D3	0.8	0.9	0.925	0.95
D4	0.775	0.875	0.925	0.95
D5	0.575	0.875	0.8375	0.925
D6	0.35	0.875	0.7	0.9125

Table 4: Post-priming data, two weeks after queen inoculation.