# The effects of propolis on the honey bee (*Apis mellifera*) immune system and mouthpart microbiome

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## Dedication

*This thesis is dedicated to Mitákuye Oyás'iŋ, all of my relations.*

## Abstract

Honey bees (*Apis mellifera*) collect and apply antimicrobial plant resins to the interior of their nest cavity to form a lining called a propolis envelope. Previous studies show that exposure to a propolis envelope within the nest cavity resulted in reduced immune system activation in adult bees, however the mechanism for this reduction remained unclear. In Chapter 1, I tested the hypothesis that propolis exposure would reduce the general bacterial load (16S rRNA transcription) in and on honey bees, thereby reducing antimicrobial peptide (*hymenoptaecin* gene) expression by the honey bee innate immune system. The results showed that bees exposed to a propolis envelope in field colonies had significantly lower transcript levels of *hymenoptaecin*, but in contrast to previous studies had significantly greater 16S rRNA transcription, compared to bees in colonies without a propolis envelope. Bees held in cages had significantly greater *hymenoptaecin* expression and significantly greater 16S rRNA transcription compared to bees from colonies, suggesting that bees are exposed to different bacterial communities between colonies and cages. The consistent reduction in immune activation yet variable general bacterial loads upon propolis exposure, as seen in previous studies, suggests that there may be a relationship between propolis exposure and the abundance and diversity of specific bacterial species in particular microbial niches in and on the honey bee body. In Chapter 2, I hypothesized that the antimicrobial activity of a propolis envelope in bees from field colonies would influence the bacterial diversity and abundance of the worker mouthpart microbiome. The results of DNA sequencing revealed that the mouthparts of worker bees in colonies with a propolis envelope had significantly lower bacterial diversity and significantly higher bacterial abundance, compared to the mouthparts of bees in colonies

without a propolis envelope. Based on the taxonomic results, the propolis envelope appeared to reduce pathogenic or opportunistic bacteria and to promote the proliferation of putatively beneficial bacteria on the honey bee mouthparts, thus reinforcing the core microbiome of the mouthpart niche. This work suggests that the mechanism for reduced immune system activation may be due to the antimicrobial properties of propolis reducing pathogenic and opportunistic bacterial species and promoting beneficial bacterial species in the mouthparts, which may affect disease transmission throughout the colony, thus promoting colony health and wellbeing. This relationships among honey bees, propolis, and microbes likely stems from their long evolutionary history together. The differences in bacterial loads between bees from field colonies and cages suggest that the antimicrobial properties of propolis, the community of microbes, and the individual immune response may vary according to the nest environment, availability of floral resources, and social and organizational behaviors of the bees within the colony.

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## Introduction

The work in this thesis was done to clarify and test gaps in our knowledge about the contribution and effects of propolis on honey bee (*Apis mellifera*) individual and social immunity. My main finding was that the antimicrobial properties of propolis reduced the pathogenic and opportunistic bacteria on the mouthpart microbiome of honey bees and promoted the putatively beneficial microbes. The honey bee microbiome contributes to the overall health and immune function, and the mouthparts of bees are an important interface between nestmates and the external environment. Thus, these findings support the use of a propolis envelope in a colony as a novel approach to promote honey bee social immune behaviors. The long-term goals of this line of research are to reduce unsustainable colony losses and increase the sustainability of beekeeping, which in turn will enhance food security.

Honey bee health and wellbeing is important because of the economic values from their harvestable hive products such as honey, beeswax, royal jelly and propolis, and from their valuable pollination services to agriculture worldwide (Klein et al. 2007), and their social structure and behaviors have been a source of inspiration to humanity since ancient history (Crane, 1999). Unfortunately, honey bees are experiencing unsustainable losses in North America and Europe (Lee et al. 2015; Seitz et al. 2015 Kulhanek et al. 2017). Much of the recent research has focused on understanding the impact of individual stressors (Cox-Foster et al. 2007; vanEngelsdorp et al. 2009; vanEngelsdorp et al. 2010; Cornman et al. 2012; Nazzi et al. 2012; Spivak et al. 2017), rather than on natural immune responses (individual immunity) and collective behavioral defenses (social immunity (Cremer et al. 2007)) that contribute to honey bee health and

survivorship (Wilson-Rich et al. 2009; Evans & Spivak, 2010; Simone-Finstrom, 2017; Simone-Finstrom et al. 2017). My work aims to improve the resiliency of honey bee populations and to better combat the diverse threats by utilizing their natural defenses with minimal human inputs.

Honey bee colonies are chronically exposed to parasitic mites, viruses, bacterial and fungal pathogens, miticides, pesticides, and poor nutrition, which weaken the individual bee and make the innate defenses insufficient at overcoming these combined stressors. Colony resiliency is diminished by sick and weakened bees, ultimately leading to a breakdown in the social structure, production, efficiency, immunity, and reproduction of the colony and eventually, colony death (Perry et al. 2015). Chronically weakened colonies can be even more susceptible to infections and levels of pesticide exposure that might otherwise not be harmful, further contributing to a downward spiral of health (Johnson et al. 2012).

Honey bees have evolved diverse mechanisms at the individual and at the social level to control the impacts of their many parasites and pathogens (Evans & Spivak, 2010). Individual pathogen resistance is achieved through different mechanisms: physical; by means of the cuticle on the exterior of the body, physiological; as through changes in pH within the body, and immunological; with cellular defenses via circulating hemocytes or humoral defenses with antimicrobial peptides (Evans & Spivak, 2010). Social immune responses consist of behavioral defenses like grooming nest mates and removing diseased brood from the nest, and modifications of the nest environment like resin collection and application (Evans & Spivak, 2010).

Honey bees typically nest in tree hollows. Once a swarm of bees finds a suitable cavity, they create a propolis envelope; they line the nest interior and often the entrance, with a layer of propolis, which consists of honey bee collected resins from plants and often mixed with wax secreted from the tergal wax glands (Ghisalberti, 1979; Simone-Finstrom & Spivak, 2010). The propolis envelope surrounding the colony serves many mechanistic non-mutually exclusive purposes: waterproofing, preventing fungal decay of the hive walls (Visscher, 1980; Simone-Finstrom & Spivak, 2010), reducing cracks, and helping promote stable temperature and humidity (Clark, 1918).

Resins are secondary plant compounds with remarkable properties that benefit the plants that produce them. Resins are composed of lipid soluble, volatile and non-volatile terpenoids and/or phenolics that protect plants against herbivory, UV radiation, desiccation, and infection (Langenheim, 2003). Metabolomic analysis identified the botanical source of propolis collected by honey bee colonies on the St. Paul University of Minnesota campus as resins produced mostly by the tree species *Populus deltoides* and to a lesser extent by *Populus balsamifera* (Wilson et al. 2013).

Most of the studied therapeutic effects of propolis and tree resins have been in relation to human health (Ghisalberti, 1979; Marcucci, 1995). Honey bees were domesticated by Ancient Egyptians sometime before 2600 BCE (vanEngelsdorp & Meixner, 2010), the use of honey bee propolis for human health dates back to 300 BCE, around the time of Aristotle, and is described by Pliny, a Roman naturalist (~79AD), and again by Huber, a French naturalist (18$^{th}$ century) (Haydak, 1954). Peoples native to North America used *Populus* resins even prior to the introduction of honey bees to North America in the 1600s (vanEngelsdorp & Meixner, 2010). Menominee, Forest

Potawatomi, Pillager Ojibwas, and Louisiana Choctaws are North American indigenous tribes that are noted for using *Populus* species tree buds, bark, and cotton down for therapeutic uses such as wound dressings, cold medicines, and fever reducers (Vogel, 1970).

Resins are collected by other animals, including other insects and other bee species for their pharmacological properties. Wood ants, *Formica paralugubris*. deposit resin in the nest mound, which reduced the general microbial load within the nest cavity, thus reducing the production of antimicrobial peptides by the innate immune system (Christe et al. 2003; Castella et al. 2008).

*Immune support of propolis to honey bees*

Studies on wood ants (Christe et al. 2003; Castella et al. 2008) were the inspiration in the Spivak lab for the original hypothesis about the mode of action of propolis on honey bee health. The first study on propolis envelopes in honey bee colonies, tested the prediction that propolis exposure would reduce general microbial loads in the nest cavity and thus immune expression of antimicrobial peptides (Simone et al. 2009). General bacterial loads (16S rRNA expression) and transcript levels of *hymenoptaecin* and *AmEater*, genes that code for an antimicrobial peptide and cellular immunity, respectively, were significantly reduced in 7d-old bees from colonies with an experimentally applied propolis envelope compared to bees from colonies with no propolis envelope (Simone et al. 2009).

A second study confirmed that bees in colonies with a propolis envelope had reduced investment in individual immune function (Borba et al. 2015). The expression of

immune genes, particularly *hymenoptaecin* and *abaecin*, of 7d-old bees was significantly lower in colonies with a propolis envelope during the 2-year study, indicating the long-term effect of propolis on baseline expression of immune genes (Borba et al. 2015). However, no differences were found in general bacterial loads (16S rRNA expression) between bees from colonies with and without a propolis envelope.

Complementary evidence from a study where bees were maintained in surface-disinfected cages lined with propolis extract and then fed lipopolysaccharides as an immune stimulant found that immune-challenged bees in a propolis enriched environment had higher immune responses than those in a propolis free environment (Simone-Finstrom et al. 2017). These studies provide evidence that the immune system is not suppressed by propolis exposure but instead may be reduced in order to prevent costly immune activation (Schmid-Hempel, 2005).

*Antimicrobial properties of propolis to honey bees*

Propolis provides an important colony defense against both general microorganisms within the nest cavity and specific honey bee brood pathogens within the colony, thus supporting social immunity. Propolis samples from diverse regions across the US were shown to have varying *in vitro* antimicrobial activity against the honey bee pathogens *Ascosphaera apis* (the fungal agent of chalkbrood disease) and *Paenibacillus larvae* (the bacterium that causes American foulbrood disease), both of which are diseases transmitted to larvae when fed contaminated brood food by adults (Wilson et al. 2015).

*In vitro* studies at the colony level have shown that a propolis envelope resulted in reduced chalkbrood infections (Simone-Finstrom & Spivak, 2012) and reduced severity of American foulbrood infections (Borba & Spivak, 2017). The ability of larval food from *P. larvae* challenged colonies with a propolis envelope to inhibit the growth of *P. larvae, in vitro,* was significantly higher compared to larval food from challenged colonies without a propolis envelope (Borba & Spivak, 2017). It is unclear if the increased antimicrobial activity of the larval food was due to the presence of antimicrobial peptides produced by adult bees and secreted into larval food, or to the leaching of compounds from propolis into the food. A third possibility is that the antimicrobial activity was due to *Bombella apis* (formerly called *Parasaccharibacter apium* and Alpha 2.2 *Acetobacteraceae*), a bacterium known to grow on larval food and found in the mouthparts (Corby-Harris et al. 2014b; Corby-Harris et al. 2016; Dalenberg et al. 2020). Since antimicrobials (e.g., peptides, glucose oxidase) are secreted by nurse bees into brood food, the same mechanism that allows adult bees to alter investment in innate immunity may allow nurse bees to invest more in these compounds as a social immune defense.

Although the abundance of pathogenic bacteria was shown to decrease in the presence of propolis, it remained unclear if general bacterial loads are affected by propolis. Although Simone et al. (2009) found significantly lower general bacterial loads in bees with a propolis envelope, Borba et al. (2015) did not find similar differences. The goal of my thesis was to try to clarify if the propolis envelope affects general bacterial loads of bees in field colonies and in cages.

*Function of the Microbiome*

The most well characterized honey bee microbiome community is associated with the gut niche (Romero et al. 2019). The bee gut microbiome plays a role in metabolism, immune function, growth and development, and protection against pathogens. Disruption of the gut microbiome has also been shown to have detrimental effects on bee health. Overall, evidence suggests that the gut microbiome plays an important role in bee health and disease (Raymann & Moran, 2018).

To our knowledge, honey bees do not consume propolis, and so there likely is no direct contact of propolis to the gut microbes. During the course of my studies, another research team published evidence that propolis alters the diversity the bacterial microbiome at the family or genus level in whole bee samples (Saelao et al. 2020). Saelao et al. (2020) found that the microbiota of bees in propolis-rich colonies were more similar to each other and more consistent between individual bees compared to honey bee microbiota from bees from propolis-poor colonies, which exhibited greater taxonomic diversity. These results suggest that propolis may support the regulation of colony microbiota by maintaining a stable or homeostatic microbial community. However, Saelao et al. (2020) examined the microbiota of the whole bee, so the findings are not particular to a specific microbiome niche, and they quantified the bacteria only to family and genus level. As there are numerous *Lactobacillus* species strains found in the different niches throughout the body, it is important to know which species are affected by propolis in the conditions of that particular niche (Anderson et al. 2013).

My work investigated the mouthparts, a honey bee microbial niche that comes in direct contact with propolis. Honey bee mouthparts are classified as the chewing and

lapping type, consisting of paired mandibles (jaws) attached on the sides of the head that can manipulate solid material, and a proboscis (tongue) that can extend to lap up liquids (Winston, 1987). Worker mouthparts have numerous functions: collecting and ingesting pollen and nectar; exchanging food to the queen, drones, and larvae; exchanging queen pheromones among workers; auto- and allogrooming; cutting, shaping, and manipulating wax and propolis for nest construction; dragging debris and dead bees out of the nest (Winston, 1987). The honey bee mouthparts are an under investigated microbial niche, possibly influencing honey bee health and disease (Anderson et al. 2018) because they are an interface between the external environment (both ambient and within the nest cavity) and the internal physiology (environment) of the bees.

*Chapters*

This thesis is divided into two chapters and two appendices. In Chapter 1 I tested the hypothesis that the mode of action of propolis on individual immunity acts by reducing the general microbial load of whole bees, which subsequently reduces antimicrobial peptide production. I measured the immune response and general bacterial loads of adult bees held in colonies and in cages with and without propolis. I hypothesized that bees in cages compared to field colonies would show a lower general bacterial loads and lower immune response due to the potentially closer contact bees have with propolis in cages than in large field colonies. The results from the first chapter support the hypothesis that propolis reduces immune expression of the antimicrobial peptide, *hymenoptaecin*, in bees from both colonies and from cages, the immune gene

transcript that was most consistently reduced in previous studies (Simone et al. 2009; Borba et al. 2015).

However, the mechanism for reduced immune expression does not appear to be due to reduced general bacterial loads, measured by 16S rRNA gene expression using primers and qPCR alone, in or on bees (Janda & Abbott, 2007). I speculated that propolis may reduce opportunistic and pathogenic bacterial species but may allow non-pathogenic or beneficial microbes to persist. In order to fully understand the antimicrobial effects of propolis on an entire community of bacteria that would include the microbiome of the bee and other pathogenic and opportunistic bacteria, it became clear that the 16S rRNA gene needed to be sequenced, identified to bacterial species Operational Taxonomic Unit (OTU), and then the OTUs need to be quantified (abundance) and qualified (taxonomy and diversity). To focus my investigation, I chose a specific microbial niche, the mouthparts, rather than a whole-bee general microbial load analysis, as they come into direct contact with propolis in the colony.

In Chapter 2 I tested the hypothesis that the presence of a propolis envelope in field colonies would alter the diversity and abundance of bacteria in the honey bee mouthpart microbiome. Because the mouthpart microbiome of *A. mellifera* has likely adapted to the antimicrobial properties of propolis in the nest cavity over evolutionary time, I hypothesized that pathogenic and opportunistic bacteria would be reduced on the mouthparts of worker bees exposed to propolis compared to bees without a propolis envelope. I dissected out the mouthparts, extracted the DNA, and quantified the DNA using 16S rRNA primers and qPCR. The extracted DNA was sequenced at the University of Arizona Genetics Core. Sequenced DNA was analyzed by the Anderson Lab at the

USDA-ARS Carl Hayden Bee Research Lab in Tucson, AZ. The results showed that propolis both reduced the diversity of pathogenic and opportunistic bacterial species, and increased the abundance of beneficial bacterial species on the mouthparts of honey bees with a propolis envelope compared to the mouthparts of honey bees without a propolis envelope in field colonies.

Appendix 1 contains the methods and results from an assay that confirms the antibacterial properties of the propolis extract used in both Chapters 1 and 2, against *Paenibacillus larvae*, the causative agent of the brood disease American foulbrood.

Appendix 2 contains data on general bacterial load of mouthparts from caged bees analyzed using 16S rRNA primers and RT-qPCR. The data is included because the 16S rRNA abundance on the mouthparts of caged bees was significantly greater than that of colony bees, which was an unexpected and interesting finding. Future studies, beyond the scope of my thesis, could analyze the bacterial community composition to determine which species were in greater abundance in cages compared to colonies.

The work in this thesis helped clarify the contribution and mode of action of propolis on honey bee (*Apis mellifera*) individual and social immunity, and adds to the body of literature on the honey bee mouthpart microbiome (Maes et al. 2016; Anderson et al. 2018). Based on the work of Saelao et al. (2020) and Dalenberg et al. (2020), propolis appears to alter the abundance and diversity of bacteria in and on the honey bee, by reducing pathogenic and opportunistic bacteria while also increasing beneficial bacterial species. Future work could further investigate the effect of propolis on microbiota in other microbial niches such as the various gut niches (crop, ileum, hind gut) and hive niches (stored pollen, honey, and brood food). Additionally, it would be

interesting to explore how the mouthpart microbiome changes when exposed to other factors such as artificial feed, pesticides, and ecological competition. Finally, future studies could explore the physiological mechanisms and pathways underlying the three-way interaction among the antimicrobial properties of propolis, the bacterial and fungal microbiome, and the immune system of honey bees to more fully understand and improve colony health.

# Chapter 1: Toward an understanding of the mode of action of propolis on honey bee (*Apis mellifera*) individual immunity

## Overview

Honey bees collect and apply antimicrobial plant resins to the interior of their nest cavity to form a lining called the propolis envelope. Previous studies show that exposure to a propolis envelope results in reduced immune system activation in adult bees, however it is unclear if propolis lowers immune gene expression directly, or indirectly by first reducing the microbial load in the nest. We tested the hypothesis that propolis exposure would reduce the general bacterial load (16S rRNA transcription) within the nest cavity, thereby reducing the expression of antimicrobial peptides by the honey bee innate immune system. We found that bees exposed to a propolis envelope in field colonies had significantly lower transcript levels of the antimicrobial peptide *hymenoptaecin*, but in contrast to previous studies, significantly greater general bacterial loads compared to bees in colonies without a propolis envelope. Bees from the same colonies held in incubator cages with and without propolis exposure showed the same trends, but there were no statistical differences in *hymenoptaecin* expression or in general bacterial loads between propolis and control treatments. Overall, bees held in cages had significantly greater *hymenoptaecin* expression and significantly greater general bacterial loads compared to bees from colonies, suggestive of different bacterial communities between colonies and cages. The consistent reduction in immune activation yet variable general bacterial loads upon propolis exposure, suggests that there may be a relationship between propolis exposure and the abundance and diversity of specific bacterial species in particular

microbial niches in and on the honey bee body. It is possible there is an interplay among the antimicrobial properties of propolis, the community of beneficial, opportunistic and pathogenic microbes, and the individual immune response that varies according to the nest environment, availability of floral resources, and social and organizational behaviors of the bees within the colony.

**Introduction**

To remediate honey bee (*Apis mellifera)* health in the North America and Europe (vanEngelsdorp & Meixner, 2010; Kulhanek et al. 2017), it is important to understand how colonies counteract stressors naturally (Spivak et al. 2019; Tauber et al. 2019). As a social insect that lives in a densely populated group of closely related individuals with frequent social contact, honey bees have evolved collective behavioral defenses, termed social immunity, to reduce transmission of infectious diseases (Cremer et al. 2007). To protect the colony against pathogens that enter the colony, honey bees collect antimicrobial plant resins, and deposit them on the interior of the nest cavity, which is then called a propolis envelope (Ghisalberti, 1979; Simone-Finstrom & Spivak, 2010). The propolis envelope surrounds and protects the colony, and early studies suggested that it serves many non-mutually exclusive purposes: waterproofing and preventing fungal decay of the hive walls, reducing cracks, and helping promote a stable temperature and humidity level (Simone-Finstrom & Spivak, 2010).

Plant resins are composed of lipid soluble, volatile and non-volatile terpenoids and/or phenolics that protect plants against herbivory, UV radiation, desiccation, and infection (Langenheim, 2003). Insects and other animals, including humans, collect resins

for their well-known antimicrobial activity against many bacterial, fungal and viral pathogens (Ghisalberti, 1979; Marcucci, 1995; Gekker et al. 2005; Sforcin, 2016). In recent years, research has focused on the health benefits of a propolis envelope to a honey bee colony (Simone-Finstrom & Spivak, 2010; Simone-Finstrom et al. 2017).

Simone et al. (2009) was the first to pose the hypothesis that propolis would reduce the general microbial load within the nest cavity, thereby reducing the expression of antimicrobial peptides by the honey bee innate immune system. To test the hypothesis, bees were hived in boxes with an experimentally applied layer of propolis-ethanol extract painted inside the box, using either propolis from colonies at the University of Minnesota, collected mostly from plants in the genus *Populus* (Wilson et al. 2013), or from "green propolis" collected from *Baccharis dracunculifolia* shrubs in Brazil (Park et al. 2004). Bees from colonies exposed to either the *Populus* or *Baccharis* derived propolis envelope for the first week of their adult life, compared to 7d-old bees from colonies with no propolis envelope, had significantly reduced immune system expression levels: exposure to *Populus* propolis resulted in significantly reduced *hymenoptaecin*, a gene that codes for an antimicrobial peptide; exposure to *Baccharis* propolis resulted in significantly reduced *AmEater*, a gene that codes for cellular immunity. Bees exposed to the *Populus* propolis, but not the *Baccharis* propolis, compared to 7d-old bees from colonies with no propolis envelope, had significantly reduced general bacterial loads, measured by 16S rRNA gene transcription (Simone et al. 2009). The difference in general bacterial loads between bees exposed to the *Populus* propolis and the *Baccharis* propolis compared to bees without any propolis exposure suggested that propolis may not reduce general bacterial loads consistently.

Borba et al. (2015) expanded on the Simone et al. (2009) study using large field colonies exposed to propolis envelope created from naturally honey bee collected resins at apiaries in Minnesota. To stimulate resin deposition, commercial propolis traps (plastic sheets with 7 mm wide gaps) were stapled along all inner walls of the hive boxes (Figure 1.1). The expression of the immune genes *hymenoptaecin* and *abaecin*, both genes that code for antimicrobial peptides, of 7d-old bees was significantly lower in colonies with a propolis envelope during the 2-year study, indicating the long-term effect of propolis on baseline expression of immune genes (Borba et al. 2015). However, the general bacterial loads were not significantly different between propolis treatments at any timepoint from spring to late summer. These field studies demonstrated that a propolis envelope affects the adult bee immune response in a consistent manner yet has a variable effect on the general bacterial load, similar to the findings by Simone et al. (2009).

Previous propolis envelope studies examined additional antimicrobial peptides, (e.g., *hymenoptaecin, abaecin, apidaecin, defensin-1 and defensin-2)* and a candidate for cellular immunity, the Epithelial Growth Factor (EGF)-family protein member *AmEater* (Simone et al. 2009; Borba et al. 2015). *Hymenoptaecin* was the only antimicrobial peptide that was consistently reduced in bees with propolis envelopes compared to bees without propolis envelopes. Thus, for my experiments, only *hymenoptaecin* expression was measured.

In order to better understand the modulating effect of propolis on the honey bee immune system, we repeated the experimental designs of Simone et al. (2009) and Borba et al. (2015) using field colonies, but also included bees held in incubator cages to control for environmental and colony conditions that might affect bees' exposure to propolis

contact and/or volatiles. We hypothesized that propolis exposure would reduce the general microbial load within the nest cavity, thereby reducing the expression of antimicrobial peptides by the honey bee innate immune system. More specifically, we hypothesized that bees in cages compared to field colonies would show a lower general bacterial loads and lower immune response due to the closer contact bees would have with propolis in cages than they do in large field colonies.

## Materials and Methods

### *Propolis*

The propolis used to form the propolis envelope in the field colonies (P+ = field colonies with a propolis envelope; P- = field colonies without a propolis envelope) was collected from colonies located at the University of Minnesota Outreach Research and Education Park (UMore) in Rosemount, MN in the summer of 2017. Commercial propolis traps (Mann Lake Ltd, MN, USA) were placed over the top frames of the colonies and at the end of the season, the propolis-filled traps were removed and stored at -80°C. Nine of the propolis-filled traps were cut to fit (1.5 traps/P+ box for two deeps) within the three P+ experimental colonies as described above. The propolis was harvested from the remaining 15 traps to make the propolis ethanol extract. The frozen resin was twisted out of the traps and pulverized using a coffee grinder. A 30% (w/v) ethanol extract of propolis was made by dissolving 30 grams of the powdered propolis into 70 mL of 70% ethanol. Propolis was dissolved at room temperature in the dark for three days and was shaken once manually each day. Debris was removed from the propolis extract using a filter paper under vacuum filtration. The final concentration of propolis

was 196 mg/ml, determined by allowing triplicate samples of the propolis extract to dry in a laminar flow hood until the weight no longer changed. *In vitro* antibacterial activity of the propolis ethanol extract was determined using an antibacterial assay against *Paenibacillus larvae* following previous methods (Wilson et al. 2015) (see Appendix 1 - Supplementary Materials and Methods).

*Colony Design*

Six colonies of honey bees derived from Italian stock (*Apis mellifera ligustica*) were purchased as packages from a northern California beekeeping operation. The colonies were hived in standard Langstroth bee boxes, in April 2018 at the Bee Lab apiary at the University of Minnesota, Saint Paul, MN, USA. Each colony began with one deep box, new wooden frames and new plastic foundation (Mann Lake Ltd, MN, USA). All colonies were monitored for *Varroa destructor* parasitic mites, which are known to suppress immune function in bees at high loads (Gregory et al. 2005). All colonies had fewer than three mites/100 bees, which is low and below the recommended treatment threshold. Three colonies were provided a propolis envelope (treatment = P+) by both coating the interior of each box with 100 ml of a 30% propolis extract (following Simone et al. (2009)), and by fitting propolis-filled traps (following Borba et al. (2015); see section on Propolis, below) onto the inner two sides and the back wall of the box Figure 1.1). The front wall was not given a propolis filled trap to facilitate removal of the frames for colony management. Both propolis extract and propolis traps were used in order to provide the colonies with as much propolis as possible and to replicate the propolis exposure methods in the previous propolis studies. Three additional colonies were not

provided propolis envelopes (control colonies = P-) but 100 ml of 70% ethanol solvent was painted on all four inner walls. Natural propolis deposits between boxes and between frames were not removed from either the P+ or P- colonies. Second boxes with the same treatments were added to both sets of colonies in June of 2018 to accommodate colony growth.

*Cage Design*

The cages were designed to ensure that the young bees held in them would be exposed by *per os* inoculation to bacteria from older bees and nest material, allowing for the transmission of beneficial, opportunistic and pathogenic bacteria to young bees (Powell et al. 2014) (Table 1.1). Previously used wooden cages with wire mesh screened sides were washed with dish detergent, surface disinfected with a 10% bleach solution, and then dried overnight in an oven (Table 1.2). Three small pieces of wood were fitted onto the interior of the three screen walls of the cage, with gaps on the top and bottom for ventilation. Propolis treatment was applied to the wood inserts by applying 20 ml total of propolis extract or an equal volume of 70% ethanol as a control. The propolis extract used in P+ cages was the same as the propolis extract used in P+ colonies. We predicted that the proximity to the propolis extract would be sufficient exposure and so propolis traps were not included.

In cages, pollen was provided by cutting 3.8 cm x 5.7 cm sections of wax comb from plastic foundation containing stored pollen, or "bee bread," from the respective parent colonies, and gluing the comb sections onto a piece of thin wood that was then screwed to the top of the cage, allowing for movement of bees under the comb section;

each P+ cage received pollen from each P+ field colony, and each P- cage received pollen from each P- colony. Cages were given sterile sucrose solution (50% w/v) and sterile water from inverted 15 ml tubes suspended from the top of the cage and were changed periodically throughout the experiment. The sucrose solution was filter sterilized and tap water solution was autoclaved to prevent cultivating bacteria on those solutions, which may have biased the microbiome towards those bacteria growing in either solution that would not be present in colonies (Williams et al. 2013). The cages were held in an incubator (Percival 1-36NL, Geneva Scientific, LLC) at 32°C and 50% relative humidity.

*Bee Samples*

In July 2018, frames of pre-eclosion pupae were removed from the six field colonies, to allow adult bees to eclose within screened enclosures within the incubator. One set of newly eclosed bees were marked with enamel paint on the thorax and returned to their source colony. Another set were paint marked and placed in the wooden cages using a common garden experimental design: 10 newly eclosed bees from each of the three treatment colonies were place in each cage (n=10*3=30). Additionally, 10 randomly aged, unmarked adult bees collected from each parent colony (n=10*3=30) were included in each cage, totaling 60 bees per cage. Adult bees, as well as hive components described above, were added to the cages to ensure that newly emerged bees acquired typical microbial communities and other bacteria, within the cage setting from older adult bees and pollen (Powell et al. 2014). Three cages were set up per treatment (3 P+ and 3 P-). Dead bees were removed from cages on days 3, 7, and 9. Nine days later the remaining marked young bees were collected from the colonies and cages (n = 169,

Table 1.2), and were placed individually into 2 ml tubes and stored at -80°C until RNA extraction.

*Whole bee RNA extraction*

Total RNA (T-RNA) was isolated and cDNA was synthesized from 72 bees from the colonies (approximately 12 bees per colony), and 54 bees from cages (9 bees per cage, 3 per colony source). cDNA was analyzed for immune gene expression and bacterial abundance analysis using quantitative real-time PCR (RT-qPCR). T-RNA was isolated using TRIzol reagent following the manufacturer's protocol, and cDNA was synthesized using Superscript II (Invitrogen). Prior to cDNA synthesis the tRNA quality and quantity was measured using a NanoDrop2000 instrument (Thermo Scientific Inc.), samples were diluted to 100 ng/µl, and the T-RNA was subjected to DNase treatment. After transcription, cDNA was diluted (1:1.8) with RNase- and DNase-free DEPC water and stored at -20°C until qPCR.

*Immune gene quantification*

Relative abundance of the immune response using the expression of the gene *hymenoptaecin*, normalized with actin as a reference gene, was analyzed with RT-qPCR. Samples for RT-qPCR were prepared using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Primer sequences used for this experiment were hymenoptaecin (forward primer, 5′-CTCTTCTGTGCCGTTGCATA-3′; reverse primer, 5′-GCGTCTCCTGTCATTCCATT-3′ (Evans, 2006)) and actin (forward primer, 5′-TTGTATGCCAACACTGTCCTTT-3′; reverse primer, 5′-

TGGCGCGATGATCTTAATTT-3′ (Boncristiani et al. 2011)), with PCR protocol BEEPATH (Evans 2006). All reactions were carried out with a PCR protocol consisting of 5 min at 95°C, then 40 cycles of a four-step protocol consisting of 94°C 20s, 60°C 30s, 72°C 1 min, and 78°C 20 s, followed by a measurement of the melt-curve. Any qPCR amplifications with inconsistent melt curves were discarded.

*16S rRNA gene quantification*

Absolute bacterial community abundance in the whole bee for each sample was quantified with a quantitative PCR (qPCR) assay of 16S rRNA gene copies using the Universal bacteria 16S rRNA gene primer pair (forward primer, 5′-AGG ATTAGATACCCTGGTAGT-3′; reverse primer, 5′-YCGTACTCCCCAGGCGG-3′ (Stackebrandt et al. 1994)). With the qPCR protocol of a denaturation stage at 50°C for 2 min followed by 95°C for 2 min, 40 amplification cycles at 95°C for 15 s, and 60°C for 1 min. A standard curve was generated using a 10-fold serial dilution series of a plasmid standard containing the full-length *Escherichia coli* 16S rRNA gene. The logarithmic regression equation generated from the 10-fold serial dilution plasmid series was used to convert qPCR-generated cycle thresholds to 16S rRNA copy numbers. The qPCR results were expressed as the total number of 16S rRNA gene copy numbers per DNA extraction (100 µl volume elution). Any qPCR amplifications with inconsistent melt curves were discarded.

*Statistical analysis*

Data were analyzed using JMP version 14.0.0. For immune gene expression and 16S rRNA transcript abundance in whole bee samples, all data were log transformed to meet the assumptions of normalcy. Data were then analyzed using a two-way factorial analysis of variance (ANOVA) comparing the main effects of propolis treatment (P+/P-) and housing type (Colony/Cage) and the interaction between the main effects. Post-hoc analysis was done using a Tukey HSD test.

**Results**

Bees exposed to propolis (P+) had significantly lower *hymenoptaecin* expression compared to bees not exposed to the propolis (P-) ($F_{1, 118} = 11.06$, $p = 0.0012$; treatment mean ± SE: P+ = 1.83 ± - 0.04; P- = 2.01 ± 0.04) (Figure 1.4). There was also a significant effect of housing, in which *hymenoptaecin* expression was significantly lower in bees from colonies compared to bees from cages ($F_{1, 118} = 45.50$, $p < .0001$; housing mean ± SE: Colony = 1.74 ± 0.03; Cage = 2.10 ± 0.04). The interaction between propolis treatment and housing was not significant $F_{1,118} = 0.10$, $p = 0.7554$. Tukey HSD post-hoc analysis revealed that *hymenoptaecin* expression was significantly lower in bees exposed to propolis in field colonies ($p = 0.0264$), but not in cages ($p = 0.2096$) (Figure 1.4).

Bees exposed to propolis had significantly greater 16S rRNA transcript abundance throughout the whole body compared to bees not exposed to the propolis ($F_{1, 118} = 17.32$, $p < .0001$; treatment mean ± SE: P+ = 12.96 ± 0.10; P- = 12.35 ± 0.11) (Figure 1.5). There was also a significant effect of housing, in which 16S rRNA transcript abundance was significantly greater in bees from cages compared to bees from colonies ($F_{1, 118} = 31.14$, $p < .0001$; housing mean ± SE: Colony = 12.24 ± 0.09; Cage = 13.07 ±

0.11). The interaction between propolis treatment and housing was not significant $F_{1,118} = 3.12$, $p = 0.0800$. Tukey HSD post-hoc analysis revealed that 16S rRNA transcript abundance was significantly lower in bees exposed to propolis in field colonies ($p < .0001$), but not in cages ($p = 0.4072$) (Figure 1.5).

**Discussion**

The results from this experiment support the hypothesis that propolis reduces immune expression of the antimicrobial peptide, *hymenoptaecin*, in bees from both colonies and from cages. Our findings indicate that, similar to previous propolis envelope experiments, individual bees exposed to a propolis envelope had lower immune activation (Figure 1.4), which may increase colony health and productivity (Simone et al. 2009; Borba et al. 2015) because chronic immune activation is physiologically costly to organisms (Evans & Pettis, 2005). However, in contrast to the findings from Simone et al. (2009), where general bacterial loads were significantly reduced in response to propolis exposure (Figure 1.5), and Borba et al. (2015), where general bacterial loads were unaffected by propolis, we found that the general bacterial load measured by 16S rRNA transcription was significantly increased in bees from colonies with propolis exposure compared to bees without propolis exposure. Similar to bees in colonies, bees held in incubator cages had greater general bacterial loads with propolis exposure compared to bees without propolis exposure, although the difference was not significant.

The mechanism for reduced immune expression does not appear to be due to reduced general bacterial loads in or on bees. However, it could be that propolis reduces opportunistic and pathogenic bacterial species while allowing non-pathogenic or

beneficial microbes to persist. Propolis has known antimicrobial activity against the pathogenic honey bee bacteria *Paenibacillus larvae*, the causative agent of American Foulbrood, *in vitro* and *in vivo* (Bastos et al. 2008; Wilson et al. 2015; Borba & Spivak, 2017). It is not known if propolis has antimicrobial activity against beneficial honey bee microbes, although beneficial bacteria have evolved with the ever-present propolis found in the nest cavities of honey bee colonies found in natural settings (Seeley & Morse, 1976).

The range of general bacterial loads between studies may be due to two factors: 16S rRNA gene abundance measurements are not specific to bacterial species; and whole-body bacterial loads vary across particular microbial niches, and age of bees. Propolis exposure may be antimicrobial to certain bacterial species while allowing other species to flourish, which 16S rRNA abundance does not specify. Direct contact with propolis or to the volatiles may have a greater effect on bacteria located on the exterior of the bee or on the mouthparts, rather than within the various niches of the guts. Sequencing the 16S rRNA gene of the bacteria found in particular microbial niches, such as the gut or mouthparts, may clarify the mode of action of propolis on the honey bee microbial communities, which would allow for a further understanding of how propolis reduces immune gene expression, and honey bee health at the colony level.

The use of 16S rRNA gene sequences is by far the most common housekeeping genetic marker used because it is present in almost all bacteria and it is highly conserved among bacteria species, thus a primer for this gene will bind to most bacterial DNA present in the honey bee, whether pathogenic or beneficial species (Janda & Abbott, 2007). The most useful way to use the 16S rRNA gene is for bacterial phylogeny and

taxonomy studies because the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time and the 16S rRNA gene (1,500 bp) is large enough for informatic purposes (Janda & Abbott, 2007). A drawback with 16S rRNA gene analysis is that the gene is too conserved to be a useful predictor of metabolic function (Anderson et al. 2011).

Whole bee general bacteria loads are relatively easy to obtain since there are no dissections needed that could allow for biases by the dissector, but whole bee samples are not specific enough to understand the changes that occur in each specific microbial niche. For instance, the honey bee gut has several difference microbial niches (mouth, esophagus, crop or social stomach, midgut, and hindgut). Each niche has unique microbial communities that may be shaped by the particular conditions of that niche such as changes in structure, pH changes, antimicrobial properties, and nutrient availability (Anderson et al. 2011). Each niche could change in a distinct way after exposure to propolis, and so a whole bee sample does not allow for a clear understanding of how each of those microbial communities change. Whole bee samples do allow for accurate measures of immune gene expressions, since the immune genes are present throughout the entirety of the bee body. However, separating microbial niches from whole bees can be problematic because the same RNA extractions from the same bee cannot be used for both immune gene and bacterial load analyses.

Our specific hypothesis, that propolis would have a greater effect on both the immune gene expression and the general bacterial loads in bees held in cages within an incubator compared to bees from colonies, was not supported by these results. We expected the effects to be greater because of the proximity of the bees to the propolis

painted boards, compared to the potential distance a bee may have from propolis in a standard hive box. Instead, we found that bees held in cages had significantly greater immune gene expression and significantly higher general bacterial loads compared to bees from colonies, for both bees with propolis exposure and bees without. The cages were designed to simulate microbial conditions in colonies by including wax comb, stored pollen, and older bees from the colonies, however some social aspects may be missing that play an important role in microbial transmission throughout a colony; e.g., larvae to feed, a queen to groom, the ability to defecate outside of the cage, the ability to clean the cage, and the presence of incoming microbes from floral resources (Table 1.1). Because the cages were a closed system, the microbes that were initially put in the cage could proliferate in a way that they would not have been able to in a colony. Because the identity of the bacteria that were greater in cages compared to colonies is not known, it is unclear if the abundance of bacteria caused the increase in immune gene expression or if particular species of bacteria caused the increased immune expression. Future studies may consider altering the cage design to include different social factors such as open brood to feed or the presence of a queen (Figure 1.3).

This study provides evidence that the incorporation of resin from the environment into the nest architecture in the form of a propolis envelope can benefit honey bees at the colony and individual level. Social immunity behaviors, such as the collection of plant resins and their deposition in the nest as an antimicrobial propolis envelope, may have evolved to compensate for deficiencies in innate or physiological immunity (Simone-Finstrom & Spivak, 2010). The combination of antimicrobial propolis properties and beneficial bacterial species may provide a redundant or supplementary function to the

immune system, thus allowing honey bee colonies to save energy, which allowed the species greater success. Modern hive equipment may be the first time in their evolutionary history that honey bee colonies have had a nest cavity without a propolis envelope as a foundation for comb construction, thus leaving the honey bee colony deficient in immune support. Promoting honey bee natural defenses by investigating the general and specific benefits of propolis may lead to novel and sustainable ways to improve bee health and mitigate losses.

## Tables

**Table 1.1.** Similarities and differences between colonies and cages (+ = with, - = without). Colonies and cages were designed to be as similar as possible, however some of the differences may influence immune system and general bacterial load results.

| Housing Conditions | Colonies | Cages |
|---|---|---|
| Propolis Extract | + | + |
| Raw Propolis | + | - |
| Older Worker Bees | + | + |
| Queen | + | - |
| Drones | + | - |
| Brood (eggs, larvae, pupae) | + | - |
| Pollen in Comb | + | + |
| Nectar | + | - |
| Sugar Water | - | + |
| Water | + | + |
| Incoming microorganisms and pests | + | - |
| Variable Weather Conditions | + | - |

**Table 1.2.** Cage Survivorship. Number of live bees during the cage study. Some marked bees escaped the cage while removing dead bees, resulting in less than 30 bees at the start of the trial.

| Propolis Treatment (P+/P-)/Cage Number | Day 0 (# bees alive) | Day 3 (# bees alive) | Day 7 (# bees alive) | Day 9 (# bees alive) | Percent Survived (Survived/Total) |
|---|---|---|---|---|---|
| P+1 | 30 | 30 | 30 | 29 | 97 |
| P+2 | 29 | 29 | 29 | 29 | 100 |
| P+3 | 29 | 29 | 29 | 29 | 100 |
| P-1 | 30 | 30 | 29 | 28 | 93 |
| P-2 | 30 | 30 | 29 | 24 | 80 |
| P-3 | 30 | 30 | 30 | 30 | 100 |

## Figures

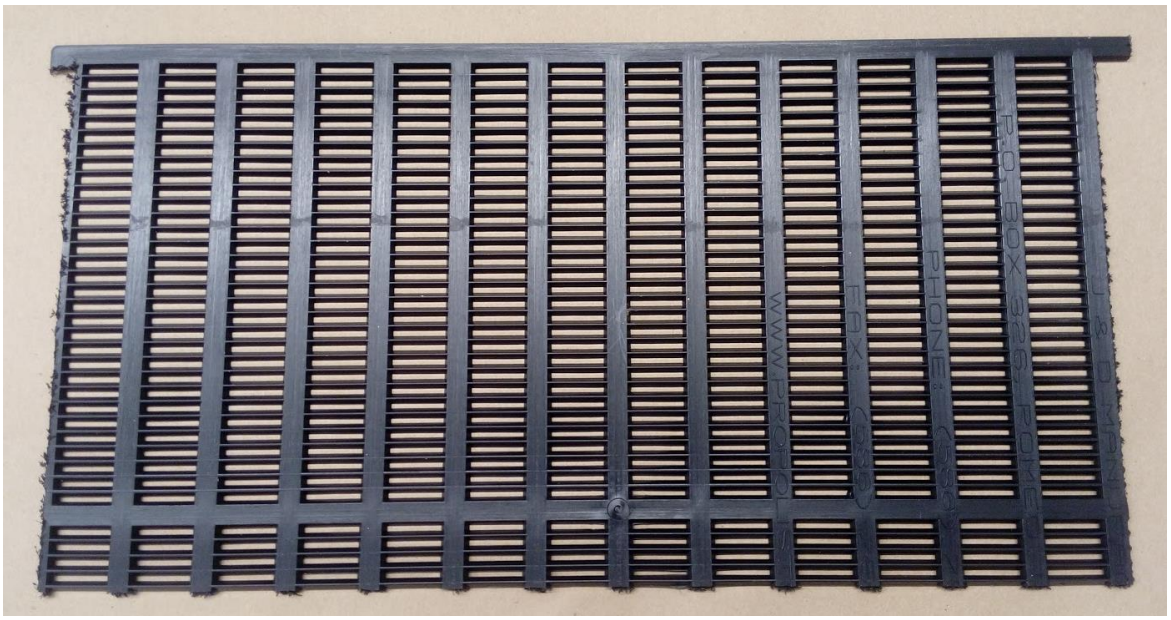

**Figure 1.1. Propolis trap.** To stimulate resin deposition, commercial propolis traps (plastic sheets with 7 mm wide gaps) were cut and stapled to fit along all inner walls of the hive boxes.

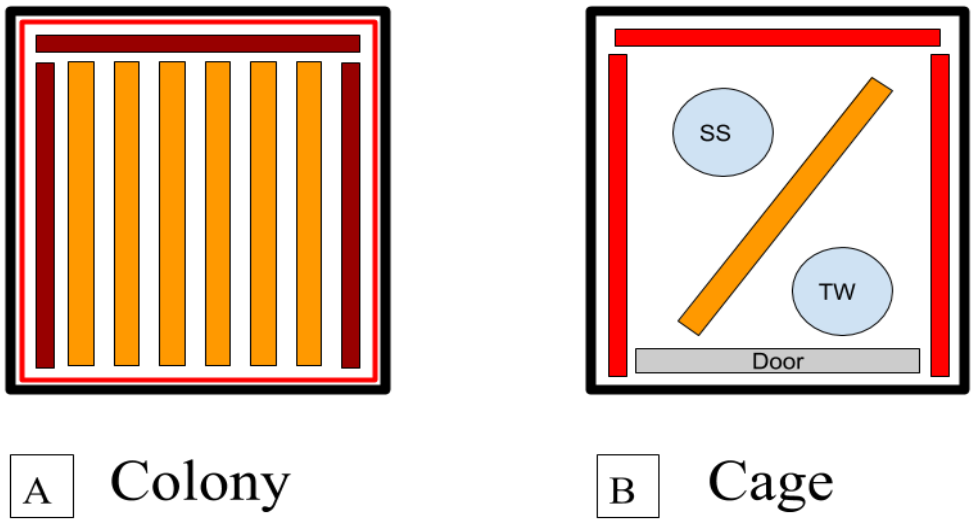


**Figure 1.2.** Colony and cage propolis treatments and setup. The field colonies (A), were given a propolis extract envelope on all four walls (bright red) and propolis traps (dark red) stapled onto the two side walls and the back wall. The front wall was not given a propolis trap to facilitate colony management. The cages (B), had wooden inserts that were first painted with propolis extract (bright red); the same propolis extract that was painted in colonies, and then the wooden boards were fitted on the interior side of the wire mesh walls. Walls in the control colonies and wooden inserts in the control cages were painted with 70% ethanol, the solvent for the propolis extract. Stored pollen (orange) was present in the field colonies (some pollen on each frame, along with brood, nectar and honey). In the cages, the combs that were suspended contained only cells containing pollen. In addition to pollen, cages were supplied with filtered sucrose solution (SS) and autoclaved tap water (TW) (both in light blue), both of which were administered through inverted tubes suspended from the top of the cage. A metal door (grey) was inserted into the fourth wall to allow the removal of dead bees throughout the experiment.

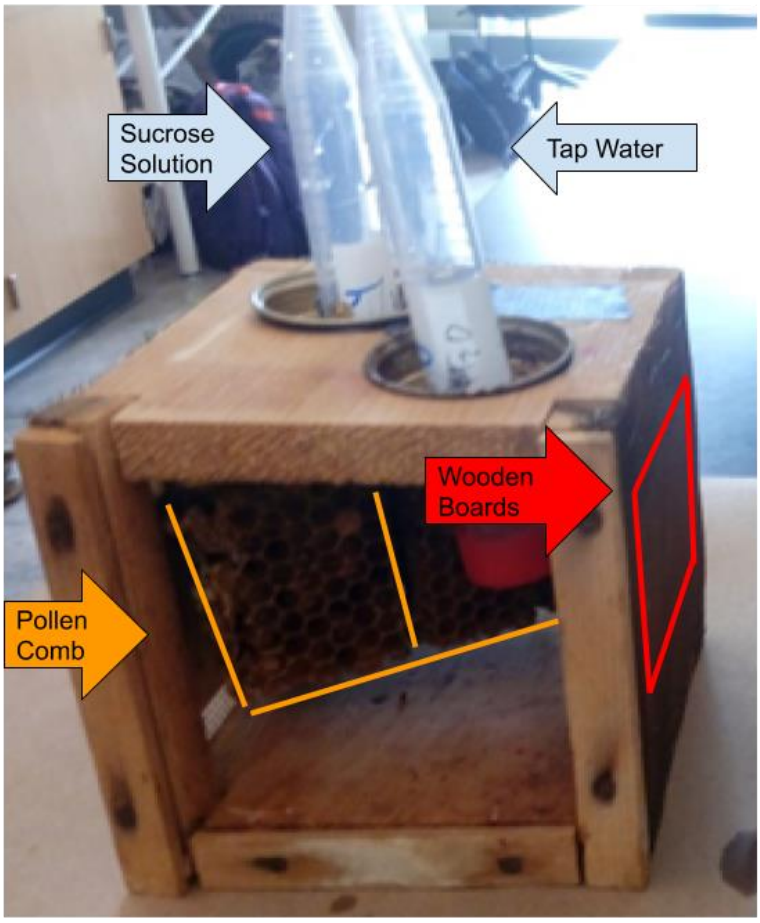


**Figure 1.3.** Cage setup. Inverted tubes containing either sterile sucrose solution or sterile tap water were suspended from the roof of the cage (blue arrows). Cut pollen comb (outlined in yellow), was suspended from the roof of the cage, allowing for bees to move freely underneath the suspended comb. Wooden boards (outlined in red) were fitted into the three wire-screened walls and painted with either 70% ethanol as a control or a propolis extract as a treatment.


**Figure 1.4.** Propolis reduces immune gene expression in bees from colonies and cages. Means +/- standard errors of expression of antimicrobial gene *hymenoptaecin*, normalized to the Cycle threshold (Ct) of the housekeeping gene *actin*, measured by RT-qPCR. Means with different letters are significantly different from each other at $p \leq 0.05$.

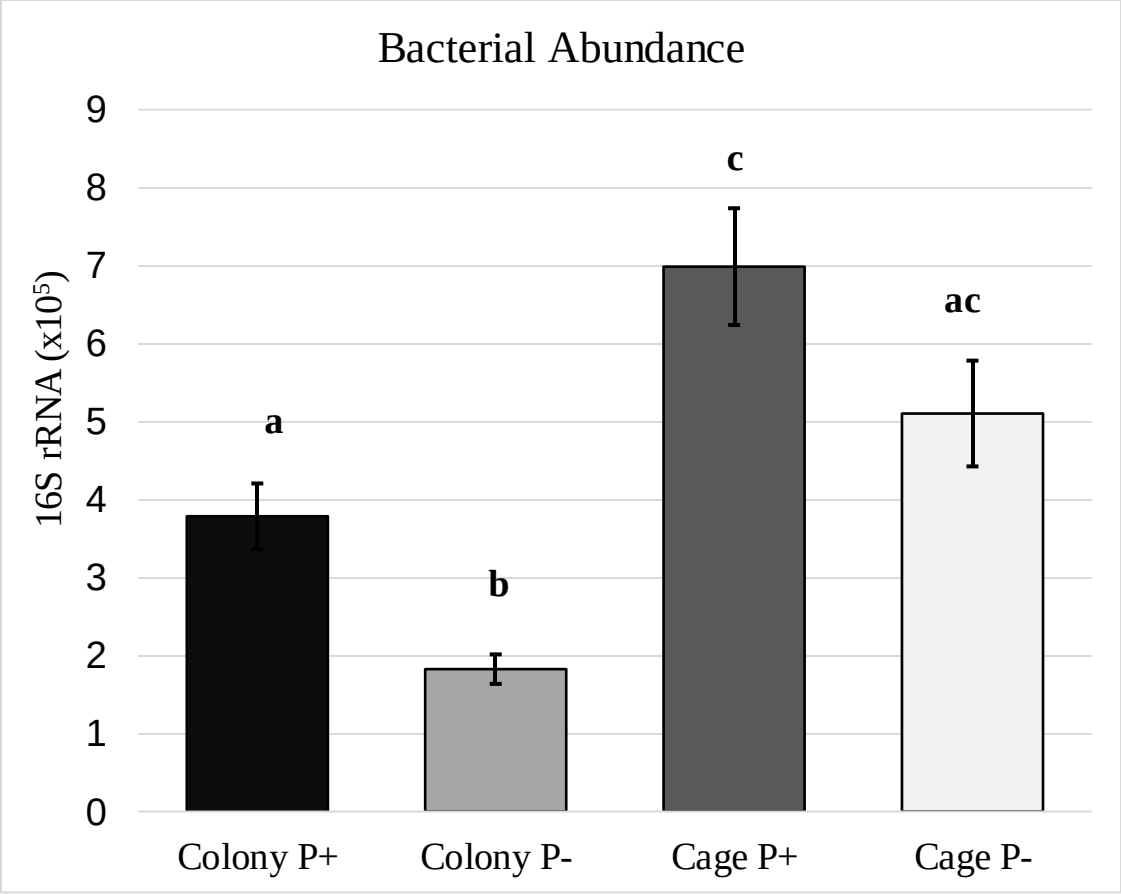


**Figure 1.5.** Propolis increases bacterial abundance in bees from colonies and cages. The independent variable was the average number of 16S rRNA copies per mouthpart, measured by RT-qPCR. Means +/- standard errors of expression of bacterial gene 16S, normalized to the Cycle threshold (Ct) with 16S rRNA standards on plasmids in *E. coli,* measured by RT-qPCR. Means with different letters are significantly different from each other at $p \leq 0.05$.

# Chapter 2: Propolis Envelope Promotes Beneficial Bacteria in the Honey Bee (*Apis mellifera*) Mouthpart Microbiome

## Publication Citation

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## Overview

Honey bees collect and apply plant resins to the interior of their nest cavity, in order to form a layer around the nest cavity called a propolis envelope. Propolis displays antimicrobial activity against honey bee pathogens, but the effect of propolis on the honey bee microbiome is unknown. Honey bees do not intentionally consume propolis, but they do manipulate propolis with their mouthparts. Because honey bee mouthparts are used for collecting and storing nectar and pollen, grooming and trophallaxis between adults, feeding larvae, and cleaning the colony, they are an important interface between the bees' external and internal environments and serve as a transmission route for core gut bacteria and pathogens alike. We hypothesized that the antimicrobial activity of an experimentally applied propolis envelope would influence the bacterial diversity and abundance of the worker mouthpart microbiome. The results revealed that the mouthparts of worker bees in colonies with a propolis envelope exhibited a significantly lower bacterial diversity and significantly higher bacterial abundance compared to the mouthparts of bees in colonies without a propolis envelope. Based on the taxonomic

results, the propolis envelope appeared to reduce pathogenic or opportunistic microbes and promote the proliferation of putatively beneficial microbes on the honey bee mouthparts, thus reinforcing the core microbiome of the mouthpart niche.

## Introduction

Honey bees and other social insect colonies have evolved behavioral, physiological, and organizational mechanisms to reduce pathogen transmission, known as social immunity (Cremer et al. 2007; Simone-Finstrom & Spivak, 2010). For *Apis mellifera*, one mechanism is the collection of antimicrobial plant resins (Simone et al. 2009) produced by some plants to defend against phytopathogens (Langenheim, 2003). Foraging bees from *A. mellifera* colonies, especially feral colonies that nest in tree cavities, collect large quantities of antimicrobial plant resins and deposit them as a thin continuous layer coating the rough interior walls of the nest cavity, called a propolis envelope (Seeley & Morse, 1976).

Previous research has established the benefits of a propolis envelope for colony health and disease resistance. Propolis in the colony is correlated or associated with increases in colony strength, vitellogenin levels, the antimicrobial activity of larval food, adult bee longevity, brood survival rates, hygienic behavior, and honey production (Manrique & Soares, 2002; Nicodemo et al. 2013; Padilha et al. 2013; Nicodemo et al. 2014; Borba et al. 2015; Borba & Spivak, 2017). Additionally, a reduction in deformed wing virus titers was found in colonies with propolis (even though Varroa destructor mite infestation did not change (Drescher et al. 2017)). After an experimental pathogen challenge, colonies with a propolis envelope had fewer clinical signs of chalkbrood

(Simone-Finstrom & Spivak, 2010) and American foulbrood (Borba & Spivak, 2017) compared to colonies without a propolis envelope. Simone et al. (2009) and Borba et al. (2015) found that colonies with a propolis envelope exhibited a reduced investment in individual bee immune function. It was hypothesized (but untested) that propolis reduced the amount of opportunistic and/or pathogenic bacteria in the nest, which subsequently reduced the need for bees to activate the physiologically costly immune system (Evans & Pettis, 2005). Recently, microbiome sequencing of bees by Borba et al. (2015) revealed that a propolis envelope significantly altered the presence of several key members of the gut microbiome and reduced the microbial diversity, suggesting that propolis may promote a healthy gut microbiome (Saelao et al. 2020).

To further test if propolis affects the bacterial community composition within a honey bee colony, we chose to investigate the bacteria associated with the mouthparts. Honey bee mouthparts serve as a horizontal transmission route for opportunistic and/or pathogenic, as well as beneficial, bacteria in the nest (Maes et al. 2016; Anderson et al. 2018). Honey bee mouthparts are used for a number of important social interactions and nutritional functions: feeding hypopharyngeal glandular secretion to developing larvae, grooming and trophallaxis between adults, collecting and storing nectar and pollen, and removing debris from the colony. Additionally, the mouthparts form the interface between the gut microbiome and various hive microbiome niches (Maes et al. 2016; Anderson et al. 2018).

The hindgut alone comprises >99% of the bacteria in adult workers; however, there are other important niches where certain bacterial species are consistently found in and around honey bees, including the upper alimentary tract of the bee (mouthparts,

hypopharyngeal glands, and crop) and the hive environment (royal jelly, larvae, fresh and stored pollen, and honey) (Anderson et al. 2013; Vojvodic et al. 2013; Anderson et al. 2014; Corby-Harris et al. 2014a; Maes et al. 2016; Anderson & Ricigliano, 2017; Anderson et al. 2018). The bacteria in the hindgut niches intimately interact with host tissue and are exposed to a refined post-digestion assortment of compounds, whereas bacteria in the hive- and mouthpart-associated niches experience many of these compounds directly, have fewer direct host–tissue interactions, and may indirectly benefit the host by outcompeting pathogens, thereby reducing pathogen transmission. These hive- and mouthpart-associated niches differ from the hindgut niche in many ways in that they typically contain far fewer bacteria, but those bacteria experience increased competition with other microbes and are more exposed to antimicrobial compounds such as oxygen, honey, and hypopharyngeal secretions. Bacteria consistently found in these foregut and hive niches are *Bombella apis* (previously named *Acetobacteraceae* Alpha2.2 and *Parasaccharibacter apium* (Corby-Harris et al. 2016; Yun et al. 2017; Smith et al. 2019)), *Lactobacillus kunkeei*, and *Fructobacillus fructosus* (Anderson et al. 2011; Anderson et al. 2013; Endo & Salminen, 2013; Anderson et al. 2014).

The mouthpart microbiome is predictive of dysbiosis occurring throughout the gut of the organism (Maes et al. 2016). When the highly predictable worker hindgut microbiome is in a state of dysbiosis, the mouthpart microbiome undergoes significant changes in the community structure. Since the honey bee gut microbiota have several functions (protection from opportunists, host adhesion and colonization, immune system and hormone regulation, and enriched metabolism of complex and toxic sugars

(Anderson & Ricigliano, 2017)), it can be inferred that a change in the mouthpart microbiome may be a reliable indicator of honey bee health (Maes et al. 2016).

A propolis envelope may result in different effects on the different honey bee microbial niches. For instance, the mouthpart microbiome may be more directly affected by the antibacterial effects of propolis compared to the gut microbiome, since the mouthparts directly come into contact with propolis during resin collection and application (Nakamura & Seeley, 2006).

We hypothesized that the presence of a propolis envelope in field colonies would alter the diversity and abundance of bacteria in the honey bee mouthpart microbiome. More specifically, because the mouthpart microbiome of *A. mellifera* has likely adapted to the antimicrobial properties of propolis in the nest cavity over evolutionary time, we hypothesized that pathogenic and opportunistic bacteria would be reduced on the mouthparts of worker bees exposed to propolis compared to bees without a propolis envelope.

## Materials and Methods

### *Experimental Design*

Six colonies of honey bees derived from an Italian stock (*Apis mellifera ligustica*) were purchased as packages from a northern California beekeeping operation. The colonies were hived in standard Langstroth bee boxes, in April 2018, at the Bee Lab apiary at the University of Minnesota, Saint Paul, MN, USA. Each colony began with one deep box, new wooden frames, and a new plastic foundation (Mann Lake Ltd., Hackensack, MN, USA). Three colonies were provided with an experimental propolis

envelope (treatment = P+) by coating the interior of each box with 100 mL of a 30% propolis extract in 70% ethanol solvent, and by fitting propolis-filled traps (see the section on propolis, below) onto the inner two sides and the back wall of the box. The front wall was not given a propolis-filled trap to facilitate removal of the frames for colony management. Three additional colonies were not provided with propolis envelopes (control colonies = P-), but 100 mL 70% ethanol, for the propolis extract was painted on all four inner walls. Natural propolis deposits between boxes and between frames were not removed from either the P+ or P- colonies. Second boxes with the same treatments were added to both sets of colonies in June of 2018 to accommodate colony growth. These were similar methods to those used in previous propolis envelope experiments (Simone et al. 2009; Borba et al. 2015).

*Propolis*

The propolis used to form the propolis envelope was collected from colonies located at the University of Minnesota Outreach Research and Education Park (UMore) in Rosemount, MN, USA, in the summer of 2017. Commercial propolis traps (Mann Lake Ltd., MN, USA) were placed over the top frames of the colonies and at the end of the season, the propolis-filled traps were removed and stored at -80 C. Nine of the propolis-filled traps were cut to fit (1.5 traps/P+ box for two deeps) within the three P+ experimental colonies, as described above. The propolis was harvested from the remaining 15 traps to make the propolis ethanol extract. The frozen resin was twisted out of the traps and pulverized using a coffee grinder. A 30% (w/v) ethanol extract of propolis was made by dissolving 30 g of the powdered propolis in 70 mL of 70% ethanol.

Propolis was dissolved in the dark for 3 days and was shaken once manually each day. Debris was removed from the propolis extract using filter paper under vacuum filtration. The final concentration of propolis was 196 mg/mL, determined by allowing triplicate samples of the propolis extract to dry in a laminar flow hood until the weight no longer changed. *In vitro* antibacterial activity of the propolis ethanol extract was validated using an antibacterial assay against *Paenibacillus larvae* following the methods used in Wilson et al. (2015) (Appendix 1 - Supplementary Materials and Methods).

*Bee Mouthpart Samples*

In July 2018, frames of an emerging brood were removed from the six experimental colonies and were placed in individual screened cages and allowed to emerge overnight in an incubator at 32°C and 50% relative humidity. The next day, the newly emerged bees were marked with enamel paint on the thorax and returned to their source colony. Nine days later, the marked bees were collected from the colonies, individually placed in 2 mL tubes, and stored at -80°C. Frozen bees from the colonies (n = 92) were individually thawed and then decapitated; the bodies were returned to the freezer. The proboscis was extended from the head using sterile tweezers and the esophagus was cut proximal of the proboscis at the cardo with sterile dissection scissors. The mandibles were not included, in order to be comparable to Anderson et al. (2018). Dissected mouthparts were placed in tubes containing 300 µL of sterile 1X TE buffer and 100 µL of 0.1 mm beads and returned to the freezer until homogenization.

*DNA extraction*

Frozen mouthparts were thawed and put in a beadbeater at 30 s intervals for a total of 2 min. A total of 100 µL lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 5% Triton X-100, 80 mg/mL lysozyme, pH 8.0) was added to each sample and the samples were incubated at 37°C for 30 min. DNA was then purified using a Thermo Fisher Scientific GeneJet Genomic DNA Purification Kit, according to the manufacturer's instructions for gram-positive bacteria. DNA was eluted with 100 uL Elution Buffer and stored at -20°C until quantification and characterization.

*16S rRNA gene sequencing for absolute community abundance*

The absolute community abundance of each sample was quantified with a quantitative real-time PCR (RT-qPCR) assay of 16S rRNA gene copies (Liu et al. 2012). A 466 bp fragment in the V3–V4 region of the bacterial rRNA gene was amplified from total DNA using the BactQuant primer pair (forward primer, 50-CCTACGGGDGGCWGCA-30; reverse primer, 50-GGACTACHVGGGTMTCTAATC-30). qPCRs were carried out on a BioRad CFX96 thermocycler in 20 µL reactions containing 6.4 µL of DEPC water, 10 µL of iTaq Universal SYBR Green Supermix (BioRad), 0.8 µL of forward primer, 0.8 µL of reverse primer, and 2 µL of DNA template. The cycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. A standard curve was generated using a 10-fold serial dilution series of a plasmid standard containing the full-length *Escherichia coli* 16S rRNA gene. The logarithmic regression equation generated from the 10-fold serial dilution plasmid series was used to convert qPCR-generated cycle thresholds into 16S copy numbers. The qPCR results were expressed as the total number of 16S rRNA gene copy numbers per

DNA extraction (100 µL volume elution). Any qPCR amplifications with inconsistent melt curves were discarded.

*16S rRNA gene sequencing for community analysis:*

The V3–V4 region of the 16S rRNA gene was amplified using PCR primers (forward primer, 341F 50-CCTACGGGNGGCWGCAG-30; reverse primer, 805R 50-GACTACHVGGGTATCTAATCC-30). Amplification was performed using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94 C for 3 min, followed by 28 cycles of 94 C for 30 s, 53 C for 40 s, and 72 C for 1 min, with a final elongation step at 72 C for 5 min. PCR products were confirmed using a 2% agarose gel. PCR products were used to prepare DNA libraries following Illumina TruSeq DNA library preparation. Sequencing was performed on a MiSeq at the University of Arizona Genetics Core. All sequence data were deposited in GenBank under Sequence Read Archive (SRA) accession PRJNA594720.

*16S rRNA gene sequence analysis*

Sequences were processed using MOTHUR v.1.39.5 (Schloss et al. 2009). Forward and reverse reads were joined using the make.contigs command. After the reads were joined, the first and last five nucleotides were removed using the SED command in UNIX. Using the screen.seqs command, sequences were screened to remove ambiguous bases. Unique sequences were generated using the unique.seqs command. A count file containing group information was generated using the count.seqs command. Sequences were aligned to the Silva SSUREF database (v102) using the align.seqs command.

Sequences not overlapping in the same region were removed using the screen.seqs command. Sequences were preclustered using the pre.cluster command. Chimeras were removed using UCHIME (Edgar et al. 2011), and any sequences that were not of known bacterial origin were removed using the remove.seqs command. All remaining sequences were classified using the classify.seqs command. All unique sequences with one or two members (single/doubletons) were removed using the AWK command in UNIX. A distance matrix was constructed for the aligned sequences using the dist.seqs command. Sequences were classified at the 97% level with the Ribosomal Database Project (RDP) Naive Bayesian Classifier (Wang et al. 2007) using a manually constructed training set containing sequences sourced from the greengenes 16S rRNA database (version gg_13_5_99 accessed May 2013), the RDP version 9 training set, and all full-length honeybee-associated gut microbiota on NCBI. Operational taxonomic units (OTUs) were generated using the cluster command. OTUs sharing exact taxonomy were merged using the merge.otu command. Representative sequences for each OTU were generated using the get.oturep command. To further confirm taxonomy, resulting representative sequences were subject to a BLAST query using the NCBI nucleotide database. For Alpha diversity analysis, rarefaction curves describing the number of OTUs observed as a function of sampling effort were generated using the rarefaction.single command. For Beta diversity, similarity calculations of the membership and structure between samples were conducted using the dist.shared and pcoa commands.

*16S abundance statistical analysis*

We compared the bacterial abundance with a parametric linear mixed-effects model. Prior to analysis, the 16S gene copy numbers were transformed using log transformation to satisfy assumptions of normality. The abundance was compared between propolis treatments, but also between colonies, in order to quantify the colony effect of the propolis treatment.

*Diversity statistical analysis*

We examined the diversity within each propolis treatment, and the relationship between diversity and OTU abundance. We first generated rarefaction curves describing the number of OTUs observed as a function of sampling effort. Next, we calculated the Inverse Simpson diversity index for each treatment condition. To investigate potential relationships between the shifts in abundance and the decrease in diversity, we ran a Spearman's correlation analysis of the bacterial absolute abundance and the inverse Simpson values.

*Relative abundance statistical analysis*

First, we conducted a MANOVA analysis of the centered log-ratio transformed (CLR) based on the relative abundance. To examine the effect of the community size, we multiplied the proportional abundance of OTUs by the group- or species-specific 16S rRNA gene copy number and total bacterial 16S rRNA gene copies determined with qPCR for each sample.

MANOVA was performed on log-converted relative abundances that account for the structure of the microbiome when comparing particular taxa between treatments. To

allow the use of parametric multivariate analyses (Pearson, 1897), we converted relative OTU abundances into ratios for all OTUs (Gloor and Reid 2016) using the software CoDaPack's centered log ratio (CLR) transformation (Comas Cufí & Thió I Fernández De Henestrosa, 2011). These transformations reflect the ratio abundance of all taxa in the data set. Nearly all of these transformed data sets were normally distributed (Gloor & Reid, 2016). A few samples slightly deviated from normal values following transformation. Because our sample size was large (n = 45 for each independent variable), these tests were robust in terms of slight deviations from normality. As an additional measure, we used Pillai's Trace test statistics, also robust in terms of violations of multivariate normality and homogeneity of covariance.

The MANOVA analyses were performed on CLR-transformed data, with OTUs 1-16 as dependent variables. False discovery rate (FDR) correction was conducted to account for multiple pairwise comparisons. It should be noted that OTUs found in only a few libraries were removed, resulting in 16 OTUs being used for all downstream statistical analyses.

*Absolute abundance statistical analysis*

Second, we tested for differences in the absolute abundance of microbial communities using pairwise Wilcoxon rank sum tests followed by FDR correction for multiple comparisons. Unlike the MANOVA, the Wilcoxon rank sum comparisons were conducted based on the calculated absolute cell number for each OTU, and do not account for the overall structure of the microbiome. We used both analyses because it is unknown how the individual bacterial species affect one another; whether they exist as an

interacting community network, or as individual species groups that occupy unique, relatively isolated mouthpart micro-niches.

We calculated the absolute cell number of community members by determining the total number of 16S gene copies in each sample via universal "bacti-quant" qPCR, and then assigning a portion of this total to each OTU based on the relative proportions generated from next generation sequencing. To obtain cell number estimates, we then corrected for the average number of 16S operon copies associated with each bacterial taxon according to the rRNA operon copy number database (Stoddard et al. 2014) (Dalenberg et al. 2020 – Supplementary Material Table S2).

An analysis of similarities (ANOSIM) was used to determine if the general similarity between groups was greater than or equal to the similarity within groups. Here, an abundance matrix for the top 94 OTUs was generated in MOTHUR using the cluster command. The matrix was then square root transformed and a Bray–Curtis dissimilarity matrix was generated. The global R and pseudo p-value were generated using 999 permutations. Analyses were conducted in Primer-e version 6.4.7, JMP_ v11 (JMP_ 1989–2007) and/or SAS_ v9.4 (2013 SAS).

## Results

### *Microbiome comparison*

Based on an ANOSIM analysis of the top 94 OTUs, the mouthpart microbiomes significantly differed by treatment condition (global test statistic: 0.23, 0.1%).

### *16S abundance*

The total 16S gene copy number significantly differed between treatments, with the propolis treatment group being significantly greater ($F_{1,4} = 27.96$, $p < 0.001$) (Figure 2.1). There was no significant difference between source colonies within the same treatment group ($F_{5,84} = 0.5008$, $p = 0.7352$). Because colonies within treatments did not differ, downstream analyses did not consider the colony source as a factor.

*Diversity*

Next generation sequencing returned 3,875,345 quality trimmed reads (<400 bp) for the 96 amplicon libraries generated, averaging 41,669 reads per library (Dalenberg et al. 2020 – Supplementary Material Table S3). A total of 1687 OTUs were resolved at 97% similarity. The top 19 OTUs and a 20th group consisting of 'other' (Σ OTUs 20-1687) represented 92% and 8% of the total sequences, respectively (Dalenberg et al. 2020 – Supplementary Material Table S4).

We found significantly less bacterial diversity on the mouthparts of workers from propolis treated colonies compared to the mouthparts of workers from colonies without a propolis envelope (Figure 2.1) ($Z = 4.05$, $p < 0.0001$). The standard error around the mean diversity was lower in the microbial communities of bees from colonies with propolis compared to the communities of bees from colonies without propolis (Figure 2.1). The results of the OTU analysis show that *Bombella apis*, *Lactobacillus kunkeei*, and *Fructobacillus fructosus* increased in absolute abundance in the samples collected from colonies treated with propolis. However, the primary effect of propolis was the restructuring of the mouthpart microbiome community by the dominant bacterial species *Bo. apis* (Figure 2.2). Given the dominance of *Bo. apis* and its correlation with diversity,

regardless of the treatment condition (Figure 2.2), we performed an ANCOVA post-hoc to explore the association of *Bo. apis* with diversity in the context of our independent variable. When the diversity explained by the relative abundance of *Bo. apis* in the mouthpart microbiome was removed from the model, the adjusted means for diversity did not differ by treatment (propolis = 4.3, control = 5.1; F1, 90 = 0.4, $p = 0.53$). The interaction term (*Bo. apis* by treatment condition) was not significant (F1, 89 = 0.03, $p = 0.86$), meeting the assumption of homogeneous regression slopes required by ANCOVA.

To investigate potential relationships between the shifts in abundance and changes in diversity, we ran a Spearman's correlation analysis for the bacterial absolute abundance of *Bo. apis*, *L. kunkeei*, and *F. fructosus* and the Inverse Simpson values obtained from the propolis treatment. We found a strong negative relationship between *Bo. apis* and *L. kunkeei* ($rs = -0.90$, $p < 0.001$) and *Bo. apis* and *F. fructosus* ($rs = -0.42$, $p < 0.003$). Additionally, while the *Bo. apis* abundance was negatively associated with the mouthpart microbiome diversity ($rs = -0.89$, $p < 0.0001$), both the *L. kunkeei* and *F. fructosus* abundance associated positively with the mouthpart microbiome diversity ($rs = 0.75$, $p < 0.001$ and $rs = 0.39$, $p < 0.006$, respectively (Figure 2.3)). From the mouthparts of non-propolis colonies, we found no significant correlation between either of the three bacteria and diversity, though we did find a similar negative relationship between *L. kunkeei* and *Bo. apis* and a positive relationship between *Fructobacillus* and *Bo. apis* ($rs = -0.41$, $p < 0.006$ and $rs = -0.31$, $p < 0.04$, respectively (Figure 2.3)).

*Relative abundance*

The multivariate analysis (MANOVA) result based on centered log ratios differed by treatment (Pillai's Trace = 0.44, F = 3.51, df = (75), $p < 0.0001$). Pairwise post-hoc comparisons resulted in ten significant differences following FDR correction for multiple comparisons. *Bo. apis* and *F. fructosus* displayed a greater abundance on mouthparts of samples collected from colonies treated with propolis ($F(1, 91) = 49.67$, $p < 0.0001$ and $F(1, 91) = 29.71$, $p < 0.0001$, respectively), whereas *Pseudomonas*, *Pseudoalteromonas*, *Streptococcus*, *Serratia*, *Microbacterium*, *Propionibacterium*, *Enterobacteriaceae*, and 'other' exhibited a lower abundance ($F(1, 91) = 37.78$, $p < 0.0003$, respectively) (Dalenberg et al. 2020 – Supplementary Material Table S5).

*Absolute abundance*

Comparisons between treatments based on Wilcoxon rank sum tests returned three significant differences (Dalenberg et al., 2020 – Supplementary Material Table S5). In agreement with the MANOVA results, *Bo. apis* and *F. fructosus* displayed a greater abundance on mouthparts of samples collected from colonies treated with propolis ($Z = -5.90$, $p < 0.0009$ and $Z = -5.11$, $p < 0.0009$, respectively). Additionally, *L. kunkeei* exhibited a greater absolute abundance on mouthparts of samples collected from colonies treated with propolis ($Z = -3.67$, $p < 0.001$).

**Discussion**

This study adds to the paradigm of social immunity in honey bees and other social groups (Evans and Pettis 2005). The presence of a propolis envelope within the nest cavity significantly altered the bacterial abundance (Figure 2.1) and diversity (Figure 2.1,

Figures 2.2 and 2.3) on the honey bee mouthparts. In colonies with a propolis envelope, the mouthparts of worker bees had a significantly greater bacterial abundance but significantly lower bacterial diversity, compared to colonies without a propolis envelope, indicating that propolis acts as a selective agent mitigating the microbiome structure and size. Accounting for the majority increase in microbiome size, growth of the dominant mouthpart bacterium significantly increased with the addition of propolis, suggesting that these two factors work in concert to promote a hygienic hive environment.

It is well-established that propolis reduces the expression of individual antimicrobial peptides (Simone et al. 2009; Borba et al. 2015), but here, we show an additional way in which propolis may improve colony health, i.e., by altering the colony microbiome, to favor beneficial or commensal bacteria that outcompete potentially pathogenic microbes (Figure 2.2). Based on the identification of the bacteria and the diversity indices of microbial communities in colonies with or without a propolis envelope (Figure 2.3), propolis significantly changed the mouthpart microbiome community structure, reducing various potentially pathogenic bacteria and promoting the proliferation of commensal and beneficial bacteria on the honey bee mouthparts. More generally, the taxonomic similarities of microbiomes found on the mouthparts of worker bees from all colonies regardless of treatment suggest a core (native) mouthpart microbiome. The mouthpart microbiome provides a first line of defense against opportunistic and pathogenic bacteria and fungi for bees, and this effect may be enhanced when bees are exposed to a propolis envelope within the nest cavity.

The major effect of propolis exposure was an increase in both the relative and absolute abundance of *Bo. apis* on the mouthparts of bees (Figure 2.2). *Bo. apis* is

abundant and prevalent on worker mouthparts of bees in general, and so may be considered the dominant core member of the mouthpart microbiome (Maes et al. 2016; Anderson et al. 2018). *Bo. apis* is consistently found in environments toxic to most bacteria, such as the aerobic, acidic, and high sugar osmolarity conditions found in food stores (stored pollen and nectar), royal jelly-associated niches (worker hypopharyngeal glands and crop, and larval gut), and queen body niches (mouthparts and throughout the guts) (Anderson et al. 2013; Vojvodic et al. 2013; Anderson et al. 2014; Corby-Harris et al. 2014a; Corby-Harris et al. 2014b; Tarpy et al. 2015; Maes et al. 2016; Anderson et al. 2018). *Bo. apis* is the most abundant bacterium on the queen mouthparts and in the midguts of healthy queens, indicating that this bacterium is non-pathogenic and may provide a protective effect, analogous to *Snodgrasella alvi* in the worker gut (Anderson et al. 2018; Saelao et al. 2020). *Bo. apis* can produce antifungal metabolites, which were shown to protect developing larvae against fungal infections (Miller et al. 2020). Exposure to propolis altered the worker bee mouth microbiome so that it more greatly resembled that of the queen mouthpart microbiome (Anderson et al. 2018), suggesting a beneficial social immune function of *Bo. apis* in a healthy honey bee colony.

Along with *Bo. apis*, there was a significant increase in the absolute abundance of the fructophilic bacterial species *Lactobacillus kunkeei* and *Fructobacillus fructosus* in the mouthpart microbiome of bees with a propolis envelope. This finding suggests that these three species have co-evolved together in the presence of highly antimicrobial hive components, such as royal jelly, honey, pollen, and propolis (Endo & Salminen, 2013; Anderson et al. 2014; Anderson & Ricigliano, 2017). As well as being putative core mouthpart microbiome species, each of these three bacterial species have demonstrated

antimicrobial activity. Feeding *Bo. apis* to worker bees was associated with a statistical decrease in Nosema spores—a microsporidian parasite that targets midgut cells (Corby-Harris et al. 2016. *L. kunkeei* displays antimicrobial activity against the bacterium *Melissococcus plutonius*—a causative pathogen of European foulbrood (Vásquez et al. 2012). *L. kunkeei* and *F. fructosus* both exhibit antimicrobial activity against the bacterium *Paenibacillus larvae*—the causative pathogen of American foulbrood (Endo & Salminen, 2013). Coupled with the demonstrated antimicrobial activity of *Bo. apis*, *L. kunkeei,* and *F. fructosus,* the addition of propolis to the hive environment could provide additional antimicrobial support to honey bees.

The abundance of *Bo. apis* correlated with a significant decrease in the overall mouthpart microbiome diversity (inverse Simpson values), suggesting that this species either drives the structure of the mouthpart microbiome or is better able to dominate the mouthpart niche because of the antimicrobial properties of propolis, or both. With the presence of a propolis envelope, the negative correlation of *Bo. apis* with *L. kunkeei,* and diversity in general was amplified (Figure 2.3). When we examined *Bo. apis* as a covariate in an ANCOVA model, the significant difference in diversity between the treatment conditions was lost. The significant negative association of *Bo. apis* with diversity, regardless of the independent variable (Figure 2.2), indicates that *Bo. apis* is the major microbial factor controlling bacterial diversity. This further supports a potential mechanism of propolis addition; propolis and *Bo. apis* may act synergistically to control bacterial diversity.

Although a few bacteria increased, most species of bacteria significantly decreased in the presence of a propolis envelope, including *Pseudomonas,*

*Pseudoalteromonas*, *Streptococcus*, *Serratia*, *Microbacterium*, *Propionibacterium*, and *Enterobacteriaceae*. *Pseudomonas*, *Pseudoalteromonas*, *Streptococcus*, *Microbacterium*, and *Enterobacteriaceae* are all genera that are found in a variety of organisms and environments world-wide and may be transient bacteria brought into the honey bee mouthpart microbiome from the pollination environment. Alternatively, many of these species may have a relatively permanent niche in honey bee colonies. For example, *Serratia marcescens* is a widespread opportunistic pathogen of many plants and animals. This well-known opportunistic bacterium was recently isolated from Varroa mites and from the hemolymph of dead and dying honey bees (Raymann & Moran, 2018). Genomic analysis revealed that this particular strain of *S. marcescens* was well-equipped for survival in honey bee hives, possessing genes not found in any other strain of *S. marcescens* (Burritt et al. 2016). This suggests that many bacteria are omnipresent in the hive environment (Anderson et al. 2013; Anderson et al. 2014; Anderson & Ricigliano, 2017), and further advocates for the generalized antimicrobial activity of propolis against transient or resident opportunistic bacteria in honey bee colonies.

**Conclusions**

We found that propolis refined the worker mouthpart microbiome, encouraging the growth of resident (core) commensal or beneficial bacteria and decreasing the relative abundance of putative opportunistic pathogens. The synergistic effect of propolis plus the dominant mouthpart bacterium suggests a variety of hypotheses, both evolutionary and ecological. This result adds to the paradigm of social immunity in honey bees, highlighting mechanisms of disease susceptibility and transmission. Enabling colonies to

enhance their own social immunity could be an asset to beekeepers and bee breeders alike. The maintenance of honey bee colonies in man-made hive boxes that do not promote the deposition of a propolis envelope within the nest cavity has occurred only relatively recently in the evolutionary history of managed honey bee colonies. The lack of a propolis envelope within managed colonies could allow for an increase in opportunistic and pathogenic microbes and present a challenge to the health of already stressed colonies. Encouraging colonies to deposit a natural propolis envelope, by using boxes with rough textured or unfinished interior surfaces, would result in multiple health benefits for the colony.

**Figures**

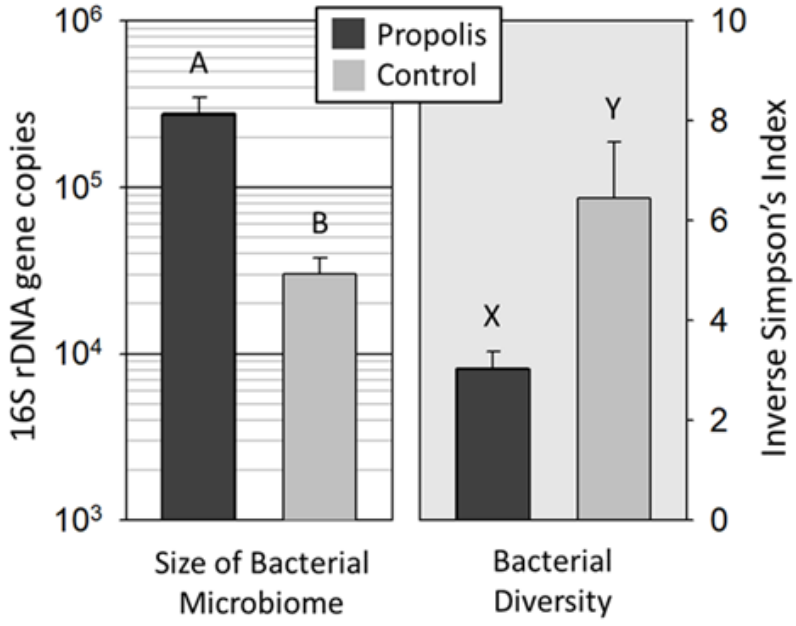


**Figure 2.1.** Propolis treatment increased the bacterial abundance but reduced the bacterial diversity. The total bacterial 16S rRNA gene copy number was significantly greater on mouthparts of worker bees in colonies with (A) a propolis envelope compared to the mouthparts of worker bees in colonies without (B) a propolis envelope. Whiskers depict the standard error of the mean. The Inverse Simpson's Index was significantly lower on the mouthparts of worker bees in colonies with a propolis envelope (X) compared to the mouthparts of worker bees in colonies without a propolis envelope (Y).

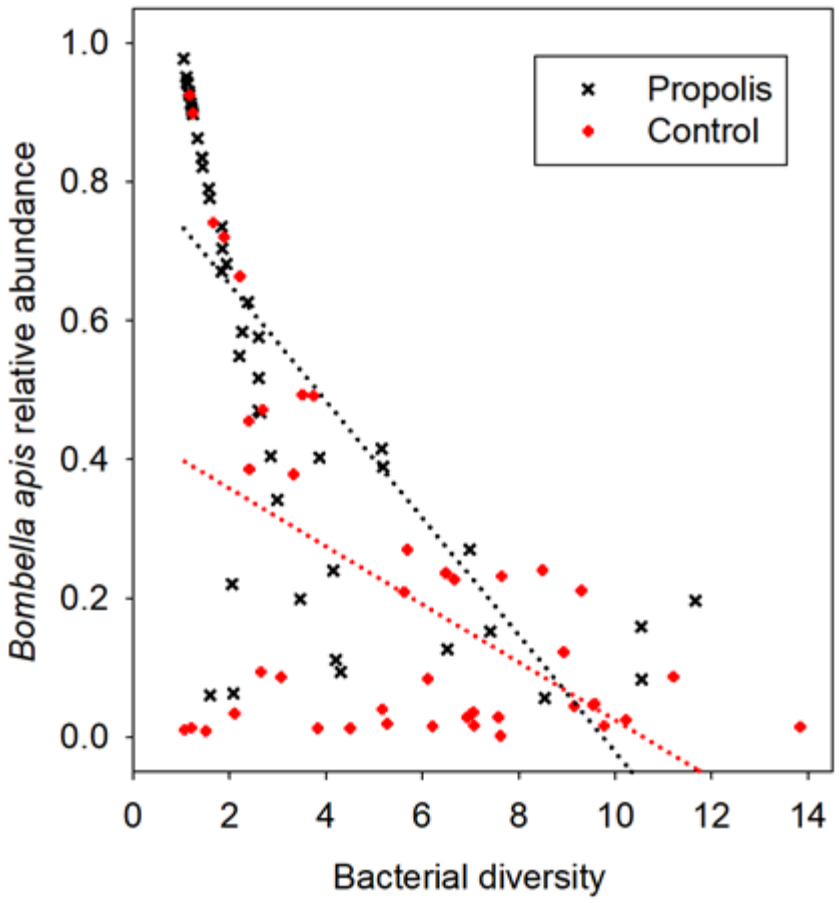


**Figure 2.2.** The primary effect of propolis in restructuring the mouthpart microbiome. The y-axis shows the variation in the relative abundance of the dominant mouthpart bacterium (*Bombella apis*). The x-axis is the total mouthpart diversity (inverse Simpson), where an increased value represents an increased diversity, and the different symbols represent propolis addition or no propolis addition (control). Considering both states, the relationship was significantly negative, but compared to the control, the relationship with added propolis explains significantly greater variation between the dominant bacterium and the diversity of the mouthpart microbiome. Linear regression of the relative abundance of *Bo. apis* with inverse Simpson values; propolis: adjusted R-sq = 0.5, $p < 0.0001$, and control: adjusted R-sq = 0.25, $p = 0.0003$.

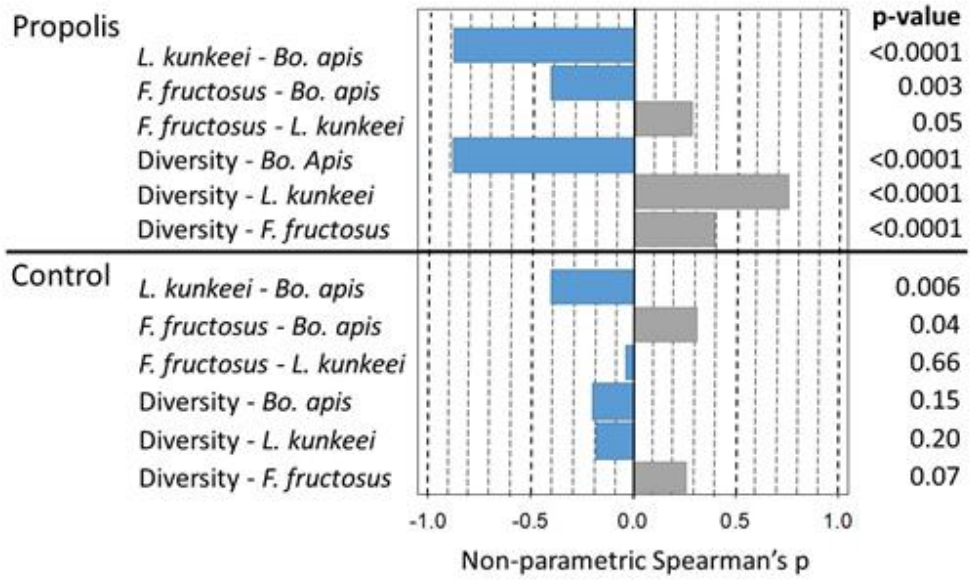


**Figure 2.3.** Spearman's correlations comparing treatments, the absolute abundance of operational taxonomic units (OTUs), and the microbiome diversity. Treatments are propolis and the control (no propolis). The pairwise species correlations depict a positive (gray) or negative (blue) association between the absolute abundance of each species. The mouthpart microbiome diversity is calculated as inverse Simpson's, which increases with numerical values. Therefore, positive correlations between species and diversity (in gray) depict a greater absolute abundance of species associated with an increased mouthpart diversity. Negative correlations (in blue) depict a lower species absolute abundance associated with an increased mouthpart diversity.

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## Appendix 1

### Supplementary Materials and Methods

The *Paenibacillus larvae* reference strain (NRRL #B-2605, ATCC 9545, LMG 9820 – ERIC type I) (de Graaf et al. 2006; Genersch et al. 2006) was obtained from the USDA Agricultural Research Service culture collection. Liquid cultures of *P. larvae* were grown overnight in a shaking incubator at 37°C in brain/heart infusion broth (BHI, Difco) fortified with 1 mg/L thiamine and diluted 1:100 with fresh BHI in each microplate well. Dilutions of propolis extract using 70% ethanol were added to 96-well microplates and dried to a solvent-free residue. Microplate wells contained 8, 10, 20, 30, 50, 60, 75, 100, 125, or 175 µg/ml of propolis, with 4 replicate wells per treatment and controls. The OD600 of each well at 0 h was subtracted as background. Total bacterial growth was measured as the optical density of the solution at an optical density of 600 run using a Synergy HTX Multi-Mode Microplate Reader (BioTek) after 6 h of shaking and incubation at 37 C, which is midway through the growth phase of *P. larvae* in our conditions. Relative bacterial growth for treated wells was calculated as the percent growth of untreated negative controls, with the absolute OD600 of untreated controls the $IC_{50}$ value was determined by growth curve analysis in JMP by fitting a four-parameter logistic equation, to the sigmoidal inhibition curves, whereas a = Growth Rate, b = Inflection Point, c = Lower Asymptote, d = Upper Asymptote: $c + \frac{d-c}{1+10^{(a*(b-concentration\ (\frac{\mu g}{mL})))}}$. The $IC_{50}$ value for the propolis sample used was 52.98 µg/ml, which was similar in activity to samples tested in Wilson et al. (2015). This result verifies

the antimicrobial character of the propolis treatment and concentrations used in this study.

# Appendix 2

## Introduction

This appendix contains supplemental data to the those presented in Chapter 1 and Chapter 2 with respect to measures of bacterial loads. Chapter 1 focused on the effect of propolis exposure on the general bacterial loads (extracted RNA) of whole bees from colonies and cages with and without propolis exposure using real-time qPCR (RT-qPCR) of 16S rRNA gene. Chapter 2 examined the general bacterial load (extracted DNA) of the mouthparts of bees from field colonies with and without propolis exposure in two ways: through RT-qPCR of 16S rRNA gene; and the bacterial OTU abundance and diversity calculated from DNA sequencing. The supplemental data presented here examines the general 16S rRNA bacterial load, using extracted DNA, of the mouthparts of bees from cages, in relation to bees from colonies, with and without propolis exposure as measured by RT-qPCR. Measures of OTU abundance and diversity of caged bee mouthparts bacteria are not included here.

The general hypothesis was that propolis exposure would reduce the general microbial load on bee mouthparts, and more specifically, that bees exposed to propolis in cages would show a lower general bacteria load compared to bees from field colonies due to the closer contact bees would have with propolis in cages than in large field colonies.

## Materials and Methods

The conditions of caged bees are summarized in Chapter 1. The mouthpart dissection and DNA extraction methods are explained in Chapter 2. The caged bee

mouthpart DNA was quantified using the same qPCR methods in Chapter 2 and the 16S rRNA is shown below in Figure 1. That DNA was sequenced at the University of Arizona Genomics lab, similar to the mouthpart DNA of colony bees in Chapter 2, but it was not yet analyzed. The Anderson lab at the USDA-ARS Carl Hayden Bee Research Laboratory has access to the DNA sequences and any preliminary analyses.

**Results**

Bees exposed to propolis had significantly lower 16S transcript abundance in the mouthparts specifically compared to bees not exposed to the propolis ($F_{1, 164} = 17.83$, $p < .0001$; treatment mean ± SE: PE+ = 13.07 ± 0.14; PE- = 12.21 ± 0.14). There was also a significant effect of housing, in which 16S rRNA transcript abundance was significantly lower in bees from colonies compared to bees from cages ($F_{1, 164} = 444.04$, $p < .0001$; housing mean ± SE: Colony = 10.51 ± 0.14; Cage = 14.76 ± 0.15). The interaction between propolis treatment and housing was significant $F_{1,164} = 17.93$, $p < .0001$. Tukey HSD post-hoc analysis revealed that 16S rRNA transcript abundance was significantly lower in bees exposed to propolis in field colonies ($p < .0001$), but not in cages ($p = 1.0000$) (Figure A.1).

**Discussion**

The data did not confirm either the general hypothesis about the effects of propolis on the microbial load of the mouthparts of bees held in cages, or the specific hypothesis that there would be lower general bacterial loads in the mouthparts of caged bees compared to bees from field colonies.

Bees from field colonies exposed to propolis had significantly higher bacterial loads compared to bees not exposed to propolis, as shown in Figure 1.5 and in Chapter 2.1. Bees that originated from the same field colonies but held in cages with propolis exposure until they were nine days of age showed no difference in bacterial load on the mouthparts compared to bees in cages without propolis (Figure A.1). The reasons for the lack of difference are not clear. It could be the mouthparts of bees with propolis exposure may have a different bacterial community, measured as OTU diversity, compared to those without propolis exposure, as was observed in bees from field colonies, summarized in Chapter 2.

Further experiments would be needed to understand why the general bacterial loads on bee mouthparts were so much higher in cages compared to colonies. Factors that are different between cages and colonies are summarized in Chapter 1, Table 1.1, and would include: the inability of bees to leave the cage to defecate, which could lead to fecal contamination, or the lack of a queen bee and larvae to feed, which would cause a buildup of hypopharyngeal gland secretions such as royal jelly.

**Figure**

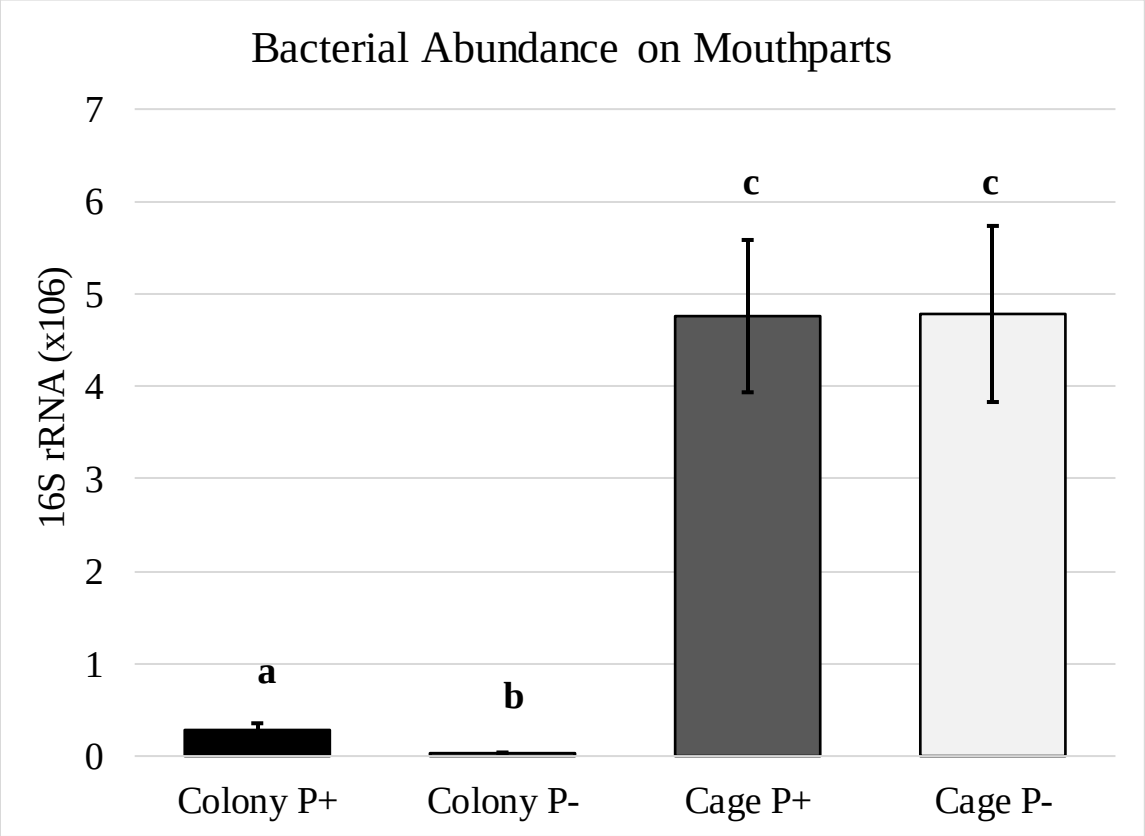


**Figure A.1.** General bacterial loads of mouthparts are significantly greater in bees from cages compared to bees from colonies. The independent variable was the average number of 16S rRNA copies, measured by RT-qPCR. Means +/- standard errors of expression of bacterial gene 16S, normalized to the Cycle threshold (Ct) with 16S rRNA standards on plasmids in *E. coli,* measured by RT-qPCR. Means with different letters are significantly different from each other at $p \leq 0.05$.