Colony-level immunity benefits and behavioral mechanisms of resin collection by honey bees

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Michael Dominick Simone

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Definitions and abbreviations

Nucleus colony — experimental colonies that contain four or five frames of comb, and so are approximately half the size of the typical managed honey bee colony

Propolis — the apicultural term for plant resins collected by honey bees, mixed with varying amounts of wax and used within a honey bee colony

Social immunity — the concept that individual behaviors of group members effectively reduce disease and parasite transmission at the colony level

AFB — American foulbrood, a larval disease caused by the bacterial agent *Paenibacillus larvae*

CB — Chalkbrood, a larval disease caused by the fungal agent *Ascosphaera apis*. Spores germinate in the larval gut and eventually the fungal mycelia penetrate the larval cuticle, turning infected larvae into fungal or CB mummies

PER — Proboscis extension response, which is used in a classical conditioning paradigm to train bees to various stimuli

SRT — Sucrose response threshold, which is the lowest concentration of sucrose that elicits an individual honey bee's proboscis extension response

Introduction

The general goal of this thesis is to understand the proximate and ultimate mechanisms of resin collection and use in honey bees, *Apis mellifera*. While there has been significant research on bee-collected resins with respect to human health and various chemical component analyses, this thesis provides the first review and studies on the direct implications of the role of resin in regard to honey bee health, and thus, pioneers a new area of research. I also provide novel information concerning the stimuli that may be involved in the recruitment of foragers and initiation of resin foraging. I tested original hypotheses that led to new questions and opportunities for further research that will be conducted by me and others for a long period of time.

This introduction briefly outlines the following sections of the thesis so that it reads as a cohesive unit. As Chapters 1, 2, 3 and 5 are currently published manuscripts, I will explain the role of my co-authors specifically in regard to these chapters.

Chapter 1 serves as the introduction to my thesis. This chapter was written as an invited review for a special issue of *Apidologie* on bee health. I reviewed the function of honey bee-collected resins related to colony-level immunity and disease resistance. Previous reviews on this subject have largely focused on the role of propolis for human health, so this was the first review focused on its significance to bee health. I also reviewed the natural history of resin use in honey bees and other social insects, which provides an effective introduction to this thesis. Although the published article was co-authored by my advisor, Dr. Marla Spivak, I was responsible for taking the lead in writing, figure design, and much of the work that resulted in its final state at publication.

Chapter 2 is the published manuscript from the *Journal of Invertebrate Pathology* that was the result of my first major experiment concerning the effect of propolis versus *in vitro* cultures of the bacterial agent of the larval disease American foulbrood (*Paenibacillus larvae*). This study tested the hypothesis that propolis would be effective against *P. larvae*, and that there would be differences in activity between samples collected throughout Brazil and those from Minnesota. This was the first study to directly test Brazilian propolis against a bee pathogen, as most research with this propolis type

focused on chemical properties for use in human health issues. The results of this study determined that extracts of Brazilian propolis were generally more effective against this bacterial pathogen as compared to propolis extracts from Minnesota, but that all samples exhibited some level of bacterial inhibition. While I took the lead on all data analysis and interpretation and authorship of the manuscript, the data used was from a combination of experiments conducted by our Brazilian collaborators under Dr. Esther Bastos and by me at the University of Minnesota. Both Dr. Bastos and I are listed as authors who contributed equally on the publication of the manuscript.

For Chapter 3, I investigated the potential of more subtle effects of propolis used within a honey bee colony. I used a novel approach to test the hypothesis that resins in a honey bee colony reduce individual investment in immune function, rather than directly affecting brood pathogens. To do this, I created resin-rich and resin-poor colonies then collected bees of known age from these colonies for analysis of immune gene transcript abundances using real-time PCR. My major finding was that adult honey bees exposed to a resin-rich environment invested less in individual immunity and this reduced investment was likely due to an associated decrease in general bacterial load in resin-rich colonies. The results of this experiment were published in *Evolution*, co-authored by Dr. Marla Spivak and our collaborator Dr. Jay Evans from the United States Department of Agriculture Bee Research Lab in Beltsville, MD. I conducted the real-time PCR analyses under the direction of Dr. Evans at his laboratory. I was responsible for experimental design, data analysis and interpretation, and preparation of the manuscript. This manuscript was featured by BBC Earth News and provided the first evidence that a component of the nest environment affects immune expression in honey bees.

The goal of Chapter 4 was to determine if resin collection is a constitutive or inducible behavior in regard to pathogen or microbial loads at the colony level, basically addressing the question of self-medication with resins. The topic of self-medication in the animal kingdom is somewhat controversial and only one other clear example has been described in insects. Evidence that individual honey bees may be recruited to increase resin collection due to colony-level pathogen loads would be particularly exciting. This

chapter presents data collected over three years of study. In 2008, resin and pollen foraging rates were monitored both before and after challenge with the fungal chalkbrood disease. In 2009, I expanded this experiment by creating colonies matched for population size, and then exposed them to either the bacterial pathogen American foulbrood, the fungal pathogen chalkbrood, or the entomopathogen *Metarhizium*. Again resin and pollen foraging rates were examined during the pre- and post-challenge periods. In 2010 I repeated the 2009 experiment with just the chalkbrood challenge. The findings suggest that resin collection may actually be induced by exposure to the chalkbrood, but not the other pathogens. This appears to be a novel and exciting case of self-medication among insects.

Lastly, Chapter 5 examined an entirely new line of research on resin use and collection in honey bees by aiming to better understand the behavioral physiology of resin foraging. The goal of this research was to understand at the most basic level what cues resin foragers may use to initiate foraging behavior. Much is known about the mechanisms behind pollen and nectar foraging, but virtually no information exists on resin foraging. This study examined the tactile and sucrose sensitivity of resin foragers as compared to pollen foragers using a conditioned learning assay (proboscis extension response). Learning rates and discrimination abilities were compared between resin and pollen foragers to two different types of tactile stimuli. The results of these experiments indicated that resin foragers are better able to learn tactile stimuli compared to pollen foragers, and suggest that assessment of tactile cues may be involved in the initiation of resin foraging. These findings were published in *Behavioral Ecology and Sociobiology*, co-authored by Joel Gardner and Marla Spivak. I was responsible for taking the lead in all aspects of experimental design, data analysis and interpretation, and manuscript preparation. Joel Gardner was the technician for this work and provided input on experimental methods. This study led to a host of new questions on this bee forager type, including the need to address how this increased sensitivity of resin foragers to tactile stimuli may influence behavior at the colony level.

Another experiment not included in this thesis expanded upon the findings of Chapter 3 and will be submitted for publication elsewhere. The experiment aimed to determine the influence of a resin-rich environment on colonies after exposure to a pathogen. I investigated the hypothesis that the reduced investment in immune function due to a resin-rich environment better enables honey bees to mount an individual defense against a pathogen, rather than propolis directly reducing pathogen loads. I maintained colonies matched for size, collected adult bees of known age from resin-rich and resin-poor colonies both before and after exposure to the fungal agent of chalkbrood disease. I analyzed these bees for levels of immune gene transcripts and compared them to bees from resin-rich and resin-poor colonies not exposed to chalkbrood. The preliminary results provided interesting and publishable information concerning the temporal changes in immune expression and the immune responses to fungal pathogens. The results, while somewhat unexpected, have opened up several avenues for future research on seasonal changes in immune investment and will be published in another format.

Overall my thesis provides the first evidence that resin collection is a form of social immunity in honey bees and may both have direct and indirect effects on individual immunity and colony health. I have also shed new light on the behavioral mechanisms that may be mediating this behavior at both the colony level (self-medication) and individual level (assessment of tactile information).

CHAPTER 1—

Propolis and bee health: natural history and significance of resin use by honey bees[1]

Social immunity, which describes how individual behaviors of group members effectively reduce disease and parasite transmission at the colony level, is an emerging field in social insect biology. An understudied, but significant behavioral disease resistance mechanism in honey bees is their collection and use of plant resins. Honey bees harvest resins with antimicrobial properties from various plant species and bring them back to the colony where they are then mixed with varying amounts of wax and utilized as propolis. Propolis is an apicultural term for the resins when used by bees within a hive. While numerous studies have investigated the chemical components of propolis that could be used to treat human diseases, there is a lack of information on the importance of propolis in regards to bee health. This review serves to provide a compilation of recent research concerning the behavior of bees in relation to resins and propolis, focusing more on the bees themselves and the potential evolutionary benefits of resin collection. Future research goals are also established in order to create a new focus within the literature on the natural history of resin use among the social insects and role that propolis plays in disease resistance.

1. INTRODUCTION

Social immunity, which describes how individual behaviors of group members effectively reduce disease and parasite transmission at the colony level, is an emerging field in social insect biology (Cremer et al., 2007; Cremer and Sixt, 2009; Wilson-Rich et al., 2009). This phenomenon is widespread across the social bees, ants, wasps and termites. The behaviors range from more common acts like grooming of nestmates (i.e. in termites, Rosengaus et al., 1998) and removal of dead material from the main nest area (i.e. in ants, Currie and Stuart, 2001; Hart et al., 2002) to "social fever" in honey bees that

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is used to kill pathogens (Starks et al., 2000) and the detection and removal of pre-infectious diseased or parasitized brood (hygienic behavior in honey bees; Rothenbuhler, 1964; reviewed in Wilson-Rich et al., 2009). Since social insects generally live in large groups of constantly interacting, related individuals, there is an increased risk of disease outbreaks and evolution of specialized parasites (Schmid-Hempel, 1998). In light of this, the finding that honey bee immune pathways have a decreased number of family members or paralogs as compared to other non-social insects with complete genomes was surprising, as it indicates that honey bees may have reduced individual mechanisms of physiological defense (Evans et al., 2006). It is interesting to consider the suite of behavioral mechanisms or other traits that may have evolved at the individual and colony levels to compensate for this (Evans and Spivak, 2010).

One possible mechanism of social immunity in honey bees is the collection and in-hive use of resins, complex plant secretions with diverse antimicrobial properties. Honey bees harvest resins from various plant species and bring them back to the colony where they are then utilized as propolis (propolis is an apicultural term for the resins when used by bees within a hive). The harvesting of antimicrobial compounds (resins) from the environment and their incorporation into the social nest architecture as propolis is an exciting but relatively unexplored colony-level defense against pathogens. Much of the current literature concerning propolis has focused on the chemical constituents and biological activity of propolis and the botanical origins of the resins from which the propolis mixtures are derived (see Banskota et al., 2001; Bankova et al., 2008). Although this work is certainly interesting due to the pharmacological benefits to humans that may be available by better understanding these compounds, we still remain largely unaware of the benefits of resin collection to honey bees and the basic mechanisms that drive resin foraging at both the individual and colony levels. This review provides a compilation of recent research concerning the behavior of bees in relation to resins and propolis, focusing more on bees themselves and potential evolutionary benefits of resin collection and not on chemical analyses of propolis and resins or implications for human health.

2. RESIN COLLECTION AND PROPOLIS USE BY HONEY BEES

Honey bees use propolis in varying degrees, some species and races rely very little on the substance, while others use resins and propolis extensively (Butler, 1949; Crane, 1990; Page et al., 1995). In fact propolis can be replaced by wax in honey bee colonies (Meyer, 1956; Crane, 1990). Colonies of *Apis dorsata*, the giant honey bee, may use resin occasionally to strengthen the site of comb attachment on a branch, while *A. cerana* colonies are not thought to use resins at all (Seeley and Morse, 1976; Crane, 1990). On the other hand, resins are thought to be essential to *A. florea* (the dwarf honey bee). To prevent ants from invading their exposed nests, *A. florea* places a ring of resin on the branches leading to a nest (Crane, 1990; Seeley et al., 1982). Very limited information exists on the use of resins by these Asian species of honey bees.

Use of resins by *A. mellifera* colonies is much more widespread. While there is considerable variation among colonies in resin collection and propolis use, all colonies do appear to use at least some (Seeley and Morse, 1976; Page et al., 1995; Manrique and Soares, 2002; M. Simone-Finstrom, pers. obs.). A feral colony nesting in a tree cavity coats the entire inner walls with a thin (0.3 to 0.5 mm) layer of propolis forming what has been termed a “propolis envelope” around the nest interior (Seeley and Morse, 1976; Fig. 1.1). Propolis is continually added to the nest walls during colony development, and is first placed at areas prior to comb attachment, which not only creates a clean, smooth surface, but may also reinforce new comb (Seeley and Morse, 1976; Visscher, 1980). Both feral colonies in tree cavities and domesticated colonies in commercial hive boxes, generally use propolis for covering holes and crevices in the nest, and narrowing the hive entrance (Huber, 1814; Haydak, 1953; Ghisalberti, 1979), which is evident from the origin of the word propolis (“pro”: in front of; “polis”: the city). Utilizing propolis in this manner is thought to function as a way for colonies to better maintain homeostasis of the nest environment. This could be a result of reducing microbial growth on hive walls, preventing uncontrolled airflow into the nest, and waterproofing walls against sap (if tree-cavity nesting) and external moisture, in addition to creating some protection against invaders (Seeley and Morse, 1976; Ghisalberti, 1979; reviewed in Visscher, 1980).

Because of the range of uses for propolis, it has been noted that propolis is essential to honey bees, particularly those in the wild (Haydak, 1953; Hoyt, 1965). However, domesticating bees has resulted in a reduction of propolis collection across races (Fearnley, 2001), likely because its use by bees often makes opening hives more difficult for beekeepers. Hoyt (1965) said that propolis "is the bane of a beekeeper's existence", so it is no surprise that apiculturists have selected lines that happened to produce less propolis.

Figure 1.1 A cross-section of a feral honey bee hive within a tree cavity found September 2009 in the residential area of Bloomington, Minnesota. The nest interior, where comb is present, is coated in a thin layer of propolis (plant resins mixed with wax) creating a "propolis envelope" around the colony. The upper portion of the cavity had not been lined with propolis, as the colony had not begun to use that space.

3. SIGNIFICANCE TO BEE HEALTH

Propolis is highly regarded for its medicinal properties for humans, especially in Eastern Europe, South America, and Asia. The antimicrobial properties of propolis against human pathogens have been known since antiquity (see Ghisalberti, 1979). A number of studies have presented evidence that propolis has strong hepatoprotective, antitumor, antioxidative, antimicrobial and anti-inflammatory properties (for recent reviews see Banskota et al., 2001; Sforcin, 2007; Viuda-Martos et al., 2008). Curiously, few studies have examined the antimicrobial properties of propolis against *bee* pathogens or on honey bee immune responses. Since much of the background on biological activity of propolis involves using propolis or components of propolis as treatments of disease, there has been a logical transition into studying propolis as a treatment to use in honey bee colonies (i.e. Samšináková et al., 1977; Garedew et al., 2004; Antúnez et al., 2008). However, there should also be a combined focus on the natural function of propolis, specifically determining if its presence in a honey bee hive either directly or indirectly affects pathogen and parasite loads. There is some evidence that it may both serve as a natural mechanism of disease resistance and have the potential to be further applied as an in-hive treatment. Here we describe completed research on the potential significance of propolis for bee health, and then discuss the future direction of this work.

3.1 American foulbrood

The majority of studies relating to the effectiveness of propolis against hive diseases have investigated propolis versus the bee pathogen *Paenibacillus larvae*, the causative agent of American foulbrood (Lindenfelser, 1967, 1968; Mlagan and Sulimanovic, 1982; Bastos, et al., 2008; Antúnez et al., 2008). This is largely because American foulbrood is a highly pathenogenic disease and in recent years has become resistant to conventional antibiotics (Evans, 2003). The main focus of this research has involved in vitro laboratory studies concerning the activity of a few propolis extracts against one or several strains of *P. larvae* grown in the laboratory. One recent study compared the antibacterial activity of a variety of propolis extracts from Minnesota and

southeastern Brazil and found that green propolis from Brazil (derived from *B. drancuncilifolia*) had significantly greater activity against *P. larvae* in vitro compared to propolis from north temperate Minnesota (derived largely from *Populus* spp.), but that both inhibited growth of *P. larvae* (Bastos et al., 2008).

Limited field studies have been conducted on the effects of treating colonies with propolis against this bacterial pathogen in colonies. Mlagan and Sulimanovic (1982) fed small, 1-frame honey bee colonies propolis extract in either an aqueous or alcohol solution. They determined that while both treatments reduced the total number of diseased larvae compared to a control colony, the propolis treatments in this manner would not be sufficient to eliminate the disease from the hive. Lindenfelser (1968) found similar results indicating that while propolis treatments may temporarily reduce the spread of disease it is not enough to cure colonies. A more recent study determined that feeding colonies ethanol extracts of propolis mixed into sugar syrup can reduce the amount of *P. larvae* spores found in colony honey stores (Antúnez et al., 2008). However, none of these colonies exhibited clinical symptoms of the disease during the course of the experiment; future experiments could determine if the reduced spore load in honey reduces pathogen transmission in colonies (Antúnez et al., 2008). Furthermore, honey bees do not appear to actively ingest propolis and it is unclear if an oral method of treatment would be at all effective against more severe infections.

3.2 *Varroa destructor*

V. destructor is currently the most damaging parasite affecting honey bee colonies, even with the current colony collapse issues (Cox-Foster et al., 2007; Johnson et al., 2009). Information on the effect of propolis against this parasitic mite has the possibility to shed light on the use of propolis as an in-hive treatment, but also on a possible natural benefit of propolis use by honey bees. A series of laboratory assays have shown that directly exposing mites held in petri dishes to relatively low concentrations of ethanolic propolis extracts caused high mortality (100% due to contact with 10% extract; Garedew et al., 2002). Furthermore exposure to extracts at concentrations as low as 0.5%

caused narcotic effects leading to reduced heat production and metabolic rates (Garedew et al., 2002, 2003). These effects could influence the ability of mites to cope with other stressors (i.e. temperature changes, Garedew et al., 2003) or to successfully infest larval cells and maintain the normal course of the parasite's population growth. Taking these findings into a field setting, we have been conducting an ongoing study on the effects of propolis on the reproductive success of *V. destructor* in field colonies. Colonies with experimentally controlled mite levels were treated by painting the inside walls with propolis extracts (collected from colonies in Minnesota and Brazil) or by spraying empty combs with the propolis extract. Frames containing developing pupae infested with the parasitic mites were removed and percent infestations and reproductive success of mites within the cells were calculated. Preliminary results (M. Simone-Finstrom and M. Spivak, unpubl. data) suggest that the propolis treatments may reduce the number of mature females produced within a single cell. Altering this level of reproductive output would effectively reduce population growth of the parasites within a colony and hopefully reduce the need for the more caustic chemicals currently utilized as treatments against *V. destructor*. Furthermore, as a single propolis sample can contain up to 300 chemical components (i.e. Salatino et al., 2005), it may be more difficult for the mites to develop resistance against a suite of combined compounds.

3.3 Other large parasites and pests

Honey bee colonies also must defend themselves against a number of larger parasites and pests. Two studies have examined the effectiveness of propolis extracts against the greater wax moth, an opportunistic parasite that mainly affects weakened hives (Johnson et al., 1994; Garedew et al., 2004). In laboratory experiments similar to those conducted with *Varroa*, propolis extracts caused larval mortality and reduced metabolic rates of wax moth larvae and adults (Garedew et al., 2004). The implication here is that contact or possibly volatile emissions from propolis may reduce the ability of the moths to effectively reproduce and develop within a hive.

With respect to other large invaders, Cape honeybees, *A. m. capensis*, have been

observed encapsulating the parasitic small hive beetle, *Aethina tumida*, in "propolis prisons" which serves to prevent the beetles from successfully reproducing (Neumann et al., 2001). The European honey bee, *A. mellifera*, will also embalm other intruders that are presumably too large to remove from the nest after being killed; Hoyt (1965) observed a mouse encased in propolis and suggested that the bees covered it in propolis to prevent odor and decay from affecting the rest of the hive (Fig. 1.2). Colonies of *A. dorsata* have also been noted to coat foreign objects in propolis (Seeley and Morse, 1976), as have the stingless bee *Trigona carbonaria* that "mummify" beetle parasites alive using a mixture of wax, plant resins and mud (also known as batumen; Greco et al., 2010). It may be that this behavior of embalming predators or parasites may be a relatively widespread phenomenon among the social bees. Particularly with respect to this entombment behavior, the use of propolis by bees can be described analogously to individual immune function. If we consider a honey bee colony as one entity or "superorganism", then this behavior would be equivalent to cellular encapsulation of foreign microbes or parasites seen at the individual level (see Cremer and Sixt, 2009). The propolis envelope itself, also fits into this analogy as it is a type of mechanical barrier to both reduce parasites from entering the nest (or superorganism) and potentially prevents parasites and microbes from developing once inside (i.e. Simone et al., 2009).

Figure 1.2. A mouse skull that was encased in propolis found within a honey bee colony in an apiary of the University of Minnesota. If a colony intruder has been killed within the hive and is too large for the bees to remove, they will embalm it using propolis to prevent the corpse from decaying.

3.4 Social immunity

Recent evidence indicates that propolis in honey bee colonies may play a more subtle role in colony level immunity than direct defense against parasites and pathogens. In 2007 we conducted a field study to determine how a resin-rich environment affects immune-gene expression in honey bees (Simone et al., 2009). Colonies were experimentally enriched with propolis by painting the interior walls with extracts of either Brazilian green propolis or propolis collected from Minnesota. Age-marked bees were collected from these colonies and analyzed for immune-gene expression using real-time PCR. We found that 7-day old bees from propolis treated colonies had significantly lower expression of two immune-related genes as compared to bees collected from control colonies (hymenoptaecin, an antibacterial peptide, and AmEater, a gene involved in cellular immunity). Furthermore, this reduction in immune expression appeared to be due to a reduction in the overall bacterial loads of these colonies, as determined by 16S eubacterial gene transcript levels. This finding was significant because an elevated immune response has an associated fitness cost, as demonstrated by reduced colony productivity in honey bees (Evans and Pettis, 2005) and decreased individual survival in bumble bees (Moret and Schmid-Hempel, 2000). This was also the first study to document effects of a component of the nest environment on individual immunity in honey bees. The results of this study support those of previous laboratory studies on another social insect, the wood ant *Formica paralugubris*, which collects and distributes resin from spruce trees throughout its nest material. Soil collected from ant nests experimentally enriched with this resin had significantly fewer pathogenic bacteria and fungi (Christe et al., 2003), and adult ants in colonies experimentally made resin-rich did not invest as much in individual immunity (indicated by decreased antibacterial ability of the hemolymph) compared to ants in resin-deprived colonies (Castella et al., 2008a), which resulted in increased survival during a pathogen challenge (Chapuisat et al., 2007). Therefore, as with *F. paralugubris* ants, it is likely the presence of propolis in a honey bee colony may reduce the investment in the innate immune response by acting as an external immune defense mechanism.

3.5. Self medication

In light of all of this information, one obvious question concerns the idea of "self-medication." Resin collection may be constitutive (i.e., collected regardless of physiological demand or pathogen level) or inducible (i.e., a conditional response to infection; Schmid-Hempel and Ebert, 2003). If it is inducible, it might be considered a form of self-medication, defined as the "defense against [pathogens and] parasites by one species using substances produced by another species" (Clayton and Wolfe, 1993). There are number of vertebrates that self-medicate by ingesting, absorbing, topically applying or living in proximity to plants with medicinal compounds (reviewed in Clayton and Wolfe, 1993; Lozano, 1998). Examples of self-medication in the insect literature, particularly with respect to social insects are less common. When *F. paralugubris* ant colonies were challenged with the fungal pathogen *Metarhizium anisopliae*, they did not respond by increasing the rate or quantity of resin collection, and the authors concluded that the use of resin by this species was a constitutive rather than inducible response, and therefore not an example of self-medication (Castella et al., 2008b). Honey bee colonies infected with diseases or parasitic mites do not appear to respond by collecting more resin (M. Simone-Finstrom, M. Spivak, pers. obs.) but studies to quantify resin collection after pathogen challenge are ongoing (see Chapter 4). The trade-off between the energetic costs to individual bees of collecting resin may have been offset by the antimicrobial properties of the resins which benefited the individuals' immune systems and increased colony fitness, leading to continued selection for resin collection regardless of pathogen or parasite levels.

3.6. Future studies on bee health

There have been a number of studies on the effectiveness of propolis against bacterial pathogens. Further studies should be conducted with respect to propolis against hive diseases both alone and in combination with other disease resistance mechanisms (i.e. hygienic behavior) to better determine how valuable propolis could be as a direct treatment. In Europe, there are currently plans to study how propolis may be used against

bee pathogens and parasites as a form of treatment (see Moritz et al., 2010). Research at the University of Minnesota currently underway has a similar, but more specific focus. We are completing a series of studies to identify specific fractions and components of propolis extracts that are active against the bacterial agent of American foulbrood and common honey bee viruses (M. Wilson, J. Cohen, G. Gardner, J. Burtness, M. Spivak, unpubl. data). Propolis extracts in general have been shown to be active against some human viruses in vitro (i.e., HIV-1, Gekker et al., 2005), and the results of this work on honey bee viruses could have implications for human health by identifying possible compounds for further study.

Future research should also be directed toward the natural use of propolis by honey bees as a disease resistance mechanism. Propolis will occasionally be used for tasks other than smoothing hive walls and reducing entrances. Huber (1814) observed honey bees embedding strands of propolis in cleaned and polished cells. Ribbands (1953) believed that bees used propolis in this manner to prevent disease transmission when reusing cells. It is unclear how common this behavior is, but at least feral colonies can be found with propolis on the rims of cells (Fig. 1.3). Recent evidence also indicates that honey bees may “entomb” chemically contaminated pollen in cells with propolis, but the frequency of this behavior and subsequent e ect on colony health is currently unclear (vanEngelsdorp et al., 2009).

It is possible that the antimicrobial properties of materials used and stored in combs (e.g. royal jelly, honey) are enhanced by the addition of propolis (Visscher, 1980; Tautz, 2008). In particular, the modes of action of propolis against microbes and parasites are currently unknown and could be due to contact (e.g. Garedew et al., 2002) and/or volatile emissions (e.g. Messer, 1985). The two modes are not necessarily mutually exclusive and could have varying effects depending on the organism, and must be considered when investigating the use of propolis both as a colony treatment and its natural effectiveness in the hive. Additionally the persistence of the activity of propolis in the hive is something that needs to be known, particularly if the goal is to utilize propolis for colony treatments.

While knowledge concerning the role that propolis plays in disease resistance in honey bee colonies is growing, studies are needed on the behavioral ecology of resin collection to fully understand how it can impact bee health. We need more information concerning the mechanisms of resin collection and use as propolis within the colony, and the regulation of resin foraging, both at the individual and colony levels. The remainder of this review will focus on these issues, discussing what is currently known and identifying major areas for future study.

Figure 1.3. Particularly in feral colonies nesting in tree cavities, honey bees secure the site of comb attachment on the hive wall with propolis. In some cases, as can be seen here (same feral colony as in Fig. 1.1), the rim of comb cells will also have a thin coating of propolis. The function and significance of this behavior is currently unknown.

4. SOURCES OF RESIN AND THE PROCESS OF RESIN COLLECTION

In tropical climates honey bees mostly collect resins from *Clusia minor* and *Clusia rosea* flowers and from alecrim plants (e.g. *Baccharis dracuncufolia*), which is similar to other tropical bee species (Pereira et al., 2003; Salatino et al. 2005). Recently a leguminous species (*Dalbergia* sp.) has also been identified as a common source in tropical regions (i.e. Silva et al., 2008). In temperate climates poplar trees (*Populus* sp.) appear to be the primary source for resins (Popravko and Sokolov, 1980; Nagy et al., 1986; Greenaway et al., 1987; Bankova et al., 1992; 2006; Markham et al., 1996; Salatino et al., 2005). However, it is clear that other trees, like pine, birch, elm, alder, beech and horse chestnut species, are adequate resin sources for temperate honey bees, particularly when poplar species are unavailable (Alfonsus, 1933; Ghisalberti, 1979; Crane, 1990). Additionally, honey bees in Uganda appear to forage for resins selectively on *Alnus* sp. and can actually defoliate these trees; whether there are other possible sources in the region remains unclear (Nyeko et al., 2002).

Honey bees will forage for resins from droplets appearing on the bark of the trunks or limbs of trees (Alfonsus, 1933), from the surfaces of some fruits (i.e. *Macaranga tanarius*; Kumazawa et al. 2008), or more typically on the vegetative apices (buds, leaf primordia and young leaves). The bees must extract the resins from the trichomes and ducts by fragmenting these early leaves using their mandibles (Meyer, 1956; Nyeko et al., 2002; Teixeira et al., 2005). Resin foragers have shown a preference for young leaves and vegetative buds over more expanded leaves (Park et al., 2004).

The cues that honey bees rely on to find resinous plant sources are currently unknown. Huber (1814) placed a bunch of poplar branches “that had very large buds coated both on the outside and inside with a viscous, reddish and odoriferous sap” in front of his honey bee colonies and observed bees collecting resins within 15 minutes. It is clear that foragers select specific sources, and rely on currently unknown cues. Honey bees have been observed probing the apex of one plant with their antennae then moving to another one, probing it and subsequently collecting resin from it (Teixeira et al., 2005). The same study also provided evidence that the resin foragers preferred female versus

male *Baccharis dracuncufolia* as resin sources. The young leaves and buds have a similar chemical composition that changes as the leaves become more expanded (Park et al., 2004), which implies that there may be a chemical cue released by the resin source that the foragers are able to detect.

Once the bees find the resin source, they then have to collect it. Huber (1814), Haydak (1953), and Meyer (1956) have described this process in great detail. There are four basic steps (taken from Meyer, 1956) that a resin forager follows to pack her corbicula: (1) Break off a particle of propolis with the mandibles; (2) work it with the mandibles and take it with the forelegs; (3) transfer it from the forelegs to the middle leg; (4) transfer it from the middle leg to the corbicula on the same side. This sequence is repeated until there is a full resin load on both corbicula (see Fig. 1.4). No corbiculate bees can collect resin and pollen during a single foraging trip because of this behavior (Armbruster, 1984; Roubik, 1989). After completing the four steps, bees have been observed flying around for a few seconds above the resin source, then landing again to add more to each corbicula (Alfonsus, 1933; Haydak, 1953). The purpose of these flights is unknown but may be used to assess the weight of the current corbicular load. The process of obtaining a full corbicular load of resin has been noted to take about seven minutes (Teixeira et al., 2005; Kumazawa et al., 2008), but can take from 15 min to an hour depending on the weather (Haydak, 1953).

Once the bee has a full load, she returns to her colony to unload the resin from her corbiculae. The unloading process typically takes approximately 15 minutes, but can extend from one to seven hours or even overnight (Alfonsus, 1933; Haydak, 1953; Ratnieks and Anderson, 1999; Nakamura and Seeley, 2006). A resin forager cannot unload her corbiculae herself, but rather must rely on her nestmates to take the resins off of her. Once the resin forager returns with a full load, she will go to a site within the hive where propolis is needed, where she waits until other bees, known as cementing bees, bite off chunks of resin from her corbiculae (Betts, 1921; Alfonsus, 1933; Haydak, 1953; Meyer, 1956; von Frisch, 1993; Nakamura and Seeley, 2006). Cementing bees immediately attach the resin to a site along the hive wall. The cementing bee then

smoothes the resin, now officially propolis, with her mandibles in a manner that is similar to that of wax construction (Alfonsus, 1933; Nakamura and Seeley, 2006). The resins may also be placed in a storage area where bees can grab chunks of propolis to later place in comb cells or other areas (Huber, 1814; Haydak, 1953; Fearnley, 2001; Tautz, 2008). Many of the few resin foragers in a colony will perform cementing behavior, but not all cementing bees will forage for resins (Huber, 1814; Meyer, 1956; Nakamura and Seeley, 2006). Meyer (1956) found that forager-aged bees with atrophied wax glands do most of the cementing work. Recent evidence from Nakamura and Seeley (2006), however, indicated that the bees they observed using resin in the nest performed these behaviors prior to foraging. This suggests that cementing and other in-hive resin activities are performed by the middle-aged bees that typically perform nest construction tasks in addition to those bees foraging for resins.

Figure 1.4. As a resin forager returns to the nest with a load of resin on her corbicula, they go deep into the hive at a “cementing” site, where the resin will be used. Pictured in a colony located at the University of Minnesota, a resin forager within a hive must wait for other bees to remove the resin from her hind legs. Propolis is used in this area and can be seen here on the tops of the two frames.

During the cementing process, the resins do not appear to be chemically modified. While there is some evidence that the general chemical profiles of resins collected directly from a forager and in-hive collected propolis can vary slightly from the leaf buds of the plant source (i.e. Ghisalberti, 1979; Peev et al., 2009), it is likely that some variation could occur to due volatilization of some chemicals during the course of the return foraging trip. In addition, propolis sampled from a single colony likely contains an amalgam of various sources at least to some degree in addition to wax and is essentially a concentration of some of the compounds collected directly from the plants. Thus, some compounds would be expected to be more or less represented in propolis samples, but the general chemistry would remain similar as has been found (i.e. Greenaway et al., 1990; Park et al., 2004; Teixeira et al., 2005; Vardar-Ünlü et al., 2008). For other bee species, however, there is some suggestive evidence that bees add secretions to the resins. Workers of the stingless bee *Plebeia emerina* reach maximum development of the head and intramandibular glands during the age of most frequent resin handling, which may be utilized to maintain the viscosity of resins during use (dos Santos et al., 2009). How this may change the chemical properties of the resins has yet to be investigated.

Honey bee resin foragers follow a fairly strict diurnal pattern in foraging and cementing behaviors. Foraging for resins is typically observed between 10 am and 3:30 pm on sunny days, likely due to the increased pliability of resins at higher temperatures (Alfonsus, 1933; Meyer, 1956; Hoyt, 1965; Nyeko et al., 2002). Cementing behavior occurs most often in late afternoon with the foragers participating in the behavior once their loads have been removed (Meyer, 1956; Ratnieks and Anderson, 1999). Additionally while resin foragers can almost always be found from May through November in temperate regions (Crane, 1990), there appears to be some seasonality in resin collection and propolis use. Resin is said to be collected most frequently in late summer (end of June) through autumn when the honey flow is greatly reduced (Alfonsus, 1933; Meyer, 1956; Crane, 1990). Meyer (1956) hypothesized that more regular propolis collection in late summer and early fall is the result of a seasonal change in foraging behavior and not the result of climatic changes or the need to prepare the hive for winter,

as has been suggested (Ghisalberti, 1979). This idea is supported by the fact that honey bees can be induced to collect resin during any part of the season (Butler, 1949; Meyer, 1956; M. Simone-Finstrom, pers. obs.). However, it is also likely that higher levels of resin collection late in the season are due to the reduced nectar flows, as resin foragers are not necessarily committed to resin collection for their foraging lives. During periods of greater nectar and pollen availability a resin forager may be more motivated to forage for pollen and nectar depending on colony need. Nakamura and Seeley (2006) found that while resin foragers did not switch to other resources through the course of a single day in September, 33% switched to either pollen or nectar on subsequent days.

5. STIMULI INVOLVED IN RESIN FORAGING

In general social insect foraging has been studied extensively both in relation to the individual mechanisms involved as well as issues related to division of labor and task allocation within a colony. Although there is abundant research on the regulation of foraging behaviors at both the individual and colony levels with respect to pollen and nectar (reviewed in Page and Fondrk, 2004; Hunt et al., 2007), the behavior of foragers collecting nest-building supplies like resins is understudied. It appears that there are bees within a colony that are specialized to forage for resin (Meyer, 1956; Ranger and O'Donnell, 1999; Nakamura and Seeley, 2006) and that this likely has a genetic component similar to that of pollen and nectar specialized foragers. However the cues that resin foragers use to both initiate foraging and find a resin source are virtually unknown. One leading hypothesis is that volatile compounds released from the resin play a large role in locating resins (Armbruster, 1984; Roubik, 1989; Bankova et al., 2000; Patricio et al., 2002; Teixeira et al., 2005), though it has yet to be investigated.

5.1. Regulation at the individual level

Individual bees may detect the need for resin and then use communication signals (e.g., waggle dances, trembling) inside the nest to recruit nest mates to forage for it, as they do to recruit nest mates to food resources. The cues bees use to detect the need for

resin may be the presence of gaps, crevices or irregularities in the nest architecture that may allow the entry of microbes, intruders, air currents and sunlight (Butler, 1949; Ribbands, 1953; Seeley and Morse, 1976; Crane, 1990). Since the nest interior is completely dark, bees must rely on non-visual senses to detect stimuli within the nest environment. The bees' antennae are an integral tool for this type of information assessment (Erber and Pribbenow, 2001; Johnson, 2008). Bees, and specifically some resin handlers and foragers, have been noted to detect crevices by inserting the antenna into gaps in nest architecture (Nakamura and Seeley, 2006).

We have started to investigate whether resin foragers are more sensitive to certain stimuli as compared to other foragers to begin to understand what stimuli resin foragers may detect in order to initiate foraging behaviors. We have conducted a series of experiments using proboscis extension conditioning response to determine if resin foragers are able to learn tactile stimuli more effectively than pollen foragers (for general methods see Erber et al., 1997). Using this technique, we found that resin foragers are better able to learn a tactile stimulus (a gap between two metal plates) and may be better able to distinguish between two other tactile stimuli (rough sandpaper or smooth paper) as compared to pollen foragers (M. Simone-Finstrom, J. Gardner, M. Spivak, unpubl. data). These differences were not due to a greater ability of resin foragers to learn all stimuli, as resin and pollen foragers equally learned the odor geraniol. While this data is merely suggestive of the possible stimuli that may be involved in initiating resin foraging, it provides a general starting point for future research to examine how resin foragers assess information related to initiating their task. Use of tactile information for initiating nest construction tasks holds true for other social insects, like some species of paper wasps (*Polistes fuscatus*) and termites (*Nasutitermes costalis, Coptotermes formosanus*) that have been noted to detect nest damage and determine building sites using antennation (Jones, 1980; Downing and Jeanne, 1990; Lee et al., 2008).

5.2 Colony-level organization of resin foraging

A recent study by Nakamura and Seeley (2006) documented detailed observations

of both resin foragers and cementers (bees that manipulate resins in the hive) in order to understand how resin foraging is regulated at the colony-level. They proposed two hypotheses, and neither proved to be mutually exclusive: (1) the "unloading difficulty hypothesis", which proposes that individuals determine resin need based on how long it takes another bee to help them remove the resin from their legs; and (2) the "caulking activity hypothesis", which states that resin foragers manipulate resins within the hive and thus sense the need to forage based on available caulking sites. Of 77 resin foragers monitored closely, 26% performed tremble dances, which appeared to function as a signal to stimulate other bees to handle resin within the nest (Nakamura and Seeley, 2006). Trembling by resin foragers appears to be an unloading signal similar to how it stimulates nest bees to receive and store nectar from nectar foragers (Seeley et al., 1996). In addition, 8% of the 77 resin foragers and cementers performed "crevice-detecting" behavior, which was defined as "walking along crevices and inserting the antenna into them" (Nakamura and Seeley, 2006, p. 340). Based on their findings, they surmised that resin foraging is a "demand-driven" process in response to sensing the need for it.

After resin foraging has been initiated by one or several bees, it is possible bees use waggle dances as a colony-level recruitment signal, in a similar way to how pollen and nectar foragers use dances as communication signals to recruit other foragers to their food sources. Nakamura and Seeley (2006) found that 26% of the 77 observed resin foragers performed dances near cementing sites deep within the hive (unlike pollen and nectar dances, which are done near the hive entrance). Waggle dances by resin foragers have also been observed near cementing sites by Milum (1955), Meyer (1956) and von Frisch (1993). However the purpose and subsequent effect of these dances is unknown, and could simply be a vestige of more general foraging behaviors.

One way to better address questions concerning the mechanisms of resin foraging would be to maintain a line of bees selectively bred that consistently collects large quantities of resin and a corresponding line that consistently collects little resin. Research on lines of bees bred for hygienic and non-hygienic behavior (i.e. Spivak, 1996; reviewed in Wilson-Rich et al., 2009 and Evans and Spivak, 2010), and for high- and low-pollen

hoarding (e.g. Page and Fondrk, 1995) has been instrumental in uncovering a host of information on the genetic mechanisms regulating honey bee social behaviors.

6. TOPICS TO CONSIDER

Resins are produced by a large variety of plants across taxa worldwide. Bees around the globe collect and utilize resins as propolis for a number of purposes, including sealing cracks in the nest, creating smooth surface for comb attachment, entombing parasites and predators, and reducing in-hive microbes. We are currently at the fringe of understanding all the facets involved in this process.

While investigations on the chemical components of propolis are currently growing at an almost exponential pace, there is still a host of information lacking from our knowledge base. In particular, one area of special interest to the beekeeping community is the presence of contaminants in hive products, like propolis (i.e. Bogdanov, 2006). While commercial hives are often given a variety of chemical treatments to control various hive diseases and parasites, investigations into the residues that these may leave behind are relatively new. There is limited evidence that acaricides can occasionally be found in propolis collected from a hive (Bogdanov et al., 1998; Wallner, 1999), as well the antibiotic (tylosin) used to treat the bacterial diseases American foulbrood and European foulbrood (2 of 30 samples from China had detectable amounts; Zhou et al., 2009). Similarly low levels of pesticide residues likely from treatments on the plant sources have been detected in some propolis samples (Chen et al., 2009), but not in others (Santana dos Santos et al., 2008). Further study on the frequency and abundance of these chemicals in propolis samples needs to be conducted as well as the possible antagonistic effect that these compounds could have on the chemical constituents of propolis or possibly the synergistic effects that the residues have with those chemicals found in wax, honey and pollen stores (i.e. Frazier et al., 2008; Johnson et al., 2010).

The vast majority of current studies related to propolis, however, tend to focus on chemically identifying propolis components, while incorporating descriptions of the biological activity of samples. These studies are currently being conducted on samples

collected globally and will not only provide some comparative information on the activity of propolis from varying regions and ecosystems, but will also help to narrow focus on identifying specific components and mixtures of components required for activity against various microbes and parasites. While we know that biological activity can often be correlated with phenolic content (i.e. da Silva et al., 2006; Popova et al., 2007; reviewed in Bankova et al., 2008; Viuda-Martos et al., 2008), it is currently unclear if these compounds work in synergy with other compounds or if some are antagonistic. Both scenarios are likely. Additionally, in some cases, biological activity has been shown to be equivalent regardless of the race of the honey bee, geographical region or season, even though the chemical profiles may be dissimilar (i.e. Kujumgiev et al., 1999; Sforcin et al., 2000; Silici and Kutluca, 2005; Silici et al., 2007). However, many other studies have shown that, while propolis is generally active against most gram-positive bacteria and some fungi, the level of activity depends on location, likely due to differences in plant sources (i.e. Popova et al., 2007; Seidel et al., 2008; Chaillou and Nazareno, 2009). In particular, it has been hypothesized that wet tropical and lower latitude ecosystems may have plants with generally higher levels of antimicrobial compounds or at least access to a larger variety of plants that may differ in antimicrobial properties, and would thus lead to increased biological activity of propolis samples from those climates (Popova et al., 2007; Seidel et al., 2008).

The widespread use of resins by the various tropical bee species also should be investigated in greater detail. It is well known that many tropical euglossine, meliponine and megachilid bees use resins for nest construction (Armbruster, 1984; Roubik, 2006). These bees often mix resins with clay, soil and wax to form the nest itself and its supporting structures (Roubik, 1989). When resins are amalgamated with soil or clay material the resulting mixture is often termed geopropolis or batumen, whereas when it is only mixed with wax it is simply called propolis or cerumen with respect to non-honey bee species (Barth, 2004; Roubik, 2006). It is also apparent that some bees utilize these resin mixtures as protection against predators, like ants (Seeley et al., 1982; Roubik, 1989; Patricio et al., 2002; Lehmberg et al., 2008). One interesting case of resin

collection among tropical bees involves *Chalicodoma pluto*, which uses resins and wood to construct nest cells and tunnels. A *C. pluto* female harvests resin from vertical trunk fissures by loosening it with her large mandibles then scraping it off with her specialized, elongate labrum (Messer, 1983). In this case it appears that the bee has a specialized morphological feature to collect and use this necessary resource; however in other cases it may be the resin source that has specialized to attract bees. Flowers of *Dalechampia* sp. and *Clusia* sp., which are visited by a host of tropical bees, are thought to produce resins as a pollinator reward instead of nectar (Armbruster, 1984; Gonçalves-Alvim, 2002; Salatino et al., 2005). *Trigona pallens* specifically has been noted to be attracted to the resin-producing flowers of *Clusia odorata*, and other species may also be utilizing this resin source (Armbruster, 1984). Since resin is essential nesting material for a number of tropical bees it is likely that some plants evolved mechanisms to produce resins that attract pollinator species. In terms of the evolution of the behavior it would be important to understand if these bees are simply exploiting this commonly available resource in tropical climates for nest construction or if there are some other, possibly health-related benefits for nesting with resins. Limited studies have been done on the biological activity of tropical bee resins, and all have shown that, similarly to honey bee propolis, these samples are generally biologically active against gram-positive bacteria at the least (Lokvam and Braddock, 1999; Farnesi et al., 2009). The possibility that resin use as a mechanism of disease resistance is a widespread phenomenon across the social insects is certainly a topic that should warrant future study. It is not known if resin collection evolved several times among the ants and bees. The evolution of these behavioral disease resistance mechanisms is also currently unknown.

There are clearly a multitude of options for future research related to propolis and resin use by bees, ranging from the pharmacological opportunities for human health to understanding individual and colony-level mechanisms of resin foraging to the possible applicability for propolis as a treatment against bee pathogens. At the least, information on resin use and its incorporation into honey bee nest architecture is an exciting area of study concerning environmental impacts on disease resistance and social immunity.

CHAPTER 2—

An *in vitro* study of antimicrobial activity of propolis against *Paenibacillus larvae* [2]

The honey bee disease American foulbrood (AFB) is a serious problem since its causative agent (*Paenibacillus larvae*) has become increasingly resistant to conventional antibiotics. The objective of this study was to investigate the in vitro activity of propolis collected from various states of Brazil against *P. larvae*. Propolis is derived from plant resins collected by honey bees (*Apis mellifera*) and is globally known for its antimicrobial properties and particularly valued in tropical regions. Tests on the activity of propolis against *P. larvae* were conducted both in Brazil and Minnesota, USA using two resistance assay methods that measured zones of growth inhibition due to treatment exposure. The propolis extracts from the various states of Brazil showed significant inhibition of *P. larvae*. Clear dose responses were found for individual propolis extracts, particularly between the concentrations of 1.7 and 0.12 mg propolis/treatment disk, but the source of the propolis, rather than the concentration, may be more influential in determining overall activity. Two of the three tested antibiotics (tylosin and terramycin) exhibited a greater level of inhibition compared to most of the Brazilian samples, which could be due to the low concentrations of active compounds present in the propolis extracts. Additionally, the majority of the Brazilian propolis samples were more effective than the few collected in MN, USA. Due to the evolution of resistance of *P. larvae* to conventional antibiotic treatments, this research is an important first step in identifying possible new active compounds to treat AFB in honey bee colonies.

1. INTRODUCTION

The honey bee disease American foulbrood (AFB), caused by the bacterium *Paenibacillus larvae* (formerly *Bacillus larvae*, Genersch et al., 2006), is a serious

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problem in beekeeping worldwide. AFB is highly infectious and can be fatal to colonies. At a minimum it decreases honey productivity of colonies and increases costs to beekeepers for labor and treatments to control disease transmission (Message and De Jong, 1999; Hansen and Brødsgaard, 1999; Eischen et al., 2005).

The bacteria in the group *Bacillus* and *Paenibacillus* are spore forming, and the vegetative cells of *P. larvae* have become increasingly resistant to tetracycline treatments (Kochansky et al., 2001; Cox et al., 2005). Interestingly, the resistance of *P. larvae* to currently applied antibiotics appears to have developed independently across North America in the past decade (Miyagi et al., 2000; Evans, 2003), which has implications for its ability to become resistant to antibiotics worldwide. The use of new, commercial antibiotics can create strains that are unaffected by a suite of compounds (Kochansky et al., 2001) and can further affect the beekeeping industry since many antibiotics leave residues in hive products (i.e. Feldlaufer et al., 2004). In untreated colonies, spores in the combs can remain viable for a long time through extreme temperature changes. Beekeeping equipment and products from infected hives, including honey from colonies experiencing infection by AFB, can become contaminated and can promote the spread of the disease within and among colonies (Hansen and Brødsgaard, 1999; Schuch et al., 2003). As a result, beekeepers often burn infected colonies to eliminate the source of infection.

Larvae are infected by ingesting spores within the larval food provided by adult worker bees. Diseased larvae will ultimately die from infection when sporulation occurs and then will transmit spores throughout the hive. The disease can kill the colony as spores become widespread unless colonies demonstrate mechanisms of resistance. Young larvae from some colonies demonstrate physiological resistance to infection (Rothenbuhler and Thompson, 1956; Crailsheim and Riessberger-Galle, 2001; Evans, 2004). Another form of resistance is behavioral: adult bees in some colonies exhibit hygienic behavior and remove diseased larvae before they become contagious (Rothenbuhler, 1964; Spivak and Reuter, 2001).

Larval food and honey are the principal means of dispersal of this disease.

Various studies have demonstrated that commercial honey, internationally, contains live spores of this bacterium, which generally constitutes a risk of contamination (i.e. Lauro et al., 2003; Iurlina and Fritz, 2005). While AFB is fairly common across the US, Europe and Australia, it has only recently affected South American countries. AFB was not documented in Argentina until 1992 (Alippi, 1992) and has only just been detected in a few honey, pollen and adult bee samples in southern Brazil. However no clinical symptoms of the disease have been detected in live bee colonies in Brazil (Schuch et al., 2003) or in the bordering regions of Uruguay (Antúnez et al., 2004). There is also some evidence that the more recently introduced strains of AFB show resistance to some conventional antibiotics (i.e. oxytetracycline), but not to others (i.e. tylosin; Schuch et al., 2003; Alippi et al., 2005). If the disease does spread through Brazil, beekeepers may try to control it with antibiotics with limited effectiveness, and possibly lose the emerging and very promising honey export market.

As an additional possible natural mechanism of disease resistance, bees collect resin from trees and shrubs and typically use it as a form of cement, called propolis, to seal any cracks or openings in the nest. Propolis is known for its antimicrobial properties and is commonly used by people especially outside of the US to treat a number of bacterial and viral infections (see Ghisalberti, 1979). Brazilian propolis has been widely documented for its properties as an antimicrobial, antifungal, antitumor and antiviral agent (Grange and Davey, 1990; Dobrowolsky et al., 1991; Fernandes et al., 1995; Park et al., 1995, 1997, 2002; Ota et al., 2001; Bastos, 2003). Because of the range of its antimicrobial properties, propolis may be essential to honey bees, particularly those in the wild (Haydak, 1953; Hoyt, 1965; Ghisalberti, 1979; Seeley, 1985). Propolis from temperate regions has been tested against AFB both in cultures and in field studies, although not in recent years (Lindenfelser, 1967, 1968; Mlagan and Sulimanovic, 1982). Until now, no one has tested the antimicrobial properties of Brazilian propolis against *P. larvae*, even though it is well known for its strong inhibitory effects against other Gram-positive bacteria in laboratory cultures (Aga et al., 1994; Sforcin et al., 2000; Bastos, 2001).

The objective of this study was to investigate the *in vitro* activity of propolis collected from various states of Brazil against *P. larvae* and to compare the activity of propolis with conventional antibiotics and, as a reference, two propolis samples from Minnesota, USA.

2. MATERIALS AND METHODS

2.1. Propolis extracts

2.1.1. Brazilian propolis

Two sets of propolis samples were collected from colonies from a variety of states within Brazil. In the first set, 16 samples of crude propolis of different colors were collected from colonies located in the states of Minas Gerais, Goiás, Paraná and Bahia, Brazil. A second set of 16 samples of crude propolis was collected from different colonies only in the states of Minas Gerais and Paraná.

The green propolis in both sets that originated from Minas Gerais was derived from *Baccharis dracunculifolia* (Bastos, 1998, 2001, 2003; Bastos and Oliveira, 1998; Bastos et al., 2000; Santos et al., 2003). The green propolis from Parana had a distinct botanical origin from that of green propolis in Minas Gerais, although the specific plant source remains unknown. Black propolis produced in Minas Gerais has been identified as a mixture of resins from *B. dracunculifolia* and *Vernonia polyanthes*. The plant sources of the other colors of propolis were not known.

Ethanol extractions of the two sets of Brazilian propolis samples were done in Brazil as follows: 50 g of crude propolis from each sample was added to 100 mL of 70% ethyl alcohol. This solution was kept in the dark at ambient temperature for 14 days and agitated once per day. After this time, the solution was filtered through filter paper.

The first set of samples was tested in Minas Gerais, Brazil and the second set was shipped to MN where they were tested at the University of Minnesota.

2.1.2. Minnesota propolis

As a reference for the results of tests done with Brazilian propolis, propolis was collected from colonies located in two locations within MN: the first near the University

of Minnesota, St. Paul campus, and the second in the southeastern part of the state, near the town of Houston, MN. These were the same propolis samples used by Gekker et al. (2005). The botanical origin of the MN propolis has not been identified, but is probably comprised to some degree from poplar trees, *Populus* spp. (see Ghisalberti, 1979).

Ethanol extractions of MN propolis were done at the University of Minnesota. 50 g of crude propolis was ground and placed in 100 mL of 95% ethyl alcohol. The solution was covered from light and kept under constant, moderate agitation for 8 days after which point the extracts were filtered following Gekker et al. (2005). After extraction, all propolis extracts (from Brazil and MN) were stored in amber flasks and analyzed for the amount of dissolved solids to obtain the real concentration (Instructional Norms No. 3, 2001). A portion of these extracts was tested in MN and another was shipped to Brazil for testing there.

Dose-response tests were done on four samples (two Brazilian extracts—2 and 19b—and the two MN extracts). Serial dilutions were made from the initial extracts so that the concentrations ranged from approximately 260 to 2 mg/mL (see Gekker et al., 2005). The dilutions made were 3-fold and a total of four dilutions were made for each extract. The concentrations of the propolis extracts determined by dissolved solids was 16.9%, 26.6%, 24.6%, and 26.0%, respectively, which correspond to 169, 266, 246, and 260 mg/mL of propolis in ethanol. The 3-fold serial dilutions were made from these initial concentrations. While the exact concentrations tested across extracts were slightly different (refer to Fig. 2.1), the magnitude of change for each dilution was equivalent.

2.2. *In vitro* assay in Brazil

2.2.1. Obtaining microorganisms in Brazil

An ampule containing lyophilized cells of *P. larvae* CCT 4443, originating from a collection of Argentinean spore cultures from the Tropical Foundation of Research and Technology Andre Tosello, Campinas, Sao Paulo, Brazil, was opened according to technical specifications, adding 0.2mL sterile distilled water. The cellular suspension obtained in this manner was transferred to two test tubes containing 5 mL of Brain Heart

Infusion (BHI), and incubated at 37°C for 24 h.

Once activated, the *P. larvae* culture was transferred and incubated for 48 h at 37°C in tubes with agar J (5.0 g triptone, 15.0 g yeast extract, 3.0 g K_2HPO_4, 20.0 g agar, 1 L of water, and 2.0 g glucose at a pH of 7.5) following Gordon et al. (1973). The tubes were stored at 5°C.

With the objective of maintaining the cultures of *P. larvae* for future research, the *P. larvae* were removed from colonies grown in agar J, and these were transferred to glass flasks containing 4 mL of semi-solid agar J and incubated at 37°C for 48 h. After this time, the flasks were sealed and stored at 5°C.

2.2.2. Sporulation technique

The inoculum that was previously stored was transferred into a tube containing 0.5 mL of BHI. It was incubated at 37°C for 72 h. After the incubation time, it was streaked in four tubes of agar J, inclined and incubated at 37°C for 72 h. This procedure was repeated with the same lines two more times. The growth obtained was rinsed with 0.5 mL sterile distilled water and transferred into an inclined Roux flask containing Bailey and Lee agar specifically for sporulation (Bailey and Lee, 1962). It was incubated at 37°C for 21 days.

At the end of the incubation time, the superficial growth was rinsed using a pipette and sterile distilled water. All of the rinsed growth was transferred into centrifuge tubes. This material was centrifuged for 20 min at 3000 rpm at 4°C five times, resuspended and exposed to thermic shock at 80°C for 10 min. This spore suspension was stored at 5°C and used as tests of pure antimicrobial activity or to prepare serial dilutions.

2.2.3. Test of antimicrobial activity of propolis extracts against **P. larvae**

This resistance assay was adapted from the technique of radial diffusion on two layers of perforated agar following Grove and Randall (1990). For the first layer, 15 mL of BHI-T agar (commercial BHI agar from Merck with 0.1% thiamine added) was used in Petri dishes. For the second layer, a flask with 3 mL BHI-T semi-solid medium

(commercial BHI from Merck with 0.75% agar and 0.1% thiamine added) was added to 10 µL spore suspension (2 x 10^9 CFU/mL). This medium was poured over the first layer of solidified BHI-T agar. The agar was perforated using a metal cylinder of 7 mm diameter, and 50 µL of propolis extract was inoculated into the perforated well. The amount of actual propolis in the wells varied according to the concentration of the extracts. For example, Bahia Sample 24 and Minas Gerais Sample 9a (see Table 2.2) had concentrations of 15.5% and 24.4%, which resulted in 7.75 and 12.2 mg of propolis in the well, respectively. After the wells were full, the plates were incubated at 35°C for 72 h. For the controls, commercial disks of the antibiotics, vancomycin (30 µg, Sigma) and tetracycline (30 µg, Sigma), were used and sterile filter paper disks saturated with 70% EtOH were used as a control for the propolis extracts. Vancomycin was used because it is a broad spectrum antibiotic effective against Gram-positive bacteria.

After the incubation period, the zones of growth inhibition of the bacterium were measured with a digital micrometer. Assays on each propolis extract from both Brazil and MN, USA were replicated five times.

2.3. *In vitro* assay in Minnesota

2.3.1. Obtaining microorganisms in MN

As a way to compare activity of the propolis extracts on various strains of *P. larvae*, an *in vitro* assay was also conducted in MN, USA. *P. larvae* spores were collected from several diseased colonies in MN by collecting scales of dried larvae that contain bacterial spores from combs. In this way, the spores may have included multiple strains of *P. larvae* (Evans, 2003). Five scales were macerated with 10 mL sterilized water in screw-cap tubes. This suspension was heat-shocked at 80°C for 10 min to kill non-spore forming bacteria.

2.3.2. Test of antimicrobial activity of propolis extracts against P. larvae

The resistance assay used in MN followed the protocol for the disk diffusion method as described by Shimanuki and Knox (2000). To prepare the bacterial cultures,

0.2 mL of the spore suspension was evenly spread over brain–heart infusion (BHI) agar plates (Difco Laboratories) fortified with 0.1 mg/L thiamine hydrochloride and pH adjusted to 6.6 using hydrochloric acid. A 6.35 mm filter paper disk (Whatman 7) was saturated with the antibiotic (propolis extract, tylosin, or methanol/ethanol control) and dried. Each disk absorbed approximately 20 µL of solution (see Kochansky et al., 2001). A treated disk was then placed on the center of the plates with the spore suspension. The plates were then incubated at 34°C for 72 h. The diameter of the zone of growth inhibition around the disk was then measured without correcting for disk diameter. If there was no visible zone of inhibition, the diameter was scored as 0.

For the controls, the antibiotic tylosin (Sigma) was chosen because preliminary tests provided evidence that *P. larvae* from the MN colonies was resistant to terramyacin (Miyagi et al., 2000). A solution of 400 mg/L was prepared for the antibiotic treatment. Both methanol and ethanol were used as negative controls because tylosin was in a methanol solution while the propolis extracts were prepared in ethanol (see above).

For the dose response tests, the disks were treated in the same manner. A disk was saturated with one of the diluted propolis extracts (ranging from approximately 260 to 2 mg/mL). This resulted in disks containing approximately 5, 1.7, 0.5, 0.17, and 0.06 mg of each extract/disk (for exact amounts refer to Fig. 2.1).

In summary, the assays presented here provide a robust analysis of the activity of propolis extracts collected from various areas of Brazil against multiple strains of *P. larvae*. Table 2.1 provides a summary of the sources of propolis samples and methods used in each location. By applying two different conventional methods to test for antibacterial activity, the consistency of any effects would be apparent.

Table 2.1. Summary of Methods[1].

	Minas Gerais, Brazil		St Paul, Minnesota	
Origin of propolis samples	various states in Brazil	two locations in MN	two states in Brazil	two locations in MN
Extraction Procedure	70% EtOH, 14d	95% EtOH, 8d	70% EtOH, 14d	95% EtOH, 8d
P. larvae spore origin	Culture bank, Argentina		Scales from diseased colonies in MN	
Resistance Assay Methods	Grove and Randal (1990)		Shimanuki and Knox (2000)	

[1]Tests of propolis extracts were conducted in Brazil and in Minnesota, USA. Two different methods were used to extract the propolis samples in ethanol (although see Discussion). In addition, spores of *Paenibacillus larvae* originated from different locations for each of the two resistance assays.

2.4. Statistical analysis

Differences among the activity of the propolis extracts as measured by the zones of inhibition were analyzed using one-way ANOVA, followed by post-hoc Tukey's HSD tests. A Pearson correlation was calculated to determine if the concentration of propolis was related to the zones of inhibition.

3. RESULTS

3.1. *In vitro* assay in Brazil

The results obtained on the susceptibility of *P. larvae* to the propolis extracts and the controls are shown in Table 2.2. The zones of inhibition of all the Brazilian samples varied between 20.5 ± 2.1 and 15.5 ± 2.2 mm demonstrating some antimicrobial activity of all the Brazilian propolis samples against *P. larvae*. An ANOVA of the Brazilian-derived samples revealed significant differences in their inhibitory activity ($F = 12.29$; $df = 13, 56$, $p = 0.02$). However, post-hoc pairwise comparisons of means indicated the

differences were only between the two samples that showed the highest average zones of inhibition (samples 9a from Minas Gerais and 24 from Bahia) and the two that showed the lowest (samples 34 and 3T, both from Minas Gerais).

The negative control (ethanol) did not inhibit bacterial growth, and vancomycin and tetracycline had zones of inhibition of 19.1 and 31.3 mm, respectively. An ANOVA comparing the two extracts with the highest average inhibition, the two with the lowest average inhibition and the two antibiotics was significant ($F = 17.57$; $df = 5, 16$; $p < 0.001$). The Tukey's test showed that tetracycline had a significantly larger zone of inhibition than vancomycin and all four propolis extracts ($p < 0.01$ for each comparison).

An ANOVA comparing the two high and two low Brazilian samples with the two MN propolis samples revealed significant differences in inhibitory activity ($F = 22.38$; $df = 5,24$; $p < 0.001$) (Table 2.2). Tukey's comparisons showed that both MN samples had significantly less inhibitory activity than the two Brazilian extracts with highest average inhibition (samples 9a and 24, $p < 0.001$ for each comparison). The MN propolis collected from the University had the same inhibitory activity as the two Brazilian samples with lowest average zones of inhibition (samples 34 and 3T, $p > 0.238$). The MN sample from southeastern MN was significantly less effective than the Brazilian sample 3T ($p = 0.015$) but not different from the lowest, Brazilian Sample 34 ($p = 0.089$).

Among the Brazilian propolis extracts, there was a significant correlation ($r = 0.575$, Bonferroni $p = 0.031$) between the concentration of soluble solids and zones of inhibition; that is, the higher the propolis concentration, the greater the antimicrobial activity (refer to Table 2.2).

Table 2.2. Average zones of inhibition of two antibiotics and the Brazilian and Minnesota propolis extracts tested against *Paenibacillus larvae* cultures in Brazil.

State of Origin (Sample#)	Botanic Origin	Color	Concentration (Percent)	Zone of Inhibition mm ± s.d. (n)
Vancomycin	Antibiotic			19.06 (1)
Tetracycline	Antibiotic			31.27 (1)
Brazil				
Bahia (24)	Unknown	Red	15.5	20.5 ± 2.1 (5)
Minas Gerais (9a)	*B. drancunculifolia*	Green	24.4	20.2 ± 1.0 (5)
Bahia (23)	Unkown	Red	24.8	20.1 ± 2.1 (5)
Minas Gerais (19a)	*B. drancunculifolia*	Green	25.2	19.8 ± 0.7 (5)
Minas Gerais (13)	*B. drancunculifolia*	Green	23.9	19.3 ± 1.5 (5)
Goiás (5)	Unknown	Brown	5.4	18.3 ± 1.4 (5)
Minas Gerais (11)	*B. drancunculifolia* and *V. polyanthes*	Black	17.2	18.1 ± 3.6 (5)
Minas Gerais (3)	*B. drancunculifolia*	Green	18.2	17.7 ± 1.6 (5)
Minas Gerais (35)	*B. drancunculifolia* and *V. polyanthes*	Black	15.0	17.6 ± 2.2 (5)
Minas Gerais (10a)	*B. drancunculifolia*	Green	20.8	17.5 ± 4.5 (5)
Paraná (6)	Unknown	Green	7.5	17.3 ± 2.6 (5)
Minas Gerais (4a)	Unknown	Brown	10.4	16.6 ± 3.0 (5)
Minas Gerais (3T)	*B. drancunculifolia*	Green	14.6	16.3 ± 1.4 (5)
Minas Gerais (34)	*B. drancunculifolia*	Black	14.1	15.5 ± 2.2 (5)
United States				
University of MN	Unknown	Red	31.4	14.1 ± 0.5 (5)
Southeastern MN	Unknown	Red	28.6	12.9 ± 1.0 (5)

3.2. *In vitro* assay in Minnesota

The results of the tests conducted in MN are shown in Table 2.3. The range of the zones of inhibition for the Brazilian propolis extracts was from 34.7 ± 1.0 to 29.3 ± 5.9 mm. There were no statistical differences in the zones of inhibition among the Brazilian propolis extracts ($F = 1.58$; $df = 15, 40$, $p = 0.124$), and so they were pooled for subsequent analysis. There was a highly significant difference among the zones of inhibition for tylosin, the two MN propolis extracts, and the pooled Brazilian extracts ($F = 93.89$; $df = 3, 62$, $p < 0.001$). The Tukey's post hoc comparison of means indicated that tylosin had a significantly larger zone of inhibition compared to all the Brazilian extracts ($p < 0.001$) and each of the MN propolis extracts had significantly smaller zones compared to both the antibiotic ($p < 0.001$) and the Brazilian extracts ($p < 0.001$). The solvents (ethanol and methanol) showed no growth inhibition with zones of inhibition of 0.

To study the correlation between the concentration of the propolis extracts and their antimicrobial activity we used dose response tests. No significant differences in the zone of inhibition were observed for any propolis samples between the highest two concentrations (5 mg propolis/ disk and 1.7 mg propolis/disk; Fig. 2.1). In all cases, significant differences were observed between the concentrations of 1.7 and 0.12 mg propolis/disk. The middle concentration, 0.5 mg propolis/disk, showed intermediate zones of inhibition between the highest and lowest concentrations for each propolis extract indicative of a clear dose response.

Table 2.3. Average zones of inhibition of the antibiotic tylosin and the Brazilian and Minnesota propolis extracts tested against *Paenibacillus larvae* cultures in Minnesota.

State of Origin (Sample#)	Botanic Origin	Color	Concentration (Percent)	Zone of Inhibition mm ± s.d. (n)
Tylosin	Antibiotic			43.3 ± 1.0 (4)
Brazil				
Minas Gerais (19b)	*B. drancunculifolia*	Green	26.6	34.7 ± 1.0 (3)
Minas Gerais (17)	*B. drancunculifolia*	Green	21.1	34.0 ± 1.2 (3)
Minas Gerais (2)	*B. drancunculifolia*	Green	16.9	34.0 ± 2.8 (4)
Minas Gerais (4b)	*B. drancunculifolia*	Green	17.4	33.5 ± 3.8 (4)
Minas Gerais (5)	*B. drancunculifolia*	Green	15.9	33.5 ± 3.0 (4)
Paraná (3)	Unknown	Green	16.7	33.3 ± 1.2 (4)
Minas Gerais (10b)	*B. drancunculifolia*	Green	21.9	33.0 ± 1.4 (4)
Paraná (11)	Unknown	Green	21.6	32.8 ± 1.0 (4)
Minas Gerais (8)	*B. drancunculifolia*	Green	22.6	32.8 ± 1.3 (4)
Minas Gerais (20)	*B. drancunculifolia*	Green	17.1	32.3 ± 1.7 (3)
Minas Gerais (18)	*B. drancunculifolia*	Green	18.0	32.0 ± 0.6 (3)
Minas Gerais (1)	*B. drancunculifolia* and *V. polyanthes*	Black	16.7	30.8 ± 2.5 (4)
Minas Gerais (14)	*B. drancunculifolia* and *V. polyanthes*	Black	19.6	30.3 ± 2.1 (3)
Minas Gerais (9b)	*B. drancunculifolia*	Green	20.9	30.0 ± 1.7 (4)
Minas Gerais (12)	*B. drancunculifolia*	Green	21.7	29.3 ± 5.9 (3)
Minas Gerais (21)	*B. drancunculifolia*	Green	9.6	29.3 ± 0.6 (3)
United States				
University of MN	Unknown	Red	26.0	19.0 ± 1.0 (3)
Southeastern MN	Unknown	Red	24.6	15.0 ± 1.0 (3)

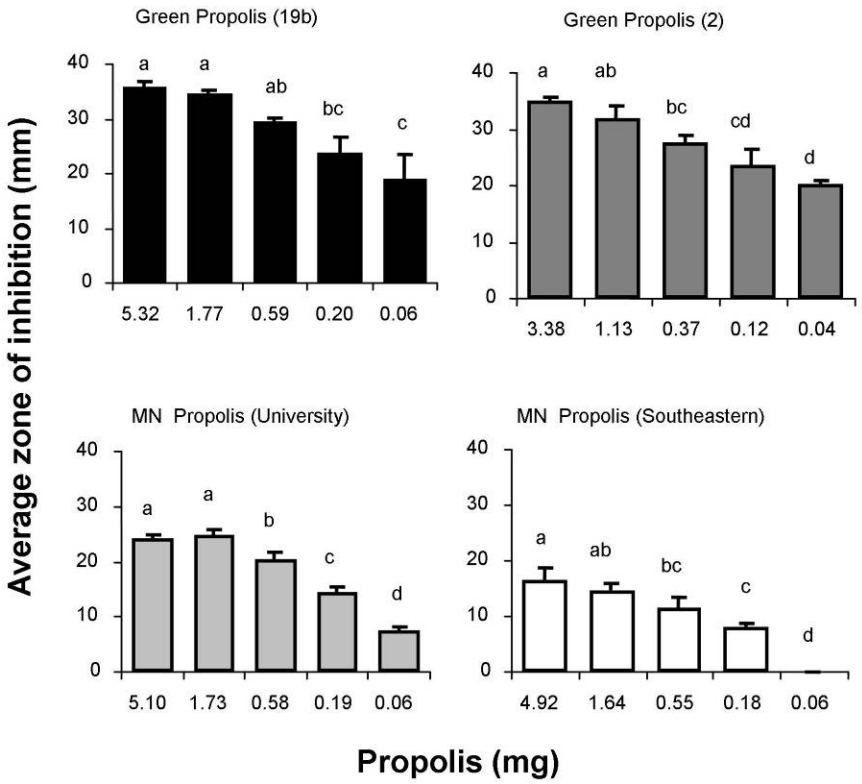

Figure 2.1. Antimicrobial activity of four different propolis extracts at different dilutions. Three replicates for each extract were done for every concentration. Dose responses to Brazilian- and MN-derived propolis extracts listed in Table 2.3. Significant differences ($p < 0.05$) are indicated by the letters above the bar, determined by ANOVA and Tukey's tests. (a) Brazilian extract 19b ($F = 17.43$, df = 4, 10, $p < 0.001$). (b) Brazilian extract 2 ($F = 23.20$, df = 4, 10, $p < 0.001$). (c) MN, University extract ($F = 126.84$, df = 4, 10, $p < 0.001$). (d) MN, Southeastern extract ($F = 46.29$, df = 4, 10, $p < 0.001$). Standard deviations are indicated on graphs.

4. DISCUSSION

The propolis extracts derived from the various states of Brazil significantly inhibited the growth of multiple strains of *P. larvae* in cultures. The antibiotics terramycin and tylosin had significantly more activity against *P. larvae* compared to the most active propolis samples from Brazil and MN, respectively. However vancomycin had similar effects as the Brazilian propolis samples. It is important to note that the doses of antibiotic versus propolis were significantly different, especially in regards to the active components. For the Brazilian study, 30 µg of antibiotic was used, whereas the concentration of propolis in the wells ranged from 2.7 to 12.6 mg. In the MN study, 8 µg of antibiotic and doses ranging from 1.92 to 5.32 mg of propolis were used. With the antibiotics, 100% of the material is active against the bacteria. However, the concentration of active components in propolis has not been determined. In fact, it is not known what the active components acting against *P. larvae* are, and in general active components of propolis extracts are understudied.

Propolis samples consist of many (80–300) different chemical compounds in complex and varied mixtures (Bankova et al., 2000, 2002; Salatino et al., 2005). The active portion of propolis extracts is often in quantities less than 3% of the total mixture (da Silva et al., 2006). Additionally it is clear that in different samples, different combinations of compounds, which widely range in activity levels and could act synergistically, are required for significant biological activity (Kujumgiev et al., 1999). So despite the obvious difference in the dose of antibiotic versus total propolis extract used in this study, it is possible that the amount of active compounds tested was less than the dose of antibiotic. Therefore, the finding of strong activity of various propolis samples against *P. larvae*, even at low doses of active compounds, is quite promising.

Identification of the chemical profiles of propolis and the resins from which they are derived is currently a popular area of research. Propolis from temperate regions throughout the world is often identified as poplar-type propolis, and the compounds of propolis derived from various poplar species are quite similar across the globe (Greenaway et al., 1990; Bankova et al., 2000; Bankova, 2005a). In general, propolis

from temperate regions (poplar-type propolis) is typically characterized by the presence of flavonones, flavones, phenolic acids and their esters. The green Brazilian propolis from Minas Gerais is derived from *Baccharis dracunculifolia* (Bastos, 1998, 2001, 2003; Bastos and Oliveira, 1998; Bastos et al., 2000; Santos et al., 2003), and its chemical properties are well documented (i.e. Salatino et al., 2005). Brazilian green propolis is identified as mainly consisting of diterpenes and a specific group of phenolic acids, prenylated p-coumaric acids (for general review see Bankova et al., 2000; Bankova, 2005a; Salatino et al., 2005).

With respect to the two MN, USA propolis extracts used in this study, the majority of the Brazilian extracts exhibited a higher level of antibacterial activity. The only exceptions were two samples of Brazilian propolis with the lowest average zones of inhibition that did not display significantly different activity compared to the MN propolis extracts when tested in Brazil. General differences in chemistry of temperate propolis (flavonoids) and tropical propolis (terpenes) may account for the differences in activity against *P. larvae*; however, more temperate samples should be tested to confirm this idea. Additionally the results of this study indicate that: (1) Brazilian propolis has similar antimicrobial properties against *P. larvae* independent of geographic origin and (2) plant source may determine level of activity, as green propolis derived from *B. dracunculifolia* appeared to be more effective in general.

Significant dose responses were found for individual propolis extracts, particularly between the concentrations of 1.7 and 0.12 mg propolis/disk. However, it is clear that the propolis source was more important in determining its overall activity against *P. larvae* than the concentration. For example, Sample 5 from the state of Goiás, Brazil (Table 2.2) had the lowest concentration (5.4%) but showed just slightly less activity against the bacterium compared to Sample 13 from Minas Gerais with a concentration of 23.9%. Similarly, propolis extracts from MN with higher concentrations than any extracts from Brazilian propolis (31.4% and 28.6%, Table 2.2) were only as active as two Brazilian samples with the lowest activity and relatively low concentrations (14.6% and 14.1%).

These findings appear to be rather robust considering that the difference between the activity between the Brazilian and MN propolis samples was observed using two different sets of methods. The first difference in methods was that the propolis samples from MN were extracted in 95% EtOH for 8 d whereas the samples from Brazil were extracted in 70% EtOH for 14 d; this may have led to slight differences in the presence or amounts of some of the chemical components (Krell, 1996). However, in a subsequent trial, the extraction procedure used in Brazil was replicated on the same propolis sample from the University of Minnesota and found that the zone of inhibition increased from 19.0 ± 1.0 mm (n = 3) in 95% EtOH 8 d extraction to 25.8 ± 3.3 mm (n = 4) in the 70% EtOh 14 d extraction. However, the increased activity was still lower than the Brazilian samples tested in MN, confirming our initial findings. Sawaya et al. (2002) examined the effectiveness of propolis extracted in different concentrations of ethanol and water against strains of the fungus *Candida* and found that extracts with 50% EtOH or higher performed similarly, which also supports our results.

The other differences in methods concerned the origin of the *P. larvae* spores (cell culture from Argentina vs. scales collected from several AFB diseased colonies in MN), and the resistance assay methods [based on Grove and Randall (1990) or Shimanuki and Knox (2000)]. These differences may account for the overall greater zones of inhibition observed in all samples tested in MN compared to samples in Brazil. Small differences in the results from these techniques were also noted in Sawaya et al. (2002).

Lindenfelser (1967) examined the activity of 15 propolis samples collected from across the US against *P. larvae* using the tube dilution method. He found that all 15 samples showed a similar level of bactericidal action at concentrations less than 100µg/mL; this is the same order of magnitude at which the dose response data presented for this study were most clear. Additionally, Mlagan and Sulimanovic (1982) tested two temperate propolis samples from Yugoslavia against eight strains of *P. larvae* using the disk diffusion method (the same that was used for the MN assays). They found that 5% and 10% propolis extracts all inhibited the growth of the bacteria and had an average zone of inhibition of 15.1 and 15.6 mm, respectively, which is similar to what we have

shown for the two MN propolis samples.

Although these results are promising, more studies are needed to confirm the findings. For example, it may be important to test propolis samples against a variety of strains of *P. larvae*. In the present study, it is possible that multiple *P. larvae* strains were tested in MN since the scales originated from several colonies from different sites (Evans, 2003), but this was not confirmed. While Mlagan and Sulimanovic (1982) tested propolis against eight *P. larvae* strains in laboratory cultures and found similar results, strain differences may be more important when considering the effect of treatments in the field where different strains can have vastly different virulence properties (Ashiralieva and Genersch, 2006).

Further studies should also be conducted to isolate the active compounds of propolis and then to use appropriate concentrations of just the active substances to determine its effectiveness in treatments. Furthermore, the mode of action for propolis against Gram-positive bacteria needs to be understood in order to determine the potential value of propolis as a natural treatment. Field studies have been conducted by Lindenfelser (1968) and Mlagan and Sulimanovic (1982) who fed bees a low dose of temperate propolis extract in solution and found that the treatments only temporarily prevented the spread of the disease in infected colonies. However, as has been shown here, it appears that Brazilian propolis and its active compounds may be a more effective treatment option. It is also possible that feeding the bees a propolis solution may not be the optimal form of treatment, but this needs to be investigated further (Simone, Soares, & Spivak, unpublished data).

Due to the evolution of resistance of *P. larvae* to conventional antibiotic treatment, this research is an important first step in identifying possible new active compounds within propolis to treat AFB in honey bee colonies. This study succeeded in making the initial step to understand the general bioactivity of various propolis extracts in order to determine that further research should be conducted concerning its active compounds and its potential as an antibacterial agent against American foulbrood. Field bio-assays are underway to test the effectiveness of Brazilian propolis extracts on AFB

infected colonies to determine if the difference in effectiveness between conventional antibiotics and propolis is biologically important.

CHAPTER 3—

Resin collection and social immunity in honey bees[3]

Diverse animals have evolved an ability to collect antimicrobial compounds from the environment as a means of reducing infection risk. Honey bees battle an extensive assemblage of pathogens with both individual and social defenses. We determined if the collection of resins, complex plant secretions with diverse antimicrobial properties, acts as a colony-level immune defense by honey bees. Exposure to extracts from two sources of honey bee propolis (a mixture of resins and wax) led to a significantly lowered expression of two honey bee immune-related genes (hymenoptaecin and AmEater in Brazilian and Minnesota propolis, respectively) and to lowered bacterial loads in the Minnesota (MN) propolis treated colonies. Differences in immune expression were also found across age groups (third-instar larvae, 1 day-old and 7 day-old adults) irrespective of resin treatment. The finding that resins within the nest decrease investment in immune function of 7 day-old bees may have implications for colony health and productivity. This is the first direct evidence that the honey bee nest environment affects immune-gene expression.

1. INTRODUCTION

Although social living can be of a benefit for many organisms, it also creates the possibility for high costs, particularly due to an increased chance of disease outbreaks and the potential for pathogens and parasites to exploit the high concentration of individuals (Schmid-Hempel 1998). This is certainly the case in social insects, which typically live in large colonies with a constant interaction among individuals. With this in mind, the finding of the Honey Bee Genome Sequencing Consortium (2006) that honey bees (*Apis mellifera*) appear to have a sparse immune-response system was curious (Evans et al. 2006). The authors pointed out that the immune defenses of individual honey bees might

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be compensated by behavioral and colony-level mechanisms (recently termed social immunity; Cremer et al. 2007). In honey bees, the nest environment itself is often considered to be one aspect of colony-level immunity because stored honey has antimicrobial properties as does royal jelly, the larval food secreted by adult nurse bees (Morse and Flottum 1997). Several behaviors are also known to reduce parasite and pathogen loads. For example, hygienic behavior is a trait by which individual bees are able to detect and remove parasitized pupae or diseased larvae before they reach an infectious stage (Rothenbuhler 1964; Spivak and Gilliam 1998). Colonies that express this behavior are resistant to *Ascosphaera apis* (the fungal agent of chalkbrood disease), *Paenibacillus larvae* (the bacterium that causes the deadly American foulbrood), and the parasitic mite *Varroa destructor* (see Wilson-Rich et al. 2009 for a recent review).

We hypothesize that resin collection and use by honey bees provide an additional colony-level disease response. Various plant species secrete highly antimicrobial resins to protect vegetative apices and young leaves (Langenheim 2003). Several species across the animal kingdom may use these plant-produced resins to reduce effects of parasites and pathogens. One well-described example, *Formica paralugubris*, a Swiss wood ant, mixes resin globules from coniferous trees with nest material, and this resin decreases the number of total microorganisms in the nest (Christe et al. 2003). Furthermore, laboratory experiments in this species suggest that protective resin leads to increased survival of individuals when exposed to pathogens and a lowered investment of energy in immune function when unchallenged (Chapuisat et al. 2007; Castella et al. 2008a,b). Other more general examples include white-nosed coatis (*Nasua narica*) in Panama that spread resins on their coats as a hypothesized means of protection against parasites (Gompper and Hoylman 1993). The resinous, aromatic leaves used by European starlings for nest construction may decrease parasite loads and boost immunity among developing nestlings (i.e., Gwinner et al. 2000; Gwinner and Berger 2005; Mennerat et al. 2009). Female assassin bugs also harvest resin and then transfer it to eggs as they are being laid, which inhibits ant predation (Choe and Rust 2007). Among the social insects, many bees, especially in the tropics, collect and use resins as a nest-building material (Roubik 1989).

It is possible, at least in tropical regions where resinous plants are more abundant, that bees simply exploit this commonly available resource for structural benefits. However, there are other properties of the resin that may benefit all individuals in the nest. This is most likely the case in colonies of some ants and honey bees where a few individuals collect and return to the nest with loads of resin to use in the nest interior.

Honey bees collect resins on their hind legs, as they do pollen, and bring it back to the nest where it is mixed with varying amounts of wax and used mainly as a form of cement, called propolis, to seal cracks and holes in the nest architecture. Feral honey bees nesting in tree cavities line the entirety of the interior nest wall with a thin layer of resin, which has been termed the "propolis envelope" (Seeley and Morse 1976). In temperate regions, it is thought that the main sources of resins are *Populus*, *Betula*, and *Alnus* species, although others are used by honey bees less predominately (Ghisalberti 1979; Crane 1990). In tropical areas, bees have been recorded collecting resins from *Clusia* flowers and from woody plants such as *Baccharis dracunculifolia* (i.e., Salatino et al. 2005), among others. The antimicrobial properties of propolis with respect to human health and disease have received much attention (see Bankova 2005b). However, few studies have examined the roles played by resins in honey bee colony disease resistance (i.e., Mlagan and Sulimanovic 1982; Garedew et al. 2002; Bastos et al. 2008), and only one study has been conducted outside the laboratory (Antunez et al. 2008). Foraging for resins is energetically demanding and provides no clear direct, individual reward, so it is possible that resin use functions as an aspect of social immunity.

Here, we investigated the effect that resins in field colonies of honey bees have on the immune system of an individual honey bee. We hypothesized that the presence of propolis within the colony reduces the amount or diversity of pathogenic and saprophytic microbes within the nest and thus results in a lowered physiological investment in the production of antimicrobial peptides and cellular immunity in bees throughout the colony. Humoral defenses (i.e., antimicrobial peptides) and cellular defenses (i.e., melanization, phagocytosis, and encapsulation) are known to be produced in response to infection and wounding (i.e., Evans 2004). As a chronically high activation of the

immune system at the individual level can lead to decreased productivity at the colony level (Evans and Pettis 2005), factors that reduce immune investment could lead to increased productivity. With the recent sequencing of the honey bee genome (HGSC 2006), we are able to more precisely investigate questions concerning immune activity in honey bees. In light of Colony Collapse Disorder (the sudden loss of bees within colonies across the country) and the current issues relating to colony health (Cox-Foster et al. 2007; Higes et al. 2008), it is particularly important to gain a greater understanding of the mechanisms involved in reducing a physiological stress of honey bees.

For this study, we experimentally enriched colonies with either regional propolis from Minnesota or the well-studied Brazilian green propolis collected in Minas Gerais, Brazil (i.e., Salatino et al. 2005) to determine the effects of propolis on immune investment and microbial loads in honey bee colonies.

2. MATERIALS AND METHODS

2.1 Experimental design

Twelve four-frame experimental colonies were treated with resin whereas six control colonies were not treated. Each experimental colony was comprised of a sister queen and a mixture of bees from five "source" colonies to ensure genetic homogeneity. The source colonies had no visible clinical symptoms of disease and had low levels of the parasitic mite, *V. destructor*, as high mite levels have been shown to complicate immune expression in honey bees (Gregory et al. 2005; Yang and Cox-Foster 2005). To obtain this mixture, approximately 18 kg of bees from the source colonies were put into a large, screened box. The bees were mixed in this box, then divided into 18 1 kg (≈ 7000 bees) "packages" (screened boxes built to hold bees and a caged queen). After two days, the bees and queen from the packages were transferred to new "nucleus" boxes (beekeeping equipment built to hold four combs). Each nucleus box was started with two honey frames, one frame of empty comb, and one frame with foundation on which the bees could build a new comb.

Six of the nucleus boxes were treated with green propolis from Brazil (BR); six

were treated with propolis from Minnesota (MN); and six were used as controls. To treat with propolis, the inside surfaces of the box were painted with 300 mL propolis extract (13% propolis in 70% ethanol, following the extraction procedure of previous experiments with the exception that crude propolis was extracted in 70% ethanol; Gekker et al. 2005; Bastos et al. 2008) simulating a propolis envelope around the brood nest. The six control boxes were painted only with 70% ethanol, the solvent used to make the propolis extract. In addition, the frame of empty comb was sprayed with 100 mL of a 7% propolis extract for the BR-treated boxes, a 6% propolis extract for the MN-treated boxes, and 70% ethanol for the controls. The colonies were inspected weekly to insure the queen was laying eggs, there were no clinical symptoms of disease, and that the colonies were functioning normally.

Seven day-old bees were collected for subsequent analysis of gene transcripts. To obtain these known-aged bees, combs containing pupae ready to emerge as adult bees were removed from each of the original five source colonies and placed in cages in an incubator (34° C, 70% relative humidity). When the bees emerged, they were color marked on the thorax to indicate their source colony and were introduced into each of the 18 colonies over the course of two days (i.e., approximately 300 marked bees/source were introduced per colony). Samples of 12 bees of each color (60 bees per colony) were collected from each colony after one week. These bees were frozen at −80° C for subsequent analysis.

For analysis of age-related differences in gene expression, third-instar larvae and 1-day and 7 day-old bees that had fully developed from egg to adult within the nucleus colonies, and thus progeny of sister queens, were also collected and frozen at −80° C.

2.2 Gene transcript analysis

RNA was extracted from whole individual adult bees and third-instar larvae using a standard RNA extraction protocol (RNAqueous, Ambion, Austin, TX). DNA was removed from this extract using DNAse I (37° C for 1 h, 75° C for 10 min). First-strand cDNA was then synthesized by incubating 8 μg total RNA per bee in a 96-well plate with

3.9 μL of a master mix containing 50 U Superscript II (Invitrogen, Carlsbad, CA), 2 nmol DNTP mix, 2 nmol poly$(dT)_{18}$, and 0.1 nmol poly$(dT)_{12\text{-}18}$ at 42° C for 50 min followed by 15 min at 70° C as described in Evans (2006). Transcript abundances for cDNA were assayed by real-time PCR using primer pairs that amplify 120-300 bp sections of the target genes (Table 3.1). Genes encoding four antimicrobial peptides (abaecin, apidaecin, defensin1, and hymenoptaecin) and a candidate for cellular immunity (the EGF family protein member AmEater) were used. Gene transcript levels of vitellogenin were analyzed as an indicator of general robustness (see Amdam et al. 2005). Lastly transcripts of eubacterial 16s RNA were measured by real-time PCR and generic primers to assess bacterial loads of the colonies. Gene transcripts were normalized relative to expression levels for the gene encoding actin, a gene with a consistent expression in honey bees.

Reactions to amplify the DNA products were conducted in 96-well plates using a Bio-Rad Icycler (Bio-Rad Corp., Hercules, CA). A total of 50 ng cDNA from each of the tested bees was used as a template for PCR reactions driven by 1 U *Taq* with proscribed 1× buffer (Roche Applied Sciences, Indianapolis, IN) and final concentrations of 1 mM dNTP mix, 2 mM additional $MgCl_2$, 0.2 μM of specific primers (one bee or pathogen gene assayed/reaction), 1× concentration SYBR Green I dye (Applied Biosystems, Foster City, CA), and 10 nM fluorescein in a 25 μL reaction volume. The reactions were conducted under a fixed thermal protocol consisting of 5 min at 95° C, followed by 40 cycles of a four-step protocol that involves 94° C for 20 sec, 60° C for 30 sec, 72° C for 1 min, and 78° for 20 sec. Fluorescence measurements were taken repeatedly during the 78° C step. This procedure was followed by a melt-curve dissociation analysis to confirm product size.

Table 3.1. Ologonucleotide primers and sequence identification for real-time PCR.

Primer Name	Sequence (5' to 3')	Genbank Entry
Abaecin.f	CAGCATTCGCATACGTACCA	NP_001011617
Abaecin.r	GACCAGGAAACGTTGGAAAC	"
Actin.f	TTGTATGCCAACACTGTCCTTT	NC_007076
Actin.r	TGGCGCGATGATCTTAATTT	"
AmEater.f	CATTTGCCAACCTGTTTGT	XP_001120277
AmEater.r	ATCCATTGGTGCAATTTGG	"
ApidNT.f	TTTTGCCTTAGCAATTCTTGTTG	NP_001011613
ApidNT.r	GTAGGTCGAGTAGGCGGATCT	"
Defensin1.f	TGCGCTGCTAACTGTCTCAG	NP_001011616
Definsin1.r	AATGGCACTTAACCGAAACG	"
Hymenopt.f	CTCTTCTGTGCCGTTGCATA	NP_001011615
Hymenopt.r	GCGTCTCCTGTCATTCCATT	"
VgMC.f	AGTTCCGACCGACGACGA	NP_001011578
VgMC.r	TTCCCTCCCACGGAGTCC	"
Bact774.f	GTAGTCCACGCTGTAAACGATG	Stackebrandt et al. 1991
Bact1391.r	GACGGGCGGTGTGTTCA	"

2.3 Data analysis

Threshold cycles for real-time PCR were defined as the point when well fluorescence became greater than 10 times the mean standard deviation across all samples. Threshold values for each target gene were subtracted from the actin threshold for each sample to get a measure of relative cDNA abundance.

We compared these normalized levels of the gene transcripts in 7 day-old bees from resin-treated and resin-untreated colonies using a nested analysis of variance (ANOVA) with the original source of the bee being nested within treatment. Post hoc Tukey's HSD tests were used when treatment effects showed significance.

To examine possible effects of a resin-rich environment at different ages of development, only bees that developed fully in the experimental colonies were analyzed. Age effects were determined by a nested-ANOVA with treatment being nested within age-group. Post hoc Tukey's HSD tests were used when age effects showed significance.

3. RESULTS

3.1 Effect of resin treatment

Normalized gene transcript levels were analyzed for 7 day-old bees from MN-propolis treated, BR-propolis treated, and control colonies. Six colony replicates were done for each of the three treatments with an average of 24 bees of the total 60 bees collected from each colony being analyzed for gene expression for this portion of the study. Across the treatments, there were significant differences in expression of two immune-related genes. For the antimicrobial peptide hymenoptaecin, 7 day-old bees collected from the MN-propolis-treated colonies had significantly lower relative transcript abundances than those collected from control colonies whereas bees from the BR-propolis treated colonies had intermediate levels, they were not significantly different from either (Fig. 3.1A; $F_{2,10} = 3.72$, $P = 0.025$). Similarly for eater, 7 day-old bees collected from BR-propolis treated colonies had significantly fewer transcripts than those from control colonies with bees collected from MN-propolis treated colonies intermediate but not significantly different from either (Fig. 3.1B; $F_{2,10} = 5.31$, $P = 0.005$).

In addition, the eubacterial load differed significantly across treatments. Seven day-old bees from control colonies had significantly higher bacterial transcript abundances than bees from colonies treated with MN-propolis, with the bees from BR-propolis treated colonies intermediate but not significantly different from either (Fig. 3.1C; $F_{2,10} = 3.19$, $P = 0.042$).

No significant differences were noted for gene transcript levels of abaecin, apidaecin, defensin1, or vitellogenin ($P > 0.05$ for treatment affects for each). The average gene transcriptlevels (±SE) for abaecin showed a non-significant trend for reduced levels in bees from the MN-propolis treated colonies (MN: 0.486 ± 0.48, BR: 1.377 ± 0.34, C: 1.048 ± 0.33; $F_{2,10} = 1.89$, $P = 0.152$). There were no clear trends for apidaecin (MN: 0.091 ± 0.32, BR: 0.260 ± 0.31, C: 0.461 ± 0.34; $F_{2,10} = 0.322$, $P = 0.725$), defensin1 (MN: 5.758 ± 0.38, BR: 6.106 ± 0.37, C: 5.986 ± 0.42; $F_{2,10} = 0.201$, $P = 0.818$), or vitellogenin (MN: −0.576 ± 0.27, BR: −0.895 ± 0.27, C: −0.379 ± 0.28; $F_{2,10} = 0.931$, $P = 0.395$).

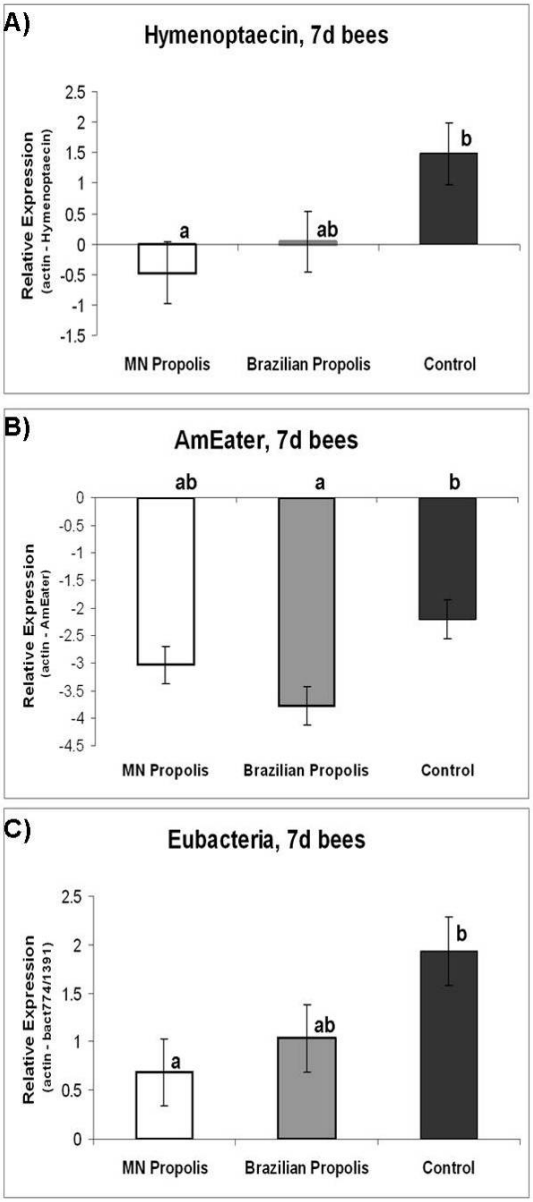


Figure 3.1. Gene transcript levels for (A) the antimicrobial peptide hymenoptaecin, (B) AmEater (a gene involved in cellular immunity), and (C) eubacteria (Bact 774/1391) normalized to the housekeeping gene actin. Six colony replicates were done for each of the three treatments with an average of 24 bees from each colony being analyzed for gene expression. Significant differences due to treatment determined by nested-ANOVA followed by post-hoc Tukey's HSD are indicated by the letters over each bar (A) $F_{2,10} = 3.19$, $P = 0.042$; (B) $F_{2,10} = 5.31$, $P = 0.005$; (C) $F_{2,10} = 3.72$, $P = 0.025$.

3.2 Effect of age

Third-instar larvae and 1 and 7 day-old bees that developed in the experimental colonies were analyzed for gene transcript levels of several immune-related genes and for the presence of eubacteria (refer to Table 3.2 for normalized means and standard errors). Six colony replicates were done for the BR-propolis treatment, five for the MN-propolis treatment, and five control colonies. An average of four bees was analyzed per age group per colony.

No significant effects due to the resin treatments were found through this analysis. In general, however, the antimicrobial peptides tested showed a general increase in abundance with age (abaecin: $F_{2,10} = 16.74$, $P < 0.0001$; apidaecin: $F_{2,10} = 7.24$, $P = 0.008$). The one gene involved in cellular immunity (eater) was lower in larvae ($F_{2,10} = 30.93$, $P < 0.0001$), but did not increase across 1 and 7 day-old bees. In addition, normalized gene transcript levels coding for general eubacteria did show a significant increase between 1 and 7 day-old bees ($F_{1,5} = 4.75$, $P = 0.031$). For vitellogenin, transcript levels increased between the larval and adult stages ($F_{2,10} = 62.08$, $P < 0.0001$) but there were no significant differences between 1 and 7 day-old bees, although there was a trend for an overall increase with age.

Gene transcript levels for hymenoptaecin were negligible for third-instar larvae and 1 day-old bees, and so were not included. Levels for apidaecin were also negligible for third-instar larvae. Defensin1 was not assayed for these samples.

Table 3.2. Average gene transcript abundances normalized to the housekeeping gene actin with standard errors of each gene for the different age groups tested.*

Gene	Age	Treatment		
		Control	**MN**	**Brazil**
Eubacteria (Bact774/1391)	3rd instar	--	--	--
	1d ***a***	0.454 ± 0.70	2.314 ± 0.75	1.069 ± 0.72
	7d ***b***	3.350 ± 0.72	3.092 ± 0.69	1.608 ± 0.72
Abaecin	3rd instar ***a***	-2.552 ± 0.54	-2.002 ± 0.48	-1.766 ± 0.54
	1d ***b***	-0.725 ± 0.45	-0.429 ± 0.52	-0.296 ± 0.48
	7d ***b***	0.306 ± 0.33	1.495 ± 0.76	0.369 ± 0.86
Apidaecin (ApidNT)	3rd instar	--	--	--
	1d ***a***	-1.155 ± 0.26	-0.116 ± 0.34	-0.509 ± 0.306
	7d ***b***	0.462 ± 0.77	0.856 ± 0.72	0.325 ± 0.88
Eater (AmEater)	3rd instar ***a***	-5.105 ± 0.52	-5.009 ± 0.48	-4.277 ± 0.58
	1d ***b***	-1.929 ± 0.35	-2.078 ± 0.41	-1.839 ± 0.41
	7d ***b***	-1.787 ± 0.77	-1.571 ± 0.74	-1.582 ± 0.93
Vitellogenin (VgMC)	3rd instar ***a***	-3.547 ± 0.51	-3.600 ± 0.51	-2.443 ± 0.53
	1d ***b***	0.109 ± 0.26	0.468 ± 0.30	0.203 ± 0.29
	7d ***b***	1.033 ± 0.46	0.781 ± 0.40	1.114 ± 0.61

***** Six colony replicates were done for the BR-propolis treatment, and 5 each were done for the MN-propolis and control treatments. An average of 4 bees was analyzed per age group per colony. Significant differences across age are indicated by letters after the age group ($p < 0.05$).

4. DISCUSSION

This is the first report that a component of the nest environment alone can influence immune expression in honey bees. Our findings indicate that individual bees in resin-enriched colonies in the field are able to invest less energy on immune function for two divergent immune-related genes, and that this effect is conceivably due to decreased bacterial loads. This decreased investment or down-regulation in immune function is the first clear evidence that the use of resins by honey bees may have implications for colony health and productivity. These field results support laboratory studies done with *F. paralugubris*, a resin-collecting ant species, which have shown that nest material enriched with resin has fewer overall microorganisms compared to resin-poor nest material (Christe et al. 2003) leading to a reduction in general immune activity (Castella et al. 2008b).

Resin foragers comprise a small percentage of the total numbers of foragers in a honey bee colony. Typically a total of 5-15 foragers will continuously collect resin during a single day (Meyer 1956; Nakamura and Seeley 2006), whereas in a 5-min period 150 foraging bees can return to the hive with pollen or nectar (i.e., Weidenmuller and Tautz 2002). Additionally, foraging for the sticky resins is a demanding process, highlighted by the unloading process, which typically takes 30 min but can take several hours (Meyer 1956; Nakamura and Seeley 2006) versus an 11-min average unloading time for a pollen forager (Nakamura and Seeley 2006). Because a very small proportion of colony members partake in the difficult task of resin foraging, the energy expended to collect propolis is likely minimal at the colony level compared to the potential energy expended by many individuals to maintain elevated expression of immune-related genes in the absence of propolis. The costs of an elevated immune system has been well-documented across bee species and include impaired learning ability at the individual level (i.e., Mallon et al. 2003; Alghamdi et al. 2008), reduced life span under stressful conditions (i.e., Moret and Schmid-Hempel 2000), and lowered colony productivity (Evans and Pettis 2005). Therefore, honey bees harvest this antimicrobial substance and incorporate it into nest architecture, which can benefit, on balance, the social immune system of

honey bees. More generally, this is a rare example of an environmental compound that can modulate immune function. It will be interesting to see whether that modulation is indirect (i.e., via decreased microbial loads as suggested here and by Castella et al. 2008b) or direct.

An important aspect of this experiment is that these colonies were not challenged with pathogens or parasites. The changes in immune expression seen here were changes in what are essentially baseline levels of immunity in field colonies. It is possible that when colonies are challenged, greater differences or differences among more immune-related genes would arise. This idea is supported by the laboratory study done with *F. paralugubris* that showed that when individual ants housed in a Petri dish with resin were challenged with a pathogen, they had higher survival rates than those without resin (Chapuisat et al. 2007). Based on this, it appears as though the presence of resin does not suppress the immune system, but merely allows for it to be down-regulated, because a pathogen challenge can still cause an up-regulation of immune proteins (see Chapuisat et al. 2007; Castella et al. 2008b).

It is clear that among the antimicrobial peptides different signaling pathways can be involved in regulating their expression, which could account for the fact that differences were seen across some genes and not others in response to the resin treatment. For example Relish, a transcription factor of the Imd pathway, appears to regulate abaecin and hymenoptaecin but not defensin1 (Schluns and Crozier 2007). There are also likely negative and positive feedback loops within the signaling pathways of the immune system, which could also influence differences across genes (Feldhaar and Gross 2008). Furthermore, individual variation in immune expression, even within closely related individuals, is extremely high, adding complexity to the relationship between the regulating pathways and the immune-related genes (Decanini et al. 2007). We had no a priori hypotheses about which genes might show differential expression in response to resin treatment. The fact that differences in transcript abundances were found for two different immune-related genes despite all of this inherent variation highlights the significance of these results.

This is the first known report to analyze gene expression across adults and larvae from the same genetic and environmental background under no pathogen challenge. The results presented here showing a relative increase in expression from larvae to 1 day and 7 day-old adults are supported by evidence indicating that immune function is reduced in larvae as compared to young adults using more standard tests of immunocompetence (Wilson-Rich et al. 2008). Bees older than seven days were not analyzed as part of this study because as bees age investment in immune function becomes extremely variable as individuals change physiological state and behavioral task and begin to immunosenesce (i.e., Amdam et al. 2005). This is particularly the case at the onset of foraging, which typically ranges from 10 day to 30 day old among nestmates (Winston 1987).

The sample sizes were likely too low for the third-instar larvae and 1 day-old bees to detect differences due to exposure to a resin-rich environment. However, it is possible that larvae would be unaffected by the propolis on the nest walls because they are surrounded by other antimicrobial substances (i.e., royal jelly, Morse and Flottum 1997). Additionally 1 day-old bees, just emerging from their cells, have possibly not yet been exposed enough to the various microbes in the nest to cause a full up-regulation of their immune systems. Future work should be done on this front, as little research has examined general differences in immune-gene expression across larvae and adults in colonies with little pathogen or parasite pressure. Additionally, the results of this study indicate that not all immune-related genes are expressed at detectable levels at all ages. Age effects on baseline levels of immune expression need to be studied further at the genetic level.

Because the honey bee genome is now fully sequenced (HGSC 2006), we have a unique opportunity to study more subtle effects of the immune system at the individual level and then follow that to the colony level. The present study is only a first step in research concerning the importance of resins in their use as propolis as a form of social immunity by honey bees.

CHAPTER 4—

Self-medication by honey bees against a fungal parasite

Across the animal kingdom, species have evolved mechanisms to resist and tolerate infection by parasites, including macroparasites (e.g. arthropods) and microparasites (e.g. bacterial and fungal pathogens). Individual defenses, such as a cuticle and inducible antimicrobial peptides, are commonly thought of as the main barriers against infection. However behavioral traits can also effectively reduce parasite transmission and infection intensity. As a specific example, when an organism changes its behavior in response to infection by ingesting or harvesting plant-produced compounds or secretions, it could constitute self-medication. Here I investigate whether resin collection and its incorporation into the nest environment is an example of self-medication in honey bees. Data is presented from three years of study on the effects of a parasite challenge on resin collection at the colony level. In 2008 and 2010 colonies were challenged with the fungal agent of the larval disease chalkbrood (*Ascosphaera apis*), and in 2009 colonies were challenged with chalkbrood, the bacterial agent of the larval disease American foulbrood (*Paenibacillus larvae*) and the entomopathogen *Metarhizium anisopliae*. The numbers of resin and pollen foragers returning to the colonies were counted during pre- and post-challenge periods. The results indicated that colonies challenged with the fungal parasite causing chalkbrood disease increased resin collection post-challenge as compared to unchallenged colonies. This increase was not due to a change in general foraging rates, since there were no significant differences in the change in pollen foraging rates. Additionally resin-collection did not increase in response to challenge with the *P. larvae* or *M. anisopliae*. The evidence shown here indicates that resin collection by honey bees is a novel example of self-medication.

1. INTRODUCTION

The constant pressure posed by parasites has caused species throughout the animal kingdom to evolve a suite of mechanisms to resist or tolerate infection (Schmid-

Hempel 1998, Evans and Spivak 2010). Parasites in this sense include macroparasites (e.g. arthropods) and microparasites (e.g. pathogens such as bacteria, fungi) that live on or in a host and reduce its overall fitness. Individual barriers that directly protect against invasion include mechanical (e.g. an insect's cuticle) and physiological defenses (e.g. inducible antimicrobial peptides). However organisms also exhibit various behavioral traits to either prevent exposure to parasites or reduce the impacts of infection (Hart 1990). One such example is the alteration of the host diet after invasion of a parasite to either combat the physiological stress imposed by the infection (e.g. Lee et al. 2006) or to directly inhibit parasite reproduction and growth (e.g. Hutchings et al. 2003, Singer et al. 2009). In some cases, these behavioral modifications in diet choice can be classified as self-medication (zoopharmacognosy or simply pharmacognosy). To truly classify a trait as self-medication in animals, it should be adaptively plastic, meaning that an individual should perform the behavior at higher rates when parasitized and at lower rates or not at all when healthy (Singer et al. 2009).

While the best-studied examples of self-medication include ingestion of whole leaves by various primate species to eliminate nematode parasites (e.g. Wrangham 1995, Huffman et al. 1996, reviewed by Hutchings et al. 2003), other cases also involve the use or ingestion of secondary plant metabolites (specifically pharmacophagy). Ingestion of nectar alkaloids by bumble bees has been shown to reduce infection intensity in laboratory experiments (Manson et al. 2010), but it is unclear if infected individuals actively prefer these type of food resources. The clearest example of self-medication by an insect was recently demonstrated with wooly bear caterpillars (*Grammia incorrupta*) that naturally feed on host plants containing non-nutritive pyrrolizidine alkaloids (PA). A series of lab studies determined that a PA-rich diet improves survival of parasitized individuals, imposes a fitness cost to non-parasitized individuals, and is preferentially consumed by infected individuals (Singer et al. 2009).

The use of antimicrobial plant resins across the animal kingdom also provides some evidence for self-medication; however most cases merely describe prophylactic behaviors rather than true self-medication (e.g. Gompper and Hoylman 1993, Choe and

Rust 2007) and can be more specifically termed pharmacophorous behaviors as the plant material is not ingested (Konig 1988). For example, several studies have determined that the aromatic leaves used in nest construction by European starlings may be negatively associated with parasite load and positively affect fledgling immunocompetence (e.g. Gwinner et al. 2000, Gwinner and Berger 2005, Mennerat et al. 2009). A series of laboratory-based studies with the Swiss wood ant (*Formica paralugubris*) have determined that resin within a nest reduces microbial load (Christe et al. 2003) and can lead to increased survival of parasitized individuals (Chapuisat et al. 2007, Castella et al. 2008a). However, it is clear that this behavior is prophylactic, as individual ants do not increase resin collection when parasite-challenged (Castella et al. 2008b).

Honey bees (*Apis mellifera*) collect plant resins from a variety of plant species worldwide. In temperate regions it is commonly thought that *Populus* spp. are the main sources (Ghisalberti 1979; Crane 1990), while in tropical regions resin-producing floral resources (e.g. *Clusia spp.*) and herbaceous shrubs (e.g. *Baccharis dracunculifolia*) are commonly used (e.g. Salatino et al. 2005). Feral honey bee colonies in tree cavities line the entire nest interior with a thin layer of resin mixed with varying amounts of wax in what has been termed the propolis envelope (Seeley and Morse 1976, Simone-Finstrom and Spivak 2010). Propolis is the apicultural term for bee-collected resins used within a hive. Since propolis is easily accessible in managed honey bee colonies, much research has been conducted concerning the chemical properties of these resin mixtures largely in aims to improve human health (reviewed in Bankova 2005b). It is known that propolis extracts are effective against a wide range of parasites, including bacteria, fungi, and other larger parasites (Mlagan and Sulimanovic 1982, Garedew et al. 2002, Antunez et al. 2008, Bastos et al. 2008, reviewed by Simone-Finstrom and Spivak 2010). Previous studies have also shown that honey bees in a resin-enriched hive are able to reduce individual investment in immune function due to an overall decrease in colony bacterial loads (Simone et al. 2009). In this way propolis use by honey bees is prophylactic and functions as a type of social immunity (Cremer et al. 2007), whereby the incorporation of resins in the nest by individual honey bees benefits colony-level immunity.

The goal of this paper was to test the hypothesis that resin collection and use by honey bees is also a type of true self-medication. The experiments presented here were done over three years of study to determine and confirm any effects of parasite challenge on the regulation of resin foraging behavior at the colony level.

2. MATERIALS AND METHODS

2.1. Experimental design

2.1.1 2008 setup

Twenty-three colonies were matched for population size and were each transferred into a new 9-frame standard commercial beekeeping box. The colonies were maintained in two apiaries in Carver County, Minnesota (12 in one apiary, and 11 in the other). Colonies at the start of the experiment (June 2008) all had 4 combs of honey and pollen, 3 combs of open and sealed brood, and 2 empty combs. Resin treatments were also applied to 12 of the colonies, six in each apiary. The inside walls of the hive box (including bottom) of 12 colonies (6 in each apiary) were painted with 92.5g of Minnesota-derived propolis (approximately 350mL of a 26.5% extract) to make them resin-rich and mimic the propolis envelope seen in feral honey bee colonies. The boxes of 11 resin-poor colonies (6 in one apiary and 5 in the other) were painted with the same volume of 70% ethanol (the solvent of the propolis extract). Extracts were prepared by dissolving propolis in 70% ethanol for at least 2 weeks and then filtered, following described methods (Simone et al. 2009). MN propolis was used in this experiment as it has been previously shown to reduce bacterial loads of colonies and affect individual immune expression of honey bees (Simone et al. 2009).

Colonies were maintained throughout the course of the experiment in one box, but additional boxes, “honey supers,” were added as colonies grew to accommodate honey storage (brood was restricted to the one original box).

The numbers of resin foragers returning to the hive during the pre-challenge (July) and post-challenge (August) periods were determined to assess any effects of a pathogen challenge on resin collection and use. If infected colonies collected more resin

post-challenge relative to the pre-challenge, with respect to the change in the unchallenged colonies, then it would support the hypothesis that honey bees are utilizing resin as a form of self-medication. Colony entrances were closed for 15 minutes for two days a week for two weeks between 1200h and 1600h both pre- and post-challenge. Honey bees returning to the hive with resin on their corbiculae within this time were placed in a wire cage to prevent repeated counts. The total number of resin foragers captured within 15 minutes was then determined. To control for any effects on general foraging levels, the number of pollen foragers that returned within the first 3 minutes was also counted. Fifteen minutes was required for resin forager counts as resin foragers represent a minority of the total foraging force (less than 10% of active foragers; Nakamura and Seeley 2006, Simone-Finstrom personal observations). A scan of the number of pollen foragers at 3 minutes was used as a reference for the total foraging force as the total number of pollen foragers at 15 minutes would likely have been too high to accurately count.

Colony population sizes were estimated at the end of the experiment (September, 2008) by counting the total number of combs of bees in the colonies (both in the bottom, brood box and in the honey supers) and the number of combs with sealed brood. A comb from the brood box covered in bees has approximately 2,430 adult bees (Burgett and Burikam 1985) and a comb from the shallower honey super box has approximately 1,200 bees. The total number of combs with worker pupae (sealed brood) was also counted with combs being divided into quarter sections. Since each comb has approximately 3600 cells per side (MAFF 1998), this was used to calculate estimated brood areas in the colonies. Percent infestations of the parasitic mite *Varroa destructor* were also determined at the end of the experiment by collecting approximately 300 bees from a comb containing brood in a jar with 70% ethanol (Lee et al. 2010). Mites were then separated from the bees and counted using the alcohol wash method (DeJong et al. 1982). Assessing levels of infestation of *V. destructor* is a helpful indicator of colony health since high infestations can cause colony death either from direct effects or indirect effects related to

the viruses they vector, but all colonies do generally have at least a low-level of *Varroa* (e.g., under 5% infestation).

2.1.2 2009 and 2010 setup

Colonies were established in four-frame "nucleus" colonies with naturally mated sister queens. Colonies were maintained for 2 months until all bees in the colonies were daughters of their respective queen. Following the methods to assess resin-foraging and pollen-foraging levels used in 2008, colony entrances were closed for 15 minutes on 6 days over a two-week period both pre- and post-challenge. The number of returning resin foragers was counted over these 15-minutes periods by placing bees with resin on their corbiculae in wire cages. Returning pollen foragers were identified by their respective corbicular loads and counted after the first 3 minutes the entrances were closed. All colonies were examined from 1230h to 1630h each day.

The parasite challenge was conducted during a three-week period between the pre-challenge (July) and post-challenge (August) foraging counts. Colonies received their challenge once weekly for three weeks. To finish the experiment, colony population sizes were determined by estimating the number of adult bees and number of capped pupae based on calibrated photographs (MAFF 1998). Percent infestations of the parasitic mite *Varroa destructor* were also determined at this time in 2010.

2.2 Parasites of interest

To test whether the frequency of resin collection increased in response to colony-level microbe and parasite loads, we chose three common parasites: the fungal agent of the brood disease chalkbrood (*Ascosphaera apis*), the bacterial agent of the larval disease American foulbrood (*Paenibacillus larvae*), and the entomopathogen *Metarhizium anisopliae*.

Chalkbrood (CB) infection occurs as an early-stage honey bee larva ingests *A. apis* spores in brood food, which results in its eventual death as the larval gut is penetrated by the growing fungal mycelia (Morse and Flottum, 1997). The fungus

undergoes rapid growth and sporulation around the time of larval defecation, overwhelming the infected larva and effectively turning it into a CB "mummy" (reviewed in Aronstein and Murray, 2010). Due to the nature of the disease, severe infections can reduce colony growth by as much as a third (Tarpy, 2003).

American foulbrood (AFB) infection follows a similar route. Larvae ingest spores in brood food and eventually die as the spores germinate and produce the vegetative form that then produces more spores (Morse and Flottum, 1997). Dead larvae eventually dry and turn into AFB scales containing millions of spores. AFB is an extremely contagious bacterial parasite that can cause colony death (Morse and Flottum, 1997).

Metarhizium anisopliae is a soil-borne fungal parasite that infects a variety of social and non-social insects. It is not, however, a recognized parasite of honey bees. We chose to use *M. anisopliae* as a control to determine if resin collection frequency changes simply in response to a more general increase in fungal load in colonies or if any changes are specific to honey bee-related parasites. *M. anisopliae* has been shown to not affect honey bee individual or colony development (Kanga et al. 2003), and so in the case of our current study merely serves to increase microbial load of the treated colonies possibly more similar to what colonies might encounter when starting a new nest in a tree cavity.

2.3 Parasite challenge

2.3.1 2008 challenge with CB

Pollen "patties" (225 g) were given to every colony during the challenge period. Control pollen patties consisted of a mixture of 25% commercially available pollen substitute (Feedbee, Bee Processing Enterprises, Ltd.), 42% frozen pollen collected from hives in 2007, and 33% of a 50% sucrose solution. For CB infection, 40 homogenized fresh white and black CB mummies collected from non-experimental colonies were combined into approximately 5500g of the pollen patty mixture used for the experimental challenge (adapted from Gilliam et al. 1988). Twelve colonies (6 resin-rich and 6 resin-poor) were given the chalkbrood pollen patties, and 11 colonies (6 resin-rich and 5 resin-poor) were given the control formulation. The respective pollen patties were given to

colonies twice within a two-week period. Level of chalkbrood infection was determined for each colony by counting the number of mummies (dead larvae covered with fungal mycelia) present in each frame or on the bottom of the hive once in the middle of treatment and again at the end of the experimental period.

2.3.2 2009 challenge using multiple parasites

Nine colonies were used for each of the three following parasite-challenge treatments, and an additional nine colonies were used as unchallenged controls located in one apiary on the St. Paul campus of the University of Minnesota. All 36 colonies were given "pollen patties" consisting of 30g of mixed-source pollen and 15mL 50% sugar. Ten fresh white and black mummies were pulverized and mixed into the pollen patty for the 9 CB-challenged colonies (adapted from Gilliam et al. 1988). The AFB-challenge was done by introducing a 7.5cm square section of comb from an AFB-infested colony that contained AFB larval scales into 9 other colonies, following the methods of Spivak and Reuter (2001). For exposure to *M. anisopliae*, the inside floors of the remaining 9 colonies were dusted with 75g of a powdered form of *M. anisopliae* ECS1 containing approximately 1×10^{10} conidia per gram. This amount has been shown to not adversely affect colony development or health (Kanga et al. 2003). All treatments were completed twice during the two-week challenge period. Levels of infection due to the challenges were measured at the end of the experiment.

2.3.3 2010 challenge with CB

This third year of study aimed to expand upon one treatment in particular, CB infection. For this experiment, again located on the St. Paul campus of the University of Minnesota, 7 colonies were exposed to CB and 7 colonies were used as unchallenged controls. CB challenge followed the same methods used in 2009.

2.4 Data analysis

The total numbers of resin and pollen foragers counted during the observational periods were summed for each colony both before and after the parasite challenge. To determine change in the foraging rates, the log differences in the total sum of foragers before and after the challenge were calculated [$\log_{10}$(after sum) – $\log_{10}$(before sum)] as a way to describe the ratio or percent change in resin foraging across the two time periods. Statistical differences were determined for an effect of the parasite challenge on number of resin foragers, number of pollen foragers, and colony demographics (including population size and parasite loads) using separate ANOVAs followed by Tukey's HSD post-hoc mean comparisons, when necessary, since the raw data was normally distributed. For the 2008 data, a two-way ANOVAs modeling resin treatment and CB-challenge as main effects were used to determine effects of the resin treatment, CB-challenge and the interaction on population size or foraging rates. When no significant differences were determined due to resin treatment, colonies were combined for analysis based on CB-challenge. Lastly data from unchallenged and CB-challenged colonies over the three years was combined and analyzed using a two-way ANOVA testing for effects of year and CB challenge, followed by post-hoc Tukey's HSD mean comparison.

For the 2008 data, one unchallenged colony that had CB symptoms during both assessment time points was removed from the analysis as were colonies that had no resin foragers during either the pre- or post-challenge periods (three unchallenged and two CB-challenged). This resulted in sample sizes of n=9 for unchallenged colonies, n=11 for CB-challenged colonies. For the 2009 data, one unchallenged colony and one AFB-challenged colony that exhibited symptoms of CB infection, and one CB-challenged colony that exhibited symptoms of another parasite-infection (deformed wing virus) were removed from all analyses. There were 8 colonies analyzed for AFB, CB and unchallenged treatments, and 9 *Metarhizium*-treated colonies. For the 2010 analyses, one control colony with mild clinical CB symptoms was removed, resulting in n = 6 for the unchallenged and n=7 for CB-challenged colonies.

3. RESULTS

3.1 2008 challenge with CB

Infection levels of CB were monitored twice during the post-challenge period for all colonies. The 5 resin-poor challenged colonies and 4 out of 6 resin-rich challenged colonies exhibited CB clinical symptoms. The average total number of mummies counted in CB-challenged colonies over two days (once in the middle of treatment and once at the end of the experiment) was 129.8 ± 53.9 (SE) CB mummies in the six resin-poor challenged colonies and 14.7 ± 7.5 (SE) mummies in the six resin-rich challenged colonies (see Figure 4.1). None of the five resin-poor unchallenged colonies exhibited CB symptoms during the course of the experiment. However three of the four resin-rich unchallenged colonies used in analyses had CB mummies present at low and non-persistent levels due to natural infection, with an average total number of mummies being 5.7 ± 2.0 (SE); two colonies were positive only at the early assessment, and one colony was positive only at final assessment. Analyzing for differences in the sums of mummies found across treatments, the resin-poor challenged colonies had a significantly higher infection intensity compared to the two resin-rich treatments and the resin-poor unchallenged treatment, which were not significantly different from each other (resin treatment and CB-challenge interaction effect: $F_{1, 16} = 4.78$, $p = 0.04$).

Resin treatment had no significant effect on the change in resin foraging rates ($F_{1, 14} = 0.01$, $p = 0.90$) or pollen foraging rates ($F_{1, 14} = 0.07$, $p = 0.79$) based on a two-way ANOVA modeling resin treatment and CB-challenge as main effects (one colony from each of the four treatments with zero sum for resin foragers either for the pre- or post-challenge periods were removed. The number of resin foragers in the resin treated and untreated colonies were combined for remainder of analysis (N=7 unchallenged colonies and N=9 challenged colonies). The mean number of resin foragers and pollen foragers counted during the pre- and post-challenge periods can be found in Table 5.1. The mean $\log_{10}$ difference in the total number of resin foragers pre- and post-challenge from the unchallenged colonies was -0.09 ± 0.10 (SE), while the mean for CB-challenged colonies was 0.21 ± 0.16 (SE); these values were not significantly different ($F_{1, 14} = 2.38$, $p = 0.14$).

The mean $\log_{10}$ difference in the total number of pollen foragers for the unchallenged colonies was 0.42 ± 0.09 (SE), and 0.24 ± 0.19 (SE) for the CB-challenged colonies; these values were not significantly different ($F_{1, 14}$= 2.53, p = 0.13).

Colony population sizes at the end of the experiment in early September were as follows (see Figure 4.2): resin-poor unchallenged colonies had on average 27,094 ± 2,172 adult bees and 9,580 ± 1,129 cm^2 of sealed brood; resin-poor challenged colonies had a mean of 18,589 ± 2,251 (SE) adult bees and 7,225 ± 980 (SE) cm^2 of sealed brood; resin-rich unchallenged colonies had on average 24,451 ± 2,517 adult bees and 7,839 ± 1,097 cm^2 of sealed brood; and resin-rich challenged colonies had a mean of 23,388 ± 1,983 (SE) adult bees and 8,851 ± 897 (SE) cm^2 of sealed brood. Based on a two-way ANOVA, there was an effect of CB-challenge on adult bee populations, with CB-challenge causing a decrease in total number of adult bees ($F_{1, 14}$= 4.42, p = 0.05). There was a trend but non-significant interactive effect with the resin-poor challenged colonies having smaller adult populations as compared to the resin rich colonies (see Figure 4.2; $F_{1, 16}$= 2.68, p = 0.12). There was also a non-significant trend for an interactive effect of resin treatment and CB-challenge on the amount of brood in colonies with resin-poor challenged colonies having less brood compared to the other colonies ($F_{1, 16}$= 2.88, p = 0.11).

Levels of the parasitic mite *Varroa destructor* were also determined at the end of the experiment. The average percent infestation was 3.33% ± 1.65 (SE) for unchallenged colonies, and 5.09% ± 1.58 (SE) for CB-challenged colonies. These infestation values are relatively low and below estimated treatment thresholds (Delaplane and Hood, 1999).

Table 4.1. Means ± SE of numbers of resin and pollen foragers counted pre- and post-challenge and the means of the Log_{10} differences ± SE between the sum of foragers at the two time points for each year of study. Significant differences within a year are indicated by letters after the values (see section 3.1 for analysis of 2008 data, 3.2 for 2009 data, 3.3 for 2010 data, and 3.4 for a combined analysis of the data).

Resin Foragers				
year	**Treatment**	**pre-challenge**	**post-challenge**	**Log_{10} difference** Log_{10} (after) - Log_{10} (before)
2008	unchallenged	9.14 ± 2.94	8.43 ± 3.72	-0.09 ± 0.10
	CB-challenged	3.78 ± 1.05	6.22 ± 1.69	0.21 ± 0.16
2009	unchallenged	10.50 ± 2.16	9.37 ± 2.38	-0.12 ± 0.11 ***a***
	CB-challenged	8.12 ± 2.68	11.75 ± 0.84	0.41 ± 0.19 ***b***
	AFB-challenged	10.75 ± 4.45	11.12 ± 4.01	0.03 ± 0.16 ***ab***
	Metarhizium	10.00 ± 3.29	5.89 ± 1.72	-0.28 ± 0.12 ***b***
2010	Unchallenged	7.33 ± 4.63	6.33 ± 3.33	-0.06 ± 0.08
	CB-challenged	6.86 ± 1.28	7.57 ± 3.50	0.06 ± 0.08
Pollen Foragers				
year	**Treatment**	**pre-challenge**	**post-challenge**	**Log_{10} difference** Log_{10} (after) - Log_{10} (before)
2008	Unchallenged	36.28 ± 3.43	105.14 ± 20.27	0.42 ± 0.09
	CB-challenged	34.78 ± 4.25	63.00 ± 11.04	0.24 ± 0.19
2009	Unchallenged	41.37 ± 4.74	66.12 ± 4.02 ***a***	0.22 ± 0.07
	CB-challenged	44.25 ± 4.97	51.87 ± 4.91 ***b***	0.07 ± 0.03
	AFB-challenged	37.50 ± 4.66	56.75 ± 6.13	0.18 ± 0.06
	Metarhizium	32.00 ± 3.61	47.22 ± 10.47	0.11 ± 0.10
2010	unchallenged	93.67 ± 9.88	63 ± 5.35	-0.17 ± 0.04
	CB-challenged	79.28 ± 11.39	63.43 ± 6.75	-0.07 ± 0.10

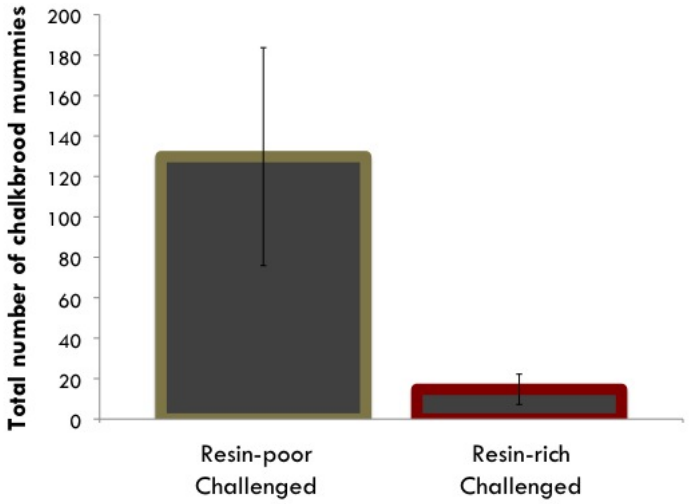


Figure 4.1. Chalkbrood infection intensity for resin-poor and resin-rich CB-challenged colonies based on the number of CB mummies found in colonies post-challenge (2008). N=5 colonies for the resin-poor and N=6 for the resin-rich challenge treatments.

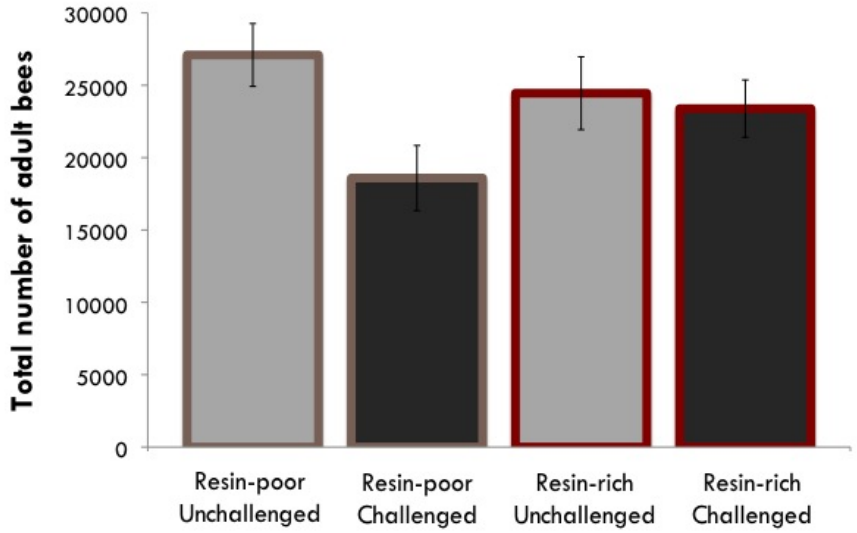


Figure 4.2. Estimated counts of the colony adult bee population in colonies in 2008. N=5 colonies for both the resin-poor unchallenged and resin-poor challenged treatments. N= 4 colonies for the resin-rich unchallenged treatment and N=6 for the resin-rich challenged treatment.

3.2 2009 challenge using multiple parasites

For infection intensities due to the treatments, only one CB-challenged colony exhibited mild clinical symptoms of CB (the presence of larval CB mummies) and two AFB-challenged colonies had symptoms of AFB infection. However other CB and AFB-challenged colonies had several partially removed larvae and pupae, which is indicative that they were infected with the disease but that bees were performing hygienic behavior, a trait in which adult bees detect and remove larvae infected with CB or AFB before they exhibit clear visible symptoms and become infectious (Rothenbuhler 1964; Spivak and Gilliam 1998; recently reviewed by Wilson-Rich et al. 2009).

The mean number of resin and pollen foragers counted during the pre- and post-challenge periods for the unchallenged, CB-challenged, AFB-challenged, and *Metarhizium*-treated colonies are shown in Table 4.1 (and see Figure 4.3). A significant difference due to treatment was determined with respect to a change in the total number of resin foragers after parasite challenge ($F_{3, 29}$= 4.27, p = 0.01). Post-hoc tests confirmed that CB-challenged colonies increased resin collection after the challenge as compared to unchallenged (Tukey's HSD post-hoc: p = 0.02) and *Metarhizium*-treated colonies (Tukey's HSD post-hoc: p = 0.008). The change in total number of resin foragers seen for AFB-challenged colonies was not significantly different from any treatment (p > 0.28 for each comparison). This difference in rate of resin collection due to the CB challenge was not simply due to an increase in general foraging rates, as there were no significant differences in the log difference of total pollen foragers before and after parasite challenge across treatments ($F_{3, 29}$= 0.85, p = 0.47, see Table 4.1).

At the end of the experiment (September 2009), the population size and parasite loads were assessed for each colony. There were no differences in the total number of bees ($F_{3, 29}$= 0.44, p = 0.72) or in the total amount of brood ($F_{3, 29}$= 0.44, p = 0.72) across treatments. Colonies had on average 5,627 ± 303 (SE) adult bees and an average number of sealed larval cells containing developing pupae of 4749 ± 321 (SE). Percent infestations of *Varroa destructor* were not determined in 2009, but the original colonies from which the starting bee populations were derived had infestation levels at

approximately 4%, which is below estimated treatment thresholds (Delaplane and Hood, 1999).

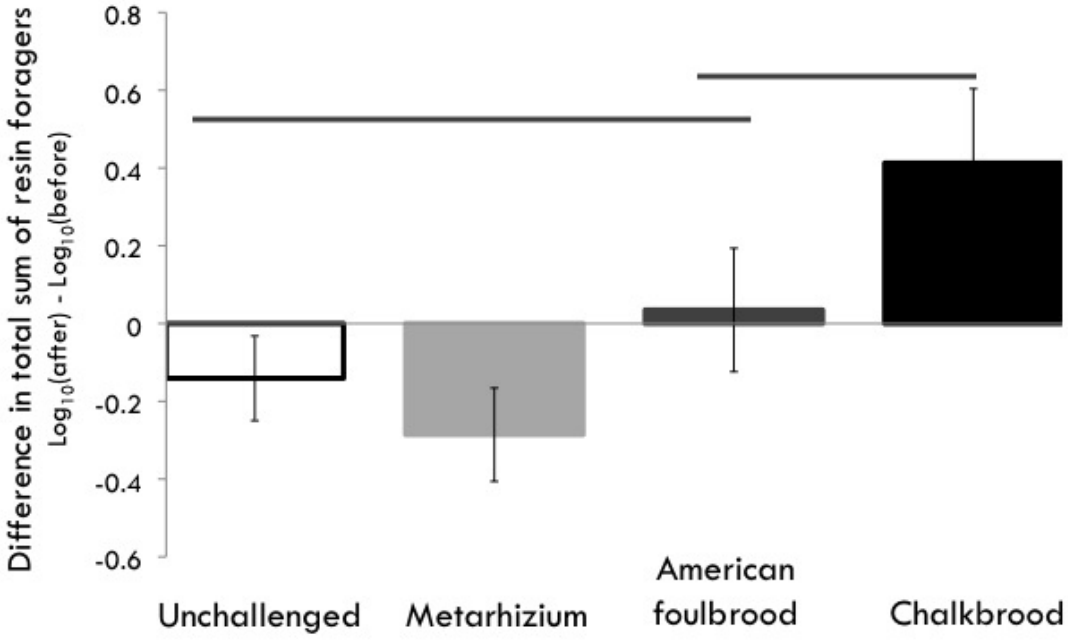


Figure 4.3. Difference in the total sum of resin foragers before and after a parasite challenge. Bars not connected by lines are significantly different ($F_{3, 29}$= 4.27, p = 0.01). N=8 colonies for the unchallenged, American foulbrood, and chalkbrood treatments. N=9 colonies for the *Metarhzium* treatment.

3.3 2010 challenge with CB

CB-challenged colonies in 2010 did not exhibit any clinical symptoms of CB-infection, despite the three doses of CB in pollen patties. The means for the number of resin foragers and the number of pollen foragers counted pre- and post challenge are shown for unchallenged and challenged colonies in Table 4.1. The mean $\log_{10}$ difference in the total number of resin foragers pre- and post-challenge from the unchallenged colonies was -0.06 ± 0.08 (SE). The mean for CB-challenged colonies was 0.06 ± 0.08

(SE). There was not a significant difference in the change in resin foraging rate after the CB challenge ($F_{1,11}$=1.03, p = 0.33). The rate of pollen foraging did not change with respect to the challenge either ($F_{1,11}$=0.63, p = 0.44). The mean $\log_{10}$ difference for pollen foragers was -0.17 ± 0.03 (SE) for unchallenged colonies and -0.07 ± 0.10 (SE) for CB-challenged colonies.

At the end of the experiment (Sept 2010), the population size and parasite loads were assessed for each colony. There were no differences in the total number of bees ($F_{1,11}$=0.82, p = 0.38) or in the total amount of brood ($F_{1,11}$=0.77, p = 0.40) due to the CB-challenge. Colonies had on average 10,565 ± 364 (SE) adult bees and an average number of sealed larval cells containing developing pupae of 5413 ± 626 (SE). The average percent infestation was 1.22% ± 0.53 (SE) for unchallenged colonies, and 0.95% ± 0.29 (SE) for CB-challenged colonies. These infestation values were very low and well below estimated treatment thresholds (Delaplane and Hood, 1999).

3.4 Combined analysis of CB challenge across years

Data across the three years of study were combined to determine if CB-challenge induces resin collection in honey bees. Using the $\log_{10}$ difference between number of resin foragers pre- and post-challenge, CB-challenged colonies increased in resin collection (mean: 0.23 ± 0.07) while unchallenged colonies decreased or stayed approximately the same (mean: -0.09 ± 0.08). A two-way ANOVA using year and CB challenge determined that CB significantly increased resin collection ($F_{1, 39}$= 8.37, p = 0.006; see Figure 4.4). The year of study was not a factor in this difference (main effect of year: $F_{2, 39}$=1.07, p = 0.35; interaction effect: $F_{2, 39}$=1.14, p=0.33).

The increase in the rate of resin foraging post-challenge was not a result of an increase in general foraging rates in CB-challenged colonies, as indicated by the pollen forager counts (main effect of CB challenge: $F_{1, 39}$= 1.73, p = 0.20; see Figure 4.5). However, analyses indicated that there were significant differences across years for the $\log_{10}$ difference of pollen foragers before and after challenge irrespective of CB-

challenge (main effect of year: $F_{2, 39}$=18.78, $p < 0.0001$; interaction effect: $F_{2, 39}$=1.99, p=0.15), with colonies in 2008 having the greatest increase in pollen foraging in August relative to July, colonies in 2009 having an intermediate increase, and colonies in 2010 having a general decrease in pollen foraging in August ($p < 0.05$ for each post-hoc comparison).

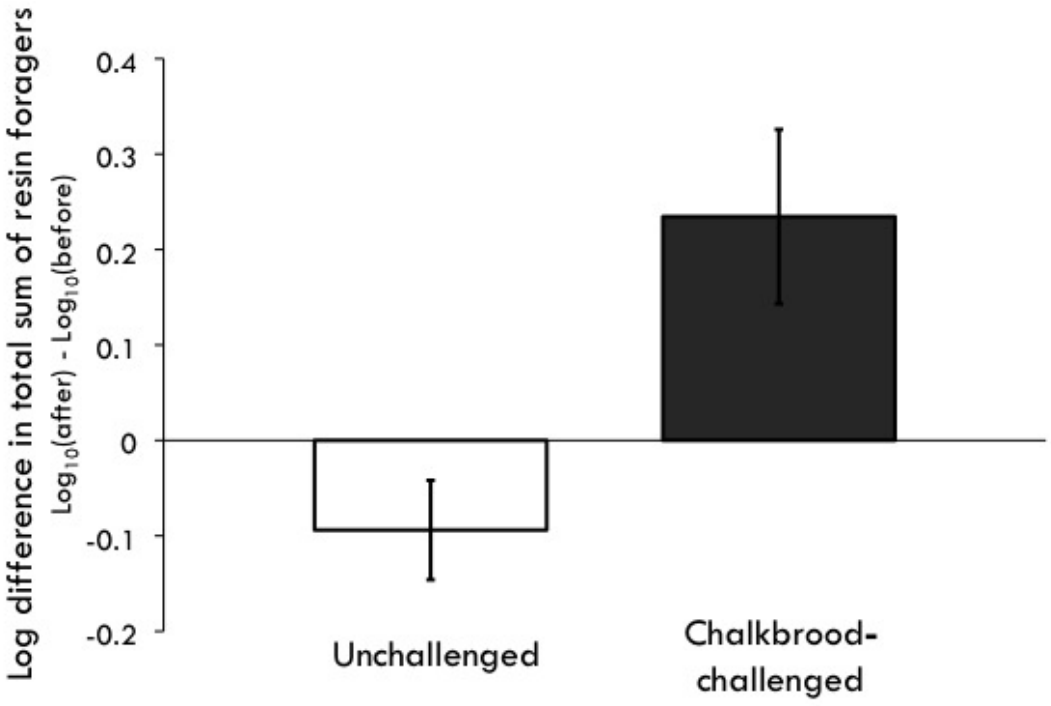


Figure 4.4. Difference in resin foraging rates between pre- and post-challenge periods for unchallenged and CB-challenged colonies combined over the three years of study. N= 21 unchallenged colonies and N=24 CB-challenged colonies.

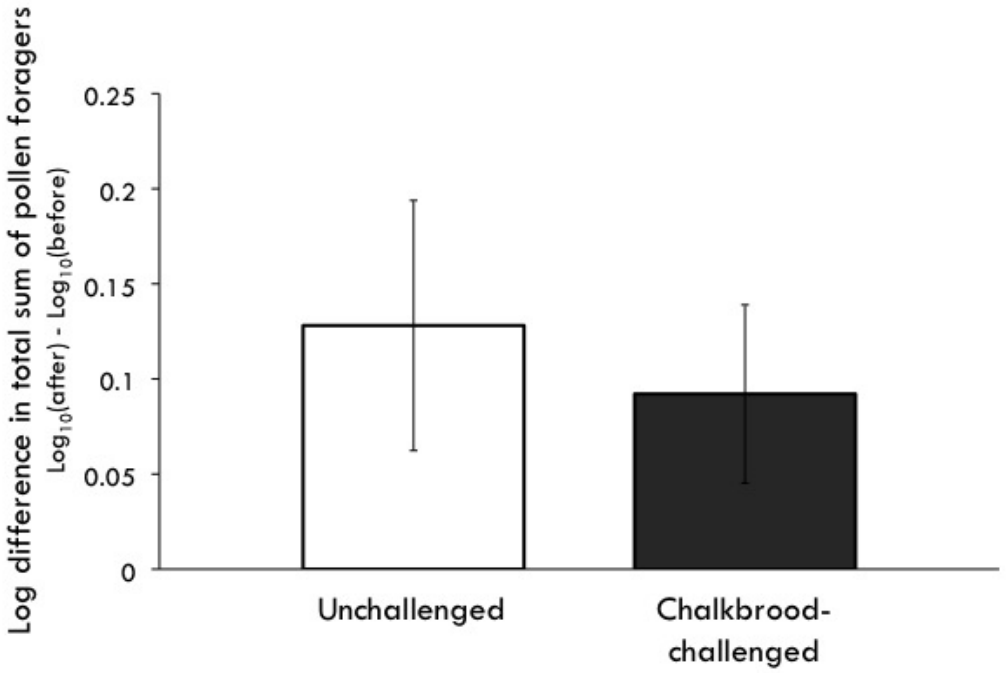


Figure 4.5. Difference in pollen foraging rates between the pre- and post-challenge periods for unchallenged and CB-challenged colonies combined over the three years of study. N= 21 unchallenged colonies and N=24 CB-challenged colonies.

4. DISCUSSION

We have determined that the rate of resin foraging increases when honey bee colonies in a field experiment are exposed to the fungal agent of the larval disease chalkbrood (CB, *Ascosphaera apis*), suggesting that honey bees may be self-medicating in response to this particular pathogen. While significant differences were not found for each year of study, trends were apparent, and when all three years were combined the difference due to CB challenge was clear. Our finding that resin collection increases in response to CB-challenge was surprising and exciting, especially considering since resin collection by another social species has been shown to be merely a prophylactic behavior (e.g. Castella et al. 2008b).

There are two unique aspects of resin foraging in honey bees that are intriguing with respect to self-medication: 1) honey bees do not ingest resin; 2) larval, not adult, honey bees are parasitized by CB. Most other instances of self-medication seen in vertebrates and invertebrates involve individuals changing their diet in response to direct infection with a parasite (pharmacophagy). In this case with honey bees, individuals are not ingesting resins (pharmacophory) and they are not infected. Chimpanzees with active nematode infections swallow whole leaves (Wrangham, 1995; Huffman et al. 1996; Fowler et al. 2007); parasitoid-infected *G. incorrupta* caterpillars ingest non-nutritive alkaloids (Singer et al. 2009); *Spodoptera littoralis* caterpillars preferentially consume high protein diets when infected with a virus (Lee et al. 2006). In the latter two examples with solitary insects, the ingestion of these compounds results in strong fitness costs when individuals are not infected. Although bees do not consume resins, foraging for resin is likely costly at the individual level because it is time-consuming to handle both at source and in the hive and provides no obvious direct food reward as does foraging for nectar or pollen. However, resin collection does function a mechanism of social immunity. The incorporation of resin in the nest environment reduces general bacterial loads in the colony, either by inhibition due to direct contact or by the volatile compounds released (e.g. Kujumgiev et al. 1999), and therefore allows individuals to invest less in immune function (Simone et al. 2009). Since high activation of individual immunity can have colony-level fitness costs (Evans and Pettis, 2005), traits that reduce chronic elevation of an individual's immune response may be of benefit to colony productivity. Therefore any costs to the individual may be offset by the benefits of resin collection to the colony, since individual fitness is largely determined by colony success in honey bees.

The data presented here suggests that in addition to the potential indirect immune effects, resins may also be collected by honey bees in response to exposure to a specific fungal parasite. This is a novel instance of self-medication where individual expression of a behavioral trait is altered due to exposure at the level of the social group. Since only larvae are affected by this particular parasite (CB), a change in adult behavior in response

to increase levels of parasite that does not directly affect them is particularly interesting. It must be noted that CB spores were dispersed within a mixture of pollen and that therefore adult bees handled and possibly ingested these spores. However, spores do not germinate within the gut of an adult bee but can remain there (Gilliam et al. 1988) and adults are the major distributor of CB spores throughout a colony through larval feeding (Morse and Flottum, 1997; Aronstein and Murray, 2010). Individual adult bees were then exposed to an overall increase in fungal spores as a result of the CB challenge even though colonies only exhibited mild disease symptoms or lacked clinical symptoms (as seen in most colonies in 2009 and 2010). In terms of a possible direct effect of resins against CB, there is evidence that propolis extracts are effective *in vivo* against a variety of fungal parasites (Dobrowolsky et al., 1991; Messer, 1995; Kujumgiev et al. 1999; Ota et al., 2001), but there is limited previous knowledge on the direct effects that propolis may have against CB in lab cultures (Samŝiňáková et al. 1977). However, the results presented here as part of the 2008 study suggest that a resin-rich environment may reduce CB-infection intensity and may therefore that resins in the hive may have an inhibitory effect on the growth of this fungus.

Our finding that resin collection only increases in response to challenge with CB and not AFB or *Metarhizium* is particularly curious and warrants confirmation through further study. A lack of a similar response in the colonies treated with the entomopathogen *Metarhizium* compared to the CB-challenged colonies could be due to the fact that honey bees are not normally exposed to this type of soil-borne fungus or that our treatment with *Metarhizium* was too heavy in comparison to the amount of the fungal parasite given to the CB-challenged colonies. Honey bees routinely remove debris from the floor of the colony rather quickly by actively taking it out of the nest (Winston, 1991). Since the *Metarhizium* was not growing on the hive walls as a fungus might inside a tree cavity it is possible that it did not induce increased levels of resin foraging. Instead it is likely that *Metarhizium*-treated colonies focused more on directly removing the powder from the hive.

With respect to the AFB-challenged colonies, there are several possible factors that may have influenced the lack of difference we saw in these colonies compared to the unchallenged colonies. One may be that individual immune responses of adult and larval bees may be a more generally effective defense against bacterial parasites than fungal parasites. While cellular immune mechanisms (e.g. cellular encapsulation) are likely involved in the individual defense against fungal parasites (Gliñski and Jarosz, 2001; Strand, 2008), the suite of physiological defenses, and particularly the antimicrobial peptides, of honey bees appear to be geared more toward controlling bacterial parasites (e.g. Evans et al. 2006). Secondly, behavioral mechanisms involved in social immunity, like hygienic behavior (the removal of diseased brood before it becomes infectious), can also control AFB infection at the colony level (e.g. Spivak and Reuter, 2001). Thus it is possible that resin collection is not significantly increased in response to AFB-infection due to the other effective defenses against this bacterial parasite. On the other hand, hygienic behavior is also an effective defense against CB (e.g. Gilliam et al., 1988), so there are multiple defenses against this fungal parasite. Additionally, propolis extracts have been shown to exhibit activity against AFB in laboratory cultures (e.g. Bastos et al. 2008) and in field colonies fed propolis extracts in sugar syrup (Antunez et al. 2009), and a resin-rich environment has been shown to reduce the general bacterial loads in colonies (Simone et al. 2009). Thus it would seem that self-medicating with resin against bacterial infection, in addition to a fungal infection, would be an adaptive response. In fact, resin collection was marginally increased in 2009, as indicated by the lack of difference between AFB-challenged colonies and CB-challenged colonies. While it is clear that bees use both individual and social immunity to fight parasitic infection, the results of this study highlight that we are just beginning to understand when and how they may use one or the other or both.

The behavioral mechanism involved in the initiation of honey bee resin foraging in response to colony-level challenge with a specific fungal parasite is currently unknown, and is also an excellent area for future research. Typically in cases of self-medication of which most concern vertebrate species, associative or social learning is

involved (Lozano, 1998; Villalba et al. 2006, Huffman et al. 2010). However the few insect cases that appear to exhibit self-medication do not necessarily involve learning. In the case of the study presented here and the work done with the caterpillar *G. incorrupta*, parasitism simply increases the rate at which a routine behavior is performed instead of the initiation of an atypical behavior (i.e. leaf-swallowing in primates; Singer et al. 2009). The mechanisms that initiate the self-medication behavior of other insects appear to be based on internal physiology. In *G. incorrupta*, infection causes changes in an individual's peripheral nervous system and heightens activity of taste receptors for pyrrolizidine alkaloids (PA), leading to increased consumption of PA-rich food sources (Bernays and Singer 2005). One possible cue that resin foragers may use to initiate resin foraging in response to CB levels could be olfactory stimuli release from infected larvae. Larvae do release specific chemical compounds in response to CB infection and these compounds do induce hygienic behavior, a type of social immunity in which bees remove diseased larvae and pupae from the nest (Swanson et al. 2009). These compounds are released prior to clear visual development of symptoms (Swanson et al. 2009), and so infected larvae would have been producing these compounds even though CB mummies were not seen in many colonies in 2009 and 2010. It is possible that resin foragers are also sensitive to this stimulus, and this is something that could be easily tested using synthetic compounds and various lab (e.g. proboscis extension response conditioning) and field techniques as done by Swanson et al. (2009). Resin foragers may also use cues related more directly to colony microbe levels as well. Since feral colonies line the entirety of the nest interior with resins prior to and during comb construction it is possible that bacteria and fungi normally found in a tree cavity may also induce the behavior. While it appears that resin-foraging honey bees are recruited in part due to colony-level parasite loads, there are likely many other stimuli involved in the behavior that are non-mutually exclusive. Future studies should focus not only on the role of parasite infection, but also on the possible influence of the type and amount of resin in the nest and factors related to the nest structure itself (i.e. air movement, incoming light sources and rough surfaces; Simone-Finstrom and Spivak, 2010; Simone-Finstrom et al. 2010).

A host of questions still exist concerning resin collection and use by honey bees, as well as resin use across the animal kingdom in general. Its true role as a mechanism of social immunity in bees and ants could be quite complex, involving direct effects against parasites and more indirect effects on individual immunity. The sequestering of resins and secondary plant metabolites appears to be a relatively common trait, and it is possible that many species are utilizing these plant defenses as a mechanism of defense against various parasites and predators. While we have shown that resin collection by honeybees represents a novel case of pharmacophorous self-medication in an insect, it is certainly possible that this phenomenon is more widespread than previously thought.

CHAPTER 5—

Tactile learning by resin foraging honey bees [4]

Honeybees harvest and use plant resins in a mixture called propolis to seal cracks and smooth surfaces in the nest architecture. Resins in the nest may be important in maintaining a healthy colony due to their antimicrobial properties. This study had two main objectives: (1) Provide initial insight on the learning capabilities of resin foraging honeybees; (2) analyze the sensitivity of resin foraging honeybees to tactile stimuli to elucidate its possible role as a mechanism behind resin foraging. The first objective provides insight into the phenotype of these bees as compared to other forager types, while the second creates a starting point for further work on behavioral mechanisms of resin foraging. Using tactile proboscis extension response conditioning, we found that resin foragers learned to associate two different tactile stimuli, the presence of a gap between two plates and a rough sandpaper surface, with a sucrose reward significantly better than pollen foragers. The results of differential tactile conditioning exhibited no significant difference in the ability of resin foragers to discriminate between smooth and rough surfaces as compared to pollen foragers. We also determined that the sucrose response thresholds (SRTs) of returning resin foragers were lower compared to returning pollen foragers, but both resin foragers and pollen foragers learned a floral odor equally well. This is the first study to examine SRTs and conditioning to tactile and olfactory stimuli with resin foraging honeybees. The results provide new information and identify areas for future research on resin collectors, an understudied foraging phenotype.

1. INTRODUCTION

The mechanisms that regulate the onset of foraging division of labor in honeybees (*Apis mellifera*) have been widely studied over the years (reviewed by Page and Erber

[4] This chapter is published as:
Simone-Finstrom, M., Gardner, J. & Spivak, M. 2010. Tactile learning by resin foraging honey bees. *Behavioral Ecology and Sociobiology*, 64, 1609-1617.

2001). However, research has largely been restricted to nectar and pollen foraging and has generally neglected other foraging tasks, such as resin foraging. Bee species around the world collect resins from the vegetative apices and other resin secreting structures of various plants (Roubik 1989; Crane 1990). Resin foraging honeybees return to the nest with a resin load on their corbiculae, and the resin is unloaded by other bees, mixed with wax, and placed within the nest interior (Meyer 1956). When the resin is used within a honeybee colony, it is known as propolis. Feral honeybee colonies, which typically nest in tree cavities, line the entire nest interior with a thin layer of this resinous mixture, creating a "propolis envelope" (Seeley and Morse 1976). The use of propolis, and particularly this propolis envelope, may have significant benefits for general immunity, colony health, and protection against hive diseases and parasites (e.g., Garedew et al. 2002; Bastos et al. 2008; Simone et al. 2009; Simone-Finstrom and Spivak 2010).

While resin collection and use by honeybees is likely quite important in maintaining healthy colonies, little research has been conducted on this subject (for a recent review, see Simone-Finstrom and Spivak 2010). Only one known study over the last 50 years has attempted to determine some of the mechanisms involved in the control of resin use behavior. Nakamura and Seeley (2006) sought to determine how resin foragers decide whether to continue or to stop foraging for resins. This study revealed much about the behaviors of resin foragers and provided insight into the role these resin foraging bees play within a colony (Nakamura and Seeley 2006; reviewed in Simone-Finstrom and Spivak 2010). It is still unclear at the most basic level how individuals assess the need for resins in the nest and decide to start foraging. This is a particularly interesting question since there is no food reward associated with resin foraging.

Resin, as propolis, is typically deposited along all inner surfaces of a tree cavity by feral colonies to smooth the walls prior to comb construction, seal cracks and crevices, and reduce the size and number of nest entrances (Seeley and Morse 1976). This propolis "envelope" with its antimicrobial properties provides important immune benefits to honeybees (Simone et al. 2009; Simone-Finstrom and Spivak 2010). In modern hives, honeybees will smooth any rough surfaces on hive walls and reduce entrances, but

largely deposit propolis on the edges of the wooden frames on which they build comb (Simone-Finstrom and Spivak 2010). Since the nest interior is mostly dark, bees mainly rely on non-visual senses to detect stimuli within the nest environment. A number of factors may stimulate bees to collect and apply resins, including the presence of light, air currents, humidity changes, and tactile cues in surfaces of the nest cavity. Recognizing that all of these stimuli may be important, we decided to begin our investigation by determining if resin foragers are particularly sensitive to tactile stimuli such as gaps, crevices and rough surfaces. The bees' antennae are an integral tool for this type of information assessment (Erber and Pribbenow 2001; Johnson 2008). Bees, and specifically some resin handlers and foragers, have been noted to detect crevices by inserting the antenna into gaps in nest architecture (Nakamura and Seeley 2006). It is possible that foragers determine the need for resins based on this kind of tactile stimuli. This idea holds true for other social insects, like some species of paper wasps (*Polistes fuscatus*) and termites (*Nasutitermes costalis, Coptotermes formosanus*) that have been noted to detect nest damage and select building sites using antennation (Jones 1980; Downing and Jeanne 1990; Downing 1992; Lee et al. 2008).

The ability of honeybees to detect and learn various tactile stimuli is well-established (i.e., Kevan and Lane 1985; Erber et al. 1998; Scheiner et al. 2005). Much of this research has been done using classical conditioning of the proboscis extension response (PER). For PER conditioning, a bee is restrained and presented with a tactile stimulus. During the presentation the bee is given a sugar reward to elicit the PER. The tactile stimulus is presented repeatedly after specific time intervals. When the bee has learned to associate the sugar reward with the tactile stimulus, she will extend her proboscis in anticipation of the coming reward. Much of this research has been done comparing nectar and pollen foragers or with pollen foragers alone and typically use a series of small metal plates with indented grooves serving as the tactile stimulus (Erber et al. 1998; Scheiner et al. 1999, 2001, 2005). While tactile information may be relevant for pollen and nectar foragers with respect to identifying appropriate food sources (as suggested by Kevan and Lane 1985), resin foragers may directly use tactile information

within the nest environment to initiate resin foraging behavior.

This study had two main objectives: (1) provide initial insight on the learning capabilities of resin foraging honeybees; (2) analyze the sensitivity of resin foraging honeybees to tactile stimuli in order to elucidate its possible role as a mechanism behind resin foraging. The former would open a window into the phenotype of these bees as compared to other forager types, while the latter may provide a starting point for further work on this important, yet understudied, group of bees.

The initial step was to conduct the first studies on PER conditioning with resin foragers. Specifically, we aimed to determine if resin foragers are more sensitive to the presence of a gap as compared to pollen foragers, and if any observed differences would also be seen for conditioning to an odorant. We followed this by performing an analysis of sucrose response thresholds (SRTs) of resin and pollen foragers, as a bee's SRT is correlated with learning performance (e.g., Scheiner et al. 1999, 2001). Finally, we utilized differential tactile conditioning using a smooth and rough substrate to determine if resin foragers are better able to discriminate between tactile cues as compared to pollen foragers. This is the first study to examine the SRTs and conditioning to tactile and olfactory stimuli with resin foraging honeybees.

2. MATERIALS AND METHODS

2.1 Animals

General methods for proboscis extension response conditioning to the tactile stimulus followed those by Erber et al. (1998). Similar methods were used for conditioning to the odor, but followed those previously used (Masterman et al. 2000, 2001). Resin and pollen foragers were collected from colonies by closing the entrance of the colony and trapping the bees in cages as they returned with their respective corbicular loads. Pollen foragers were chosen for these experiments because they have been shown to learn tactile stimuli better than nectar foragers on average (Scheiner et al. 1999, 2001). Therefore, if differences exist between resin foragers and good learners (i.e., pollen foragers), we would expect an even stronger, and more robust, difference between

resin foragers and average learners (i.e., nectar foragers).

Bees were brought into the laboratory, chilled on ice and then restrained in soda straws with duct tape. After restraining, all bees were fed 2 μl of 2 M sucrose solution by touching the antennae with the solution to elicit proboscis extension for feeding. Bees that did not respond with proboscis extension in this initial feeding were not used in the learning trials. To ensure that the individuals for testing would be acclimated to the restraints and not satiated prior to the learning trials, the bees were placed in a humidified incubator at 34°C for 1.5 h. For the sucrose response threshold assay, restrained bees were fed water until satiation before being held in the incubator. After these bees were removed from the incubator, each bee was tested for its response to water. Any bee that extended its proboscis to water was allowed to drink until satiation to control for effects of thirst per previous experiments (Goode et al. 2006).

2.2 PER conditioning to tactile stimulus

In summer of 2008, active resin foragers (n=83) and pollen foragers (n=88) were collected from six unrelated colonies. Bees from one or two colonies were tested per day over a 3-month period (July through August). Approximately zero to four resin foragers could be collected over a 20-30-min period, and due to relative rarity of resin foragers, sample sizes among colonies were not equal. Just after the bees were restrained in the laboratory (as described above), their eyes and ocelli were occluded with black enamel paint to prevent visual inputs from interfering with learning based on tactile stimulation. To perform the learning trials, an individual bee was presented with a 1 mm gap created between two 3 mm×4 mm smooth copper plates. Therefore, the entire surface area including the two plates and the gap was 4 mm×7 mm. A gap, rather than an etched plate, was used in this study to simulate a stimulus that would more likely induce resin use in colonies since gaps less than 6.35 mm are generally filled with propolis rather than wax (Crane 1990). At the start of each trial, a bee touched the tactile stimulus with the antennae for 2-3 s, and then was fed with 0.4 μL of a 2 M sucrose solution for 2 s by touching the antenna with the sucrose solution to elicit the proboscis extension. The rare

bees that spontaneously responded to first presentation of the tactile stimulus by extending the proboscis were removed from testing. If the proboscis touched the plates, they were cleaned with a cotton swab dipped in 70% ethanol and wiped dry. Six trials with a 5-min inter-trial interval were completed for each bee, and in each trial bees were rewarded with sucrose solution after the presentation of the tactile stimulus. The responses of each bee to the presentation of the tactile stimulus prior to receiving the reward were recorded as a 0 (no response) or a 1 (response). If a bee responded to the presentation of the tactile stimulus by extending her proboscis, then that bee had learned the association between the stimulus and the reward.

Since the collected data for each trial was categorical (1 or 0 indicating a response/learning or not for each bee at a given trial) and describes learning over time, the data were analyzed using a logistic growth curve analysis specifically described for associative learning data sets comparing two groups (see Hartz et al. 2001). PROC GENMOD in SAS (ver. 9.2) was used to produce generalized estimating equations (GEE) for the effect of forager type and colony.

2.3 PER conditioning to odorant

In July 2009, active resin foragers (n=29) and pollen foragers (n=29) were collected at the entrances of four unrelated colonies as described above. The floral odorant geraniol was used as the stimulus for PER conditioning following methods used previously in the lab (e.g. Masterman et al. 2000, 2001).

To perform the learning trials, a bee was placed in a constant neutral airflow for 30 s followed by the presentation of the odorant. During the 5-s odorant delivery, a syringe with 0.4 μL of a 2 M sucrose solution was touched to the bee's antennae to condition the bee to the sugar reward for 2 s. This was repeated for six trials with an inter-trial interval of 7 min. For each trial, we recorded whether or not the bee responded with a proboscis extension both when the odor was presented and the sucrose. The few bees that responded to geraniol prior to conditioning were not tested further.

The data was analyzed in the same manner as for tactile PER conditioning using a

logistic growth curve analysis designed for associative learning (see Hartz et al. 2001).

2.4 Sucrose response threshold assay

SRTs were determined for resin and pollen foragers in June 2009. Active resin foragers (n=42) and pollen foragers (n= 41) were collected at the entrances of five unrelated colonies, different from those used in 2008, and restrained in the lab as described previously.

Sucrose concentrations tested were 0.1%, 0.3%, 1%, 3%, 10%, and 30% (w/v). To determine SRTs, a drop of each concentration was touched to the antennae in ascending order over a series of six trials with 5 min between trials. Upon presentation with the sucrose solution, if the bee responded by extending the proboscis, then it was scored as a 1. If no response occurred, the bee received a score of zero for that concentration. The lowest concentration that elicits a full proboscis extension response is a direct measure of an individual bee's SRT. The sucrose concentrations were transformed to log10 values creating a linear response relationship, following previously used protocols (Goode et al. 2006). The median SRTs for resin foragers and pollen foragers were compared using a Wilcoxon rank-sum test.

2.5 Differential tactile conditioning

Differential tactile conditioning was utilized to determine if resin foragers were more able to discriminate between two tactile cues as compared to pollen foragers. Our choice to use this method was two-fold: we have previously used this method for olfactory discrimination (Masterman et al. 2000, 2001) and this is a novel method for use with tactile discrimination. For the tactile stimuli, we needed to use two distinctly different materials since this type of learning trial is inherently more difficult than a simple learning acquisition test. As shown by Erber et al. (1998) bees exhibit a great deal of generalization between stimuli. Preliminary trials using the same gap from the learning trials versus other variants of the metal plates (e.g., smooth and etched plate) were unsuccessful; the bees were not able to discriminate well between any combinations.

However, we found the bees were able to discriminate well between smooth and rough textured paper. The conditioned or rewarded stimulus (CS+) was a 2 cm square of general purpose sandpaper (3M©, 60 coarse). The unconditioned or unrewarded stimulus (CS−) was a 2 cm square of the reverse, smooth side of the sandpaper. Since honeybees use propolis to smooth hive walls (Simone-Finstrom and Spivak 2010), a rough surface is potentially another biologically relevant stimulus with respect to resin foraging.

To perform the differential conditioning tests, active resin foragers (n=26) and pollen foragers (n=29) were collected at the entrances of three unrelated colonies in late August and early September 2009, and harnessed as in the odorant-conditioning experiment. To prevent the bees from seeing and using possible visual cues, the experiment was carried out in a dark room lit only by red light. Preliminary tests found that performing this experiment under red light was equivalent to occluding the bees' eyes and ocelli with black enamel paint (data not shown). Bees were presented with either the CS+ or CS− and allowed to feel it for 4s. If they were presented with the CS+, they were then given 0.4 μL of a 2 M sucrose reward. This was done in a pseudo-random sequence of 18 trials with each stimulus being presented a total of nine times with a 7-min inter-trial interval (presentations of each CS were in the following order: (+, −, −, +, −, +, +, −, +, −, −, +, −, +, +, −). A new square of sandpaper was used for each trial to prevent accumulation of possible olfactory cues transmitted by the bees. Proboscis extension responses were recorded for each presentation.

To analyze the data, discrimination scores (DS) were calculated for each individual by subtracting the total number of responses to the CS− (unconditioned stimulus) from the total number of responses to the CS+ (conditioned stimulus). A DS of 0 would indicate that the individual responded equally to both the CS+ and CS− and therefore did not discriminate between the two. A positive number ranging to 8 would mean that the individual responded more often to the CS+ than to the CS− suggesting that discrimination occurred. We also determined the DS for the final presentations of the CS+ and CS−, to examine differences between discriminatory abilities at a point where the individuals had significant experience with the two stimuli. DS data were analyzed

using a Wilcoxon rank sum test and learning curves to the CS+ were analyzed using the previously described logistic growth curve analysis.

3. RESULTS

3.1 Tactile PER conditioning

The learning curves (Fig. 5.1) show the percentage of bees that learned to associate a 1-mm gap created between two 3 mm×4 mm copper plates with a sucrose reward at each trial for both forager types. Logistic growth curve analysis, used for accurate determination of differences in learning (see Hartz et al. 2001), indicated that resin foragers (n=83, average of 14 bees per six colonies) learned at a significantly higher rate compared to pollen foragers (n= 88, average of 15 bees per six colonies) based on the negative value for the trial × forager type parameter estimate (p=0.031; refer to Table 5.1 for GEE model parameter estimates), and that there was no colony effect (p=0.64; refer to Table 5.1). The number of non-learners across forager types was not significantly different (Fisher's exact test: p= 0.9), with 21.7% of the resin foragers and 34.1% of the pollen foragers not exhibiting any learning after six trials.

Figure 5.1. Percentage of bees that responded to the tactile stimulus, a 1-mm gap created between two 3 mm×4 mm smooth copper plates, with a proboscis extension response at each trial for resin foragers (filled circle) and pollen foragers (filled upright triangle). Based on logistic growth curve analysis (Table 5.1 and see text) resin foragers (n= 83, average of 14 bees per six colonies) learned at a significantly higher rate compared to pollen foragers (n=88, average of 15 bees per six colonies; p=0.031)

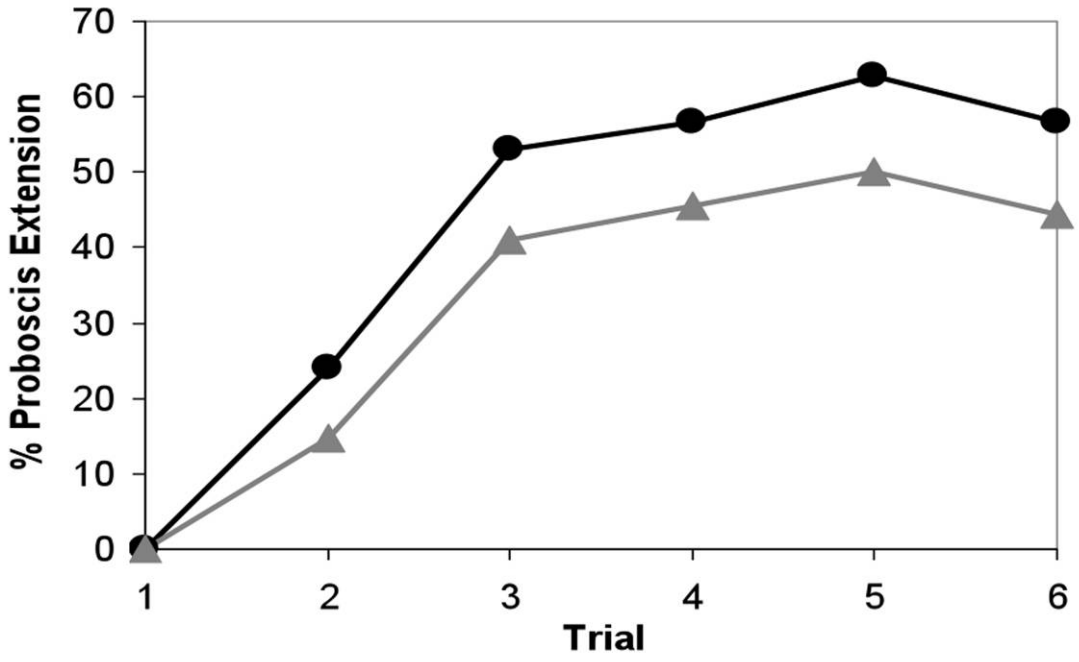


Table 5.1. Results of the logistic growth curve analysis of the responses of resin and pollen foragers to tactile PER conditioning to a gap between two metal plates (Fig. 5.1)*

Parameter	**Estimate**	**Standard Error**	***p***
Intercept	2.83	0.30	< 0.0001
Trial	-0.39	0.08	< 0.0001
Colony	-0.13	0.09	0.16
Trial*colony	-0.01	0.02	0.64
Trial*forager-type	-0.12	0.06	0.03

*The p-value for each parameter is the probability that the respective parameter is zero. The intercept value is the point at which the logistic curve turns upward. The trial value is the rate at which resin foragers learn. The trial × forager type is the difference between the rate of learning between the two groups. A negative value indicated that pollen foragers learned significantly more slowly than resin foragers

3.2 Olfactory PER conditioning

Logistic growth curve analysis based on the learning curves (see Fig. 5.2) indicated that resin foragers (n=29, average of seven bees per four colonies) and pollen foragers (n=29, average of seven bees per four colonies) learned the odorant at equivalent rates (p =0.71; Table 5.2). There was a significant colony effect (p=0.01; Table 5.2), with one of the four colonies having lower learning rates for both pollen and resin forgers as compared to the other colonies. However, this difference did not influence overall differences due to forager type.

Figure 5.2. Percentage of bees that responded to geraniol, the olfactory stimulus, with a proboscis response at each trial for resin foragers (filled circle) and pollen foragers (filled upright triangle). There were no significant differences between the rate of learning (p=0.71; Table 5.2) between resin foragers (n=29, average of seven bees from each of four colonies) and pollen foragers (n=29, average of seven bees from each of four colonies).

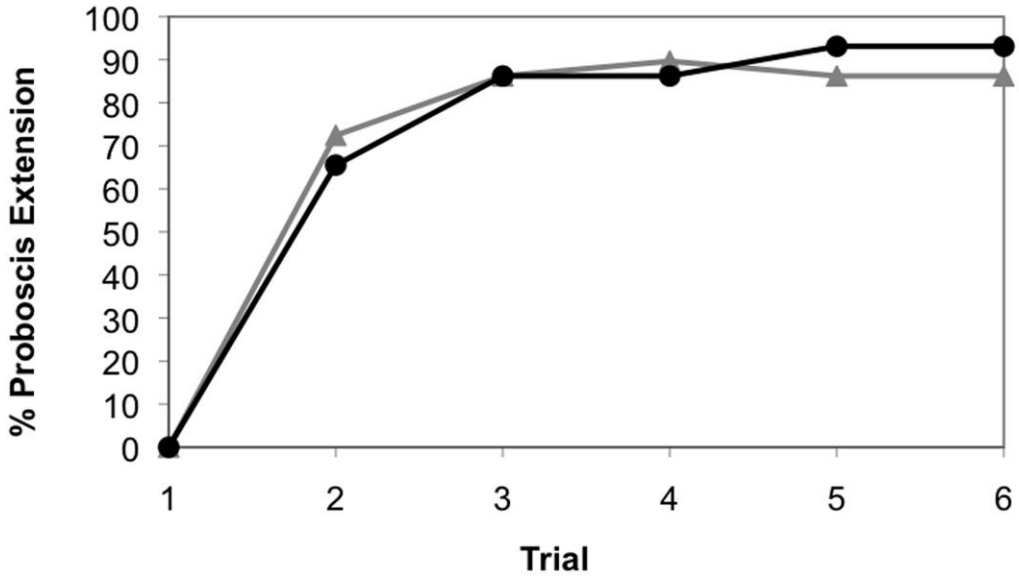


Table 5.2. Results of the logistic growth curve analysis of the responses of resin and pollen foragers to olfactory PER conditioning to the odorant geraniol (Fig. 5.2)*

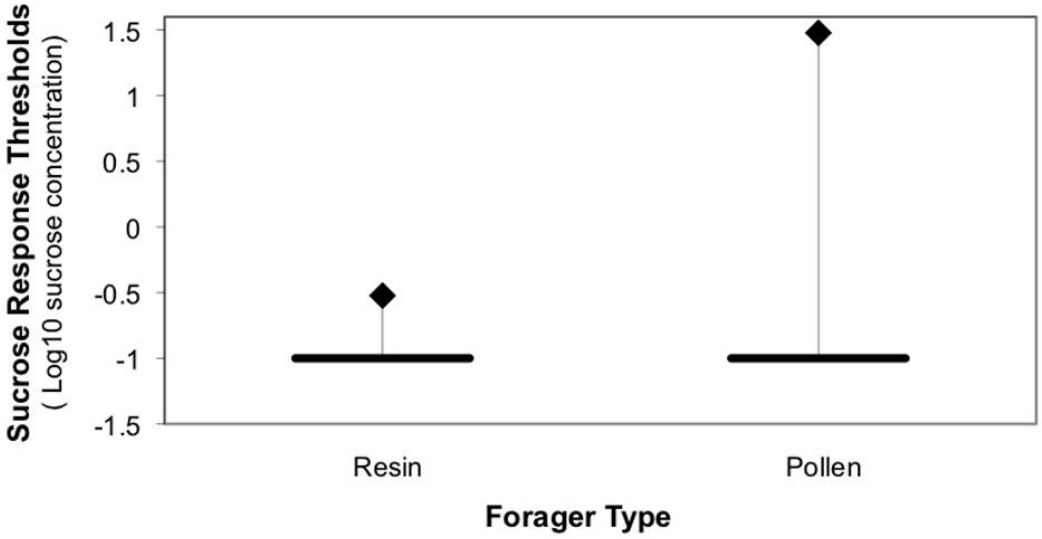
Parameter	**Estimate**	**Standard Error**	***P***
Intercept	0.49	0.83	0.56
Trial	0.61	0.50	0.22
Colony	1.38	0.70	0.05
Trial*colony	-1.08	0.42	0.01
Trial*forager-type	-0.07	0.20	0.71

*The p-value for each parameter is the probability that the respective parameter is zero. The intercept value is the point at which the logistic curve turns upward. The trial value is the rate at which resin foragers learn. The trial×forager type is the difference between the rate of learning between the two groups

3.3 Sucrose response threshold assay

The SRTs were identified for each bee by determining the lowest concentration (transformed to log10 values) that elicited a full proboscis extension response. The median SRT for resin foragers (n=42, average of eight bees per five colonies) was −1 (lower quartile, −1; upper quartile, −1) and ranged from −1 to −0.52). The median SRT for pollen foragers (n=41, average of eight bees per five colonies) was −1 (lower quartile, −1; upper quartile, −1) and ranged from −1 to 1.48. Most bees of both forager types responded to the lowest concentration of sucrose, resulting in the low SRTs. However, the range of SRTs was greater in pollen foragers as compared to resin foragers, which resulted in resin foragers having significantly lower SRTs on average than pollen foragers based on a Wilcoxon rank-sum test ($Z=2.03$, $p=0.043$; Fig. 5.3).

Figure 5.3. Median SRT. Resin foragers (n=42, average of eight bees per colony) had significantly lower SRTs on average as compared to pollen foragers (n=41, average of eight bees per colony; p=0.043). The bold horizontal lines represent the medians and the maximum values are represented by the diamonds (see text for quartile values).



3.4 Differential tactile conditioning

To analyze the ability of resin foragers and pollen foragers to discriminate between a smooth and a rough (sandpaper) tactile surface a DS was calculated for each bee by subtracting the total number of responses to the CS− (unconditioned stimulus) from the total number of responses to the CS+ (conditioned stimulus). One non-responder, a resin forager, and one colony with low sample size (five bees) were removed from all analyses. For this analysis, the DS could range from −9 to 8. A higher positive number would indicate that the bees discriminated well, while a negative value would be indicative of poor discrimination. The median DS was 1.5 (lower quartile, 1; upper quartile, 3) for resin foragers (n=26, average of eight bees per three colonies) and 1

(lower quartile, 0; upper quartile, 3) for pollen foragers (n=29, average of eight bees per three colonies). These DS values were not significantly different (Wilcoxon rank-sum, Z=0.69; p=0.48).

We followed the initial test by calculating the DS for resin and pollen foragers using just the final presentations of both the CS+ and the CS− when the ability to discriminate between the stimuli should have been most apparent. Using these final two trials, the DS could range from −1 to 1. A DS of 1 would indicate that the bee responded only to the CS+ and thus exhibited discrimination between the two stimuli. A DS of 0 would indicate that the bee extended its proboscis to both the CS+ and the CS− and therefore was unable to discriminate between the two stimuli. Resin foragers had a higher median DS compared to pollen foragers (1 [lower quartile: 0, upper quartile: 1] versus 0 [lower quartile: 0, upper quartile: 1]), respectively), but this difference was non-significant (Wilcoxon rank sum, Z=1.79; p=0.072).

However resin foragers had a significantly steeper learning curve to the CS+ based on logistic growth curve analysis (p=0.042; Table 5.3), meaning they more readily learned to associate the CS+ of rough sandpaper to the sucrose reward compared to pollen foragers (Fig. 5.4a, b). This result confirms results of the earlier tactile conditioning experiment using a gap as the CS+.

Figure 5.4. Tactile discrimination conditioning by resin foragers (a, filled circle) and pollen foragers (b, filled upright triangle). Percent proboscis extension response (% PER) over 18 trails (nine trials for each tactile stimulus), when rough sandpaper was the CS+ and smooth sandpaper was the CS−. Sample sizes: resin foragers, n=26, average of eight bees per three colonies and pollen foragers, n=29, average of eight bees per three colonies. Based on a logistic growth curve analysis (see Table 5.3 and text), resin foragers learned the CS+ at a faster rate compared to pollen foragers (p=0.046)

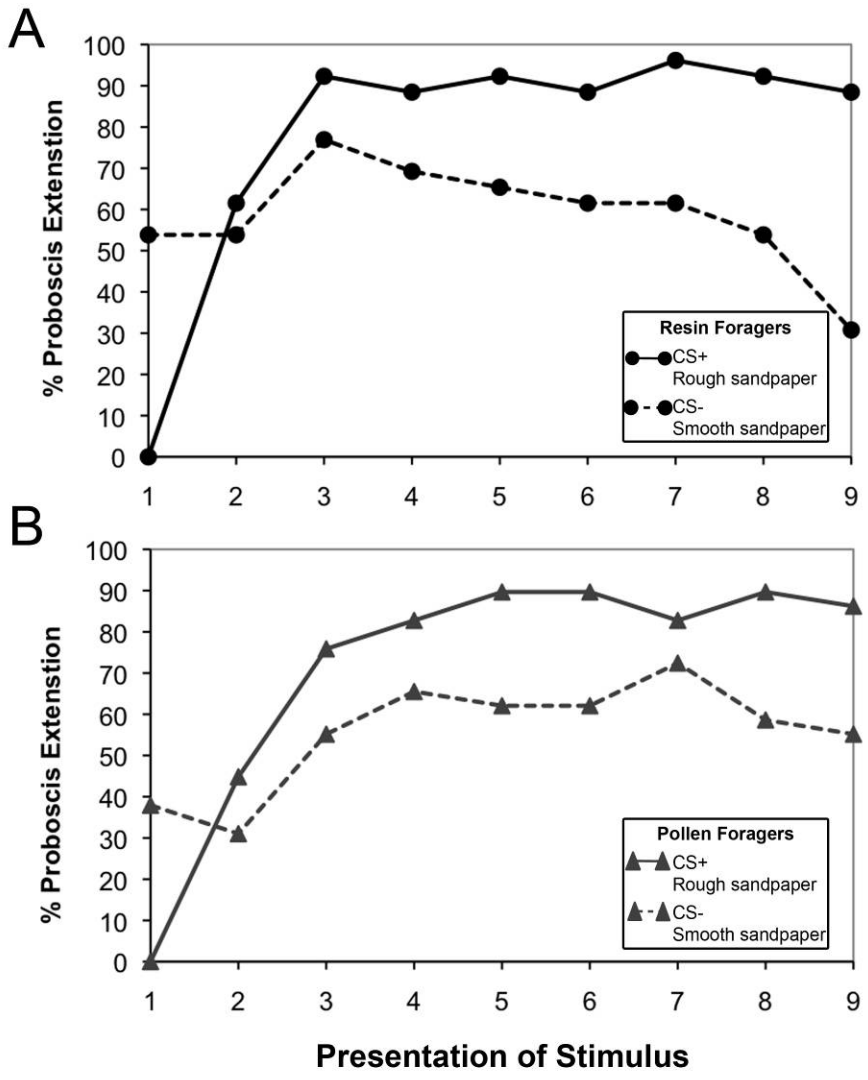


Table 5.3 Results of the logistic growth curve analysis of the responses of resin and pollen foragers to the CS+ (rough paper) during differential tactile PER conditioning (Fig. 5.4)*

Parameter	**Estimate**	**Standard Error**	***p***
Intercept	1.46	0.79	0.06
Trial	-0.74	0.30	0.01
Colony	0.02	0.34	0.96
Trial*colony	0.09	0.12	0.48
Trial*forager-type	-0.23	0.11	0.045

*The p-value for each parameter is the probability that the respective parameter is zero. The intercept value is the point at which the logistic curve turns upward. The trial value is the rate at which resin foragers learn. The trial × forager type is the difference between the rate of learning between the two groups. A negative value indicated that pollen foragers learned significantly more slowly than resin foragers

4. DISCUSSION

4.1 Learning by resin foragers

The results yielded new information about the resin forager phenotype and demonstrate areas for further research on the stimuli that honeybees use to initiate resin foraging. Specifically, we found that resin foragers have lower SRTs and learned to associate two tactile stimuli (a gap and a rough surface) with a sucrose reward at a significantly faster rate as compared to pollen foragers. In contrast, resin foragers did not learn an olfactory stimulus (the floral odor geraniol) better than pollen foragers. When presented with the tactile challenge of discriminating between a rough and smooth surface using a discrimination conditioning assay, resin foragers had a higher discrimination score than pollen foragers by the last presentation of each stimulus, but the

difference was not significant (p=0.07). Honeybees typically generalize a great deal across tactile stimuli (Erber et al. 1998), and so this learning task in particular is quite difficult. It is possible that using other types of tactile stimuli could enhance the differences seen between forager types.

Differences in response thresholds, learning, and discrimination between bees of different forager type have been shown previously for nectar and pollen foragers (i.e., Scheiner et al. 1999, 2001, Drezner-Levy et al. 2009), but this is the first study to examine sucrose responsiveness and learning with resin foraging honeybees. Various studies have determined that pollen foragers on average learn tactile and olfactory cues better than nectar foragers due to their typically lower SRTs (Scheiner et al. 1999, 2001, 2004). It has been hypothesized that these differences in SRTs between the different types of foragers (namely water, nectar, and pollen) have a strong influence on reinforcing division of labor within a hive and ensuring that the colony has the appropriate number of foragers for each task (Page and Erber 2001). SRTs can vary with age, foraging experience, and season (Pankiw and Page 1999; Pankiw et al. 2001; Scheiner et al. 2003). We found that the SRTs of returning resin foragers were slightly but significantly lower compared to returning pollen foragers. We do not know the extent to which the resin forager SRT is modified by genetics and/or recent foraging experience. Resin foragers have been noted to switch to pollen, nectar, and water foraging (Meyer 1956; Nakamura and Seeley 2006) and it would be interesting in future studies to test the SRT of a marked resin forager after she has shifted to a different resource.

The lower response thresholds of resin foragers could have contributed to the differences in learning ability that we observed. If the sensitivity to low concentrations of sucrose was the sole mechanism underlying learning performances of the bees in this experiment, we would have expected resin foragers to learn the odorant more effectively as well. If in fact SRTs were not responsible for the differences seen in this experiment, this could raise a host of new questions on the mechanisms behind individual differences in learning. Investigations of the pollen-hoarding phenotype and that behavioral syndrome began with studies on SRTs and learning of selected lines of bees (e.g., Page et

al. 1998; Pankiw and Page 1999; Pankiw et al. 2001; Roussel et al. 2009). These experiments provided much insight into the mechanisms of foraging for specific resources, and account for much of the empirical evidence for the theories behind division of labor among the foraging task force. Future experiments to better understand the mechanisms underlying the resin forager phenotype would similarly be benefited using strains of bees selected for high and low resin collection, much like the lines of high and low pollen-hoarding bees.

4.2 Cues for resin foraging

Based on our results, resin foragers were better able to learn two tactile stimuli (a gap and a rough substrate when each was presented as the CS+) as compared to pollen foragers. This finding was based on a large number of bees collected from a total of nine unrelated colonies over the course of two years of study. This study provides the first experimental insight on the possible cues used by resin foragers in initiating resin foraging. While other social insect species have been noted to be able to detect abnormalities of the nest structure and build around or fix them, this has not been studied to our knowledge in honeybees. Termites (*Zootermopsis nevadensis* and *Zootermopsis angusticollis*) have been noted to lay trails around material with sharp edges or crevices that have been introduced into the nest and actively recruit individuals to build around and bury the material (Stuart 1967). Other termite species (*N. costalis* and *C. formosanus*) are thought to use their antennae to scan the interior nest walls as a way to explore building sites (Jones 1980; Lee et al. 2008). The paper wasp *P. fuscatus* has also been known to base building decisions on antennation of various parts of the nest (Downing and Jeanne 1990; Downing 1994). But unlike these cases, in honeybees, a specialized set of foragers possibly use tactile information to determine whether or not to initiate foraging for a specific nest building material. Resin foragers may actively or passively patrol the nest interior sensing various tactile stimuli in order to make foraging decisions, as it appears pollen foragers do (Calderone and Johnson 2002; Johnson 2008). Keeping in mind that honeybees can substitute wax for resin and propolis (Meyer 1956; Crane 1990),

it is particularly interesting to investigate the mechanisms behind why and how they collect resin.

4.3. Conclusions

This study is just the starting point for a new set of research questions on the sensory and behavioral mechanisms underlying resin collection. It would be interesting to do further studies explicitly testing the learning capabilities of resin foragers as compared to other forager types (namely nectar and pollen foragers that are typically studied). This paper was a first glimpse into general learning by resin foragers and more detailed studies could be done to better understand these bees. It would be particularly beneficial to conduct a subsequent study on sucrose response thresholds and various learning tasks (e.g., Giurfa 2007) by matching resin foragers and other bees with similar thresholds. Additionally, exploration of alternative tactile stimuli, particularly for discrimination assays may provide further insight into the discriminatory abilities of these foragers. Future experiments using non-restrained bees in field and observation colonies should be conducted to further examine the role that tactile stimuli plays in resin foraging and use.

Non-tactile cues are also likely to be involved in initiating resin-related behaviors in honeybees. Antennation alone could not only serve to detect the presence of rough surfaces or gaps but it is possible that bees do this to acquire chemosensory information concerning the presence or absence of propolis (de Brito Sanchez et al. (2007) suggests that honeybees can taste resin-related compounds). More likely, the presence of moving air or odors and changes in relative humidity within the nest may also initiate resin foraging behaviors (Crane 1990). Studies of building behavior in some other social insects suggest the importance of these cues. For example, leaf-cutter ants (*Acromyrmex ambiguus*) in laboratory nests will plug tunnels with leaf material when dry air is released into it (Bollazzi and Roces 2007). Termites (*Z. nevadensis* and *Z. angusticollis*) have also been shown to seal off gaps in the nest in relation to outside air currents (Howse 1966). These cues should be investigated, as they are possibly not mutually exclusive from tactile cues in stimulating resin foraging.

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