Hidden Benefits of Honeybee Propolis in Hives

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Abstract

Honey bees (*Apis mellifera* L.), like many social insects, have collective behavioral defenses called "social immunity" to help defend and protect the colony against pathogens and parasites. One example of social immunity is the collection of plant resins by honey bees and the placement of the resins on the interior walls of the nest cavity, where it is called a propolis envelope. Propolis is known to have many antimicrobial proprieties against bacteria, fungi, and viruses and has been harvested from bee hives for use in human medicine since antiquity. However, the benefit of propolis to honey bees has not been studied until recently. This chapter focuses on how bees collect and use the antimicrobial properties of plant resins within the hive as a form of social immunity and defense against infectious bacterial and fungal pathogens. The studies presented here demonstrate the significance of the propolis envelope as a crucial component of the nest architecture in honey bee colonies. The collection and deposition of resins into the nest architecture impact individual immunity, colony health, and support honey bees' antimicrobial defenses. These studies emphasize the importance of resin to bees and show that plants are not only a source of food, but can also be "pharmacies."

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1 Benefits of Propolis to Colony Health

It is common knowledge that honey bees forage for pollen, nectar, and water. What is not well appreciated is that honey bees also forage for plant resins, but not for nutritional reasons. Resin is a sticky exudate secreted by plants to protect young leaf buds or the entire plant from disease, UV light, and herbivore attack (Langenheim 2003). Resins are composed primarily of antimicrobial compounds (e.g., terpenes and flavonoids) that play a major role in the defense and survival of the plant (Langenheim 2003). Many animals, including bees, collect these antimicrobial resins for their own health benefits. In bees, the presence of resin in the nest plays a major role in the immune defense of individual bees, improving colony health and fitness (Simone et al. 2009; Simone-Finstrom and Spivak 2012; Borba et al. 2015; Borba, 2015).

Honey bees collect resin mainly from buds and leaves of various tree species, but they also collect resins from droplets appearing on the trunks or limbs of trees (Alfonsus 1933), and from a few tropical flowers (Kumazawa et al. 2003; Armbruster 1984). Bees can extract resin by fragmenting leaves with their mandibles (mouthparts) or collecting it directly from the plant surface (Meyer 1956; Teixeira et al. 2005). Bees collect resins to varying degrees; some honey bee species and races use resins extensively, such as the African-derived subspecies *Apis mellifera scutellata* and the European-derived subspecies *A. mellifera caucasica*. At least one species of honey bees, *Apis cerana,* does not collect resin (Butler 1949; Page and Fondrk 1995). In colonies that do collect resin, the number of resin foragers depends on the needs of the colony (as discussed later in this chapter), but generally they comprise less than 1% of the total forager work force. Resin collection is a very difficult and time-consuming task to perform. After chewing pieces of resin from the plant, bees must transfer the sticky secretion from their mandibles to their hind legs before returning to the hive. Because of the sticky characteristics of resin, once back in the hive, resin foragers need the assistance of other bees to remove the resin load from their legs, which may take up to 30 min (Fig. 1; Nakamura and Seeley 2006). The bees will then carry the resin in their mandibles to the site in the hive where the resin will be deposited. Once deposited in the nest, the resin, sometimes mixed with beeswax, becomes what beekeepers know as propolis.

Honey bees naturally nest in tree cavities where they coat the entire inner surface of the nest cavity surrounding the combs with a propolis envelope (Seeley and Morse 1976). Seeley and Morse (1976) suggested that the propolis envelope had various functions, including serving as an impermeable barrier to tree sap and environmental moisture, a solid surface for comb attachment, a physical barrier to outside invaders by sealing the holes and cracks of the nest cavity, and finally, an antimicrobial layer against natural occurring fungi and bacteria in the tree cavity. When nesting in a hollow tree cavity, honey bees prepare the new nest site by removing the soft, rotten wood from the nest walls and depositing propolis in the cracks to make it solid and smooth (Seeley and Morse 1976). Beekeepers,

Fig. 1 Worker bee removing resin load from the hind leg of a resin forager. Upon return to the hive, resin foragers need the assistance of other bees to remove the resin load from their legs, which may take up to 30 min (*Photo credit* Christine Kurtz)

particularly in the USA, have selected against colonies that collect large amounts of propolis (Fearnley 2001) because its stickiness makes opening and managing colonies in standard beekeeping equipment difficult. Importantly, honey bees do not construct a propolis envelope in standard beekeeping equipment because the inner walls of the wooden boxes are already solid and smooth, which apparently does not stimulate propolis deposition. Instead, bees deposit propolis in dispersed cracks and crevices in manmade hive bodies and not as a continuous envelope as they do within a tree cavity (reviewed in Simone-Finstrom and Spivak 2010).

Honey bees are very resilient insects; they have thrived in this world for 6–8 million years (Engel 1999), relying only on their own natural defense mechanisms to survive. Although propolis has been used as a traditional and natural human medicine since biblical times (Simone-Finstrom and Spivak 2010), the benefits of propolis for honey bee health were not appreciated until the last decade. Studies have demonstrated that the presence of a propolis envelope enshrouding the nest area is a fundamental component of honey bee colony health (Simone et al. 2009; Simone-Finstrom and Spivak 2012; Borba et al. 2015; Borba and Spivak, *in review*). The propolis envelope functions as an antimicrobial, or "disinfectant" layer around the nest, and thus as an external layer of the colony immune defense. This chapter will summarize current research since the previous review (Simone-Finstrom and Spivak 2010), emphasizing research conducted by R. Borba: (1) the seasonal benefits of a propolis envelope to colony health and individual honey bee immunity; (2) the role the propolis envelope plays in bees' natural defense against brood diseases; and (3) how honey bees select and use plant resins as a form of self-medication.

1.1 Seasonal Benefits of Propolis to Bee Immunity and Colony Health Under Natural Field Conditions

A honey bee colony can be considered a superorganism, a group of related individuals living together in a nest with the ability to perform collective foraging, thermoregulatory and defensive behaviors. When collective behavioral mechanisms are used to defend the colony against parasites and pathogens, they are called mechanisms of social immunity (Cremer et al. 2007). Examples of social immunity in honey bees include hygienic behavior (the ability of adult bees to detect and quickly remove diseased and mite-infested brood from the nest, thus limiting pathogen and parasite transmission; reviewed in Evans and Spivak 2010), grooming (removal of the parasitic *Varroa* mite from a nestmate's body; Boecking and Spivak 1999), and foraging for resins to construct a propolis envelope inside the nest (Simone et al. 2009; Simone-Finstrom and Spivak 2012).

The benefits of the propolis envelope to honey bee health were first investigated by coating the inside of managed hives with a propolis extract (solution of 13% propolis in 70% ethanol) using a paintbrush and exposing bees to this propolis-enriched environment for 7 days (Simone et al. 2009). After one week, 7-d-old bees had lower immune system activation and lower bacterial loads in and on their bodies compared to same-age bees in hives without the propolis-extract coating (Simone et al. 2009). This short-term study indicated that bees in hives with the propolis-extract envelope did not have to expend as much energy turning on (activating) their immune system to fight off microbes in the nest. When the immune system of bees, or any animal, is activated, it comes with a physiological cost (e.g., reduced survival; Moret and Schmid-Hempel 2000). In fact, for insects the immune system may be the most costly physiological system to maintain (Evans and Pettis 2005; Schmid-Hempel 2005). When the immune system does not need to be highly activated, as when there is a propolis envelope in the nest cavity, bees may be able to allocate that saved energy to perform vital tasks (e.g., foraging and rearing brood) or store protein in their bodies.

Recent research from Brazil showed that Africanized bee colonies that deposited high amount of propolis had greater brood viability, longer worker lifespan, higher honey production, more rapid hygienic behavior, and larger pollen stores compared to colonies that deposited low amounts of propolis (Nicodemo et al. 2013, 2014). Even though most European-derived stocks of bees in the USA do not deposit much propolis, it is possible that they also receive the same *long-term* benefits from the antimicrobial compounds in propolis. Therefore, a research experiment was conducted to investigate the long-term benefits of a propolis envelope, but this time testing propolis naturally collected and deposited by the bees inside the nest. Colonies were encouraged to build a natural propolis envelope by cutting and stapling commercially available propolis traps to the four inner walls of each hive box in 12 colonies (propolis envelope treatment group; Fig. 2). The bees readily filled the 24 × 3 mm (height × length) gaps in the traps with resin they collected from the field. No propolis traps were provided to another set of 12 colonies, and the bees deposited propolis in the cracks and crevices within the box only where

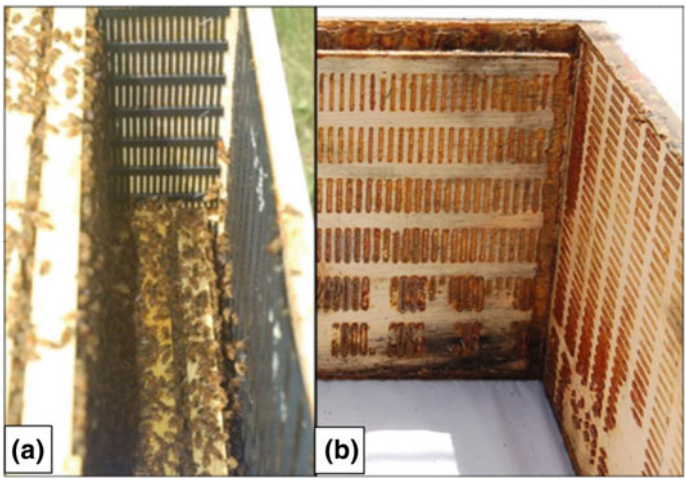

Fig. 2 Propolis envelope treatment bee box. **a** Propolis traps stapled to inside walls of a hive to encourage bees to construct a propolis envelope. **b** View of the propolis envelope when traps were removed at the end of the experiment. In each colony, the bees deposited propolis within most of the gaps of each propolis trap (*brown lines on the box* are the deposited propolis). In a tree cavity, the propolis envelope is contiguous, but bees do not tend to deposit propolis on planed wooden walls in beekeeping equipment, unless lumber is left unfinished and very rough

they could (control group). This experiment was conducted on a first set of colonies from April 2012 to May 2013 and was repeated on a new set of colonies from April 2013 to May 2014. Each year the colonies were started from package bees on unused equipment and combs.

During the active foraging season (from July to September) and the following May of both years, the following measures were taken on *colony* health: (1) adult bee population size, (2) total amount of worker brood, and (3) levels of *Varroa* mites and *Nosema* spp. Adult bee populations were estimated in each colony by counting the number of frames covered with bees in each box (following Nasr et al. 1990). Worker brood was quantified by placing a 2.6 cm^2 grid over each frame and counting the number of squares filled with sealed or unsealed brood (following Nasr et al. 1990). *Varroa* levels were measured by collecting samples of 300 adult bees from the brood area and dislodging the mites from the bees in the laboratory (following Lee et al. 2010; Spivak and Reuter 2001a). *Nosema* levels were measured by counting *Nosema* spp. spores in 100 bees using a hemocytometer (Cantwell 1970).

The presence of a propolis envelope in the colony did not appear to have an effect on adult bee population size as colonies with and without a propolis envelope had similar adult bee populations over both replicated years of the experiment. There was a potential effect of the propolis envelope on the brood area; as in the first replicate of the experiment, colonies with a propolis envelope had significantly larger brood areas in May 2013 compared to the colonies without the propolis

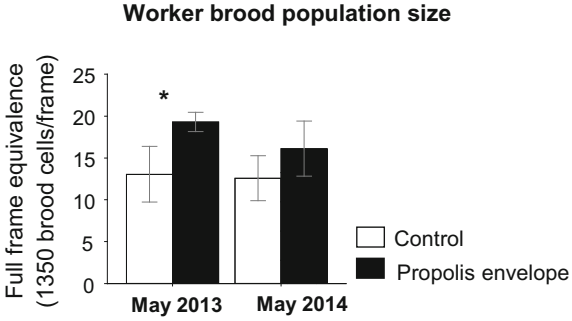

Fig. 3 A bar graph with standard errors is used to represent the worker brood population size in May 2013 and May 2014. The average (±standard error) number of full frame equivalents (1350 worker brood cells) is shown on the *y*-axis and the months are indicated on the *x*-axis. Significant differences between controls (*white*) and propolis envelope (*black*) treatment colonies are indicated with * ($P < 0.05$)

envelope. A similar trend was observed in May 2014, but the difference in brood areas was not statistically significant (Fig. 3). It was predicted that the propolis envelope might lower the levels of pathogens and parasites (Nosema spp. and parasitic mites) in the colonies. However, in both replicates of the experiment, these levels were very low and did not differ between the colonies with a propolis envelope and control colonies with no propolis envelope, likely because all colonies began as "packages" and pathogen and parasites levels do not usually rise to high levels in new colonies the first year in Minnesota. Thus, all colonies in the experiment were apparently healthy.

The effects of propolis on *individual* bee health were measured in 7-d old bees by quantifying: (1) the levels of three common viruses (DWV—Deformed Wing Virus, IAPV—Israeli Acute Paralysis Virus, and BQCV—Black Queen Cell Virus), (2) the expression of specific immune genes, and (3) the level of a blood storage protein called Vitellogenin (Vg). All three individual bee health measurements were quantified using a common (but somewhat expensive) laboratory technique called real-time, quantitative PCR (polymerase chain reaction; see explanation of this technique below).

There were no significant differences in levels of all three viruses (DWV, IAPV, and BQCV) between colonies with a propolis envelope and those without. The lack of high levels of viruses, in addition to low levels of *Varroa* and *Nosema*, support the hypothesis that colonies from both treatments were apparently healthy. Further studies will be necessary to explore the effect of propolis on viral levels, as well as other bee pathogens and parasites, when colonies are highly infected.

When infected with a pathogen, bees and humans can initiate an immune response via cellular or humoral immune pathways. The cellular immune response includes the engulfing and encapsulation of pathogens by blood cells, while the humoral immune response includes the production of small antimicrobial proteins that attack and kill pathogens. The starting point of humoral immune system

activation is called gene transcription, when a particular sequence of DNA (a gene) is transcribed to make messenger RNA (mRNA), which "message" is then translated to make a specific protein. It is possible to measure how much mRNA is being produced of a particular gene using real-time, quantitative PCR. After extracting mRNA from an individual bee, or a group of bees, one needs to backtrack the natural order of gene transcription (from DNA to mRNA) and reverse-transcribe mRNA (single-stranded sequence of a gene) to DNA (double-stranded sequence of the same gene), which can then be used in PCR reactions. PCR amplifies a specific double-stranded sequence of genes into billions of copies, which are then quantified. A specific sequence of genes is targeted using "primers," which are small segments of nucleotides that are complementary to a piece of the gene that is to be amplified. For the experiments described here, primers were designed to target honey bee immune-related genes, specific viruses (DWV, IAPV, and BQCV), and Vitellogenin.

To measure immune system activation, it is best to collect bees of the same age, preferably young nurse bees, as the immune systems of older foragers become highly variable in expression levels. To collect young bees, newly emerged bees were paint-marked with a dot of enamel paint on the thorax just after they crawled out of their cells. Six days later, when bees were 7-d old, 25 paint-marked bees were collected from each of the experimental colonies. Bees were collected in the summer, fall and the following spring in both years to measure the immune system activity.

Measures of immune gene activation revealed that bees within the colonies with naturally constructed propolis envelopes had significantly lower immune gene expression, or much "quieter" immune systems, over the summer and fall months compared to bees in control colonies. In fact, bees in colonies with a propolis envelope had less variable (more uniform) immune gene expression over the active foraging season (Borba et al. 2015). A decrease in energetic costs associated with the maintenance of an efficient immune system may help bees to allocate their energy to perform vital tasks (e.g., foraging and rearing brood) and to maintain higher storage protein levels (e.g., vitellogenin) required for overwintering success.

Immune gene expression data is often shown in scientific journals using bar graphs that are somewhat difficult to interpret. Figure 4a shows results for only one of the six immune genes measured from 7-d-old bees in September 2012 and September 2013, and the figure legend explains how to interpret the graph. Another way to show the data is through a visual representation where the low-to-high levels of gene expression are represented as colors, called a heat map. Figure 4b shows a heat map representation of the expression of all six immune genes for September 2012 and September 2013.

Surprisingly, by the following spring of both years, before the bees were actively collecting resin again, there were no significant differences in gene expression levels for most immune genes between bees from the two treatment groups (Fig. 5). This finding suggested that the bees' immune systems were not benefitting from the propolis envelope in early spring. To solve this conundrum, it became important to explore the possibility that the propolis deposited by bees in the previous summer and fall had lost some of its antimicrobial activity over the winter. Using a test

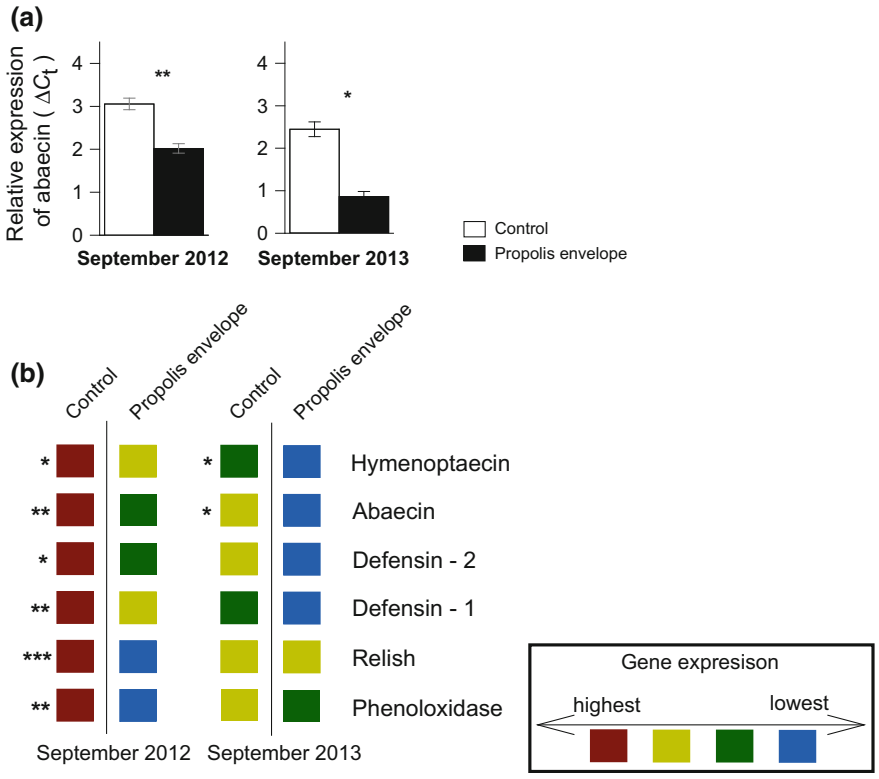

Fig. 4 **a** Relative expression levels of the immune gene abaecin in September 2012 and 2013. The expression levels are shown relative to the expression of reference genes Actin and RPS-5 $(\Delta Ct = (\bar{x}(\text{reference genes})\ Ct - \text{target gene}\ Ct))$ Reference genes are not involved in immunity but are produced in relatively equal amounts by bees over their lifetime to regulate other physiological functions. When the immune gene expression is high, the value on the vertical *y*-axis is a higher number. The height of each bar indicates the average (mean) value of the data for each immune gene, and the lines extending upward and downward from each bar represent the variation, standard error, around the mean. The *white bars* represent the control colonies and the *black bars* represent the colonies with a propolis envelope. **b** In this modified heatmap, the different colors represent low and high levels of gene expression. The color *blue* represents the lowest values of gene expression, followed by *green*, *yellow* and *red*, the highest level of gene expression. Values were statistically compared between treatment groups (propolis envelope and control colonies) for each gene separately (and thus colors can be compared only for each gene separately). Significant differences in gene expression between treatments (when results are considered statistically different) are indicated by * with increasing number of *'s indicating a higher probability of being different: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$). A P value greater than 0.05 indicates that there is no difference between the two treatment groups, while a P value lower than 0.05 means that the two treatments are significantly different

described later in this chapter (see Sect. 1.3), it was found that, indeed, the propolis within the nest in late April had lost much of its antimicrobial activity from the previous fall. The loss of biological activity of the propolis from October to April is probably due to the lack of new resins being brought in over the winter. Honey bees

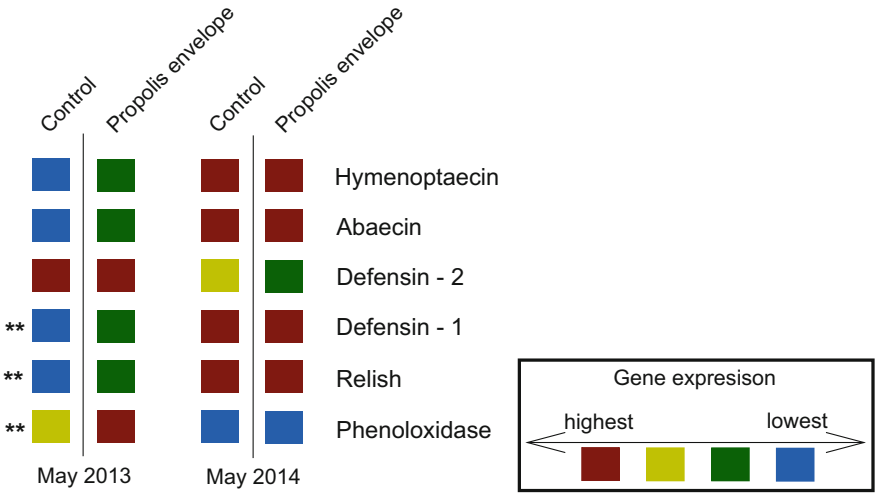

Fig. 5 Modified heatmap graph of the relative expression of immune genes in May 2013 and 2014. Significant differences in gene expression between treatments (when results are considered statistically different) are indicated by "**" ($P < 0.01$). A P value greater than 0.05 indicates that there is no difference between the two treatment groups, while a P value lower than 0.05 means that the two treatments are significantly different

in Minnesota do not forage for resin (or for any resources) during the cold temperature season, from October to April, but start collecting resin again later in May, when environmental temperatures for tree growth are favorable. With the deposition of new propolis in the nest in the spring and throughout the growing season, the benefits of the propolis envelope to the bees would return.

Even though the bees' immune systems did not benefit from the propolis envelope in early spring, measures of blood storage protein, vitellogenin (Vg), were significantly higher in spring of both years (May 2013 and May 2014) in bees from colonies with a propolis envelope compared to bees from control colonies (Fig. 6). Vg is an important protein in bees' hemolymph, and when present in high concentration is an indicator of well-nourished bees (Amdam et al. 2003, 2004; Engels et al. 1990). More recently, it has been found that Vg also contributes important priming function to the immune system of bees (Salmela et al. 2015). This high Vg level in bees from propolis envelope colonies in the spring of both years suggest that these bees had more protein storage compared to bees in control colonies and, therefore, is a possible explanation for why they were able to rear more brood compared to the control colonies (Bitondi and Simoes 1996; Mattila and Otis 2006).

The results from this experiment provided information on the long-term benefits of propolis to honey bee health, adding important new information to that reported by Simone et al. (2009).

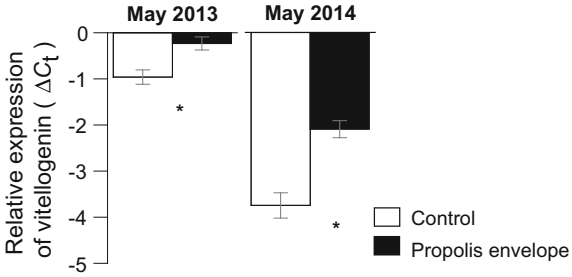

Fig. 6 Average (±standard error) of relative expression levels ($\Delta Ct = (\bar{x}(\text{reference genes})\, Ct - \text{target gene } Ct)$) of vitellogenin. As in Fig. 2a, a higher value on the *y*-axis means higher expression (e.g., –2 is higher than –4). Vitellogenin levels were higher in bees from propolis envelope colonies (*black*) compared to control colonies (*white*) in the spring of both years. Significant differences between controls and propolis envelope treatment colonies are indicated with * ($P < 0.05$)

In sum, colonies that are allowed to construct a natural propolis envelope on the inside of the hive boxes benefitted in ways that improve bee health and possibly colony strength and survivorship. The propolis envelope creates an antimicrobial layer around the bees that, remarkably, serves as an environmentally derived component of the bee's immune defense. Propolis could help bees' immune system either by reducing the microbe load in the nest cavity, as suggested by Simone et al. (2009), or by having a direct and beneficial effect on bees' immune system (Borba et al. 2015).

A human analogy. To fully understand the function and benefits of the propolis envelope to bees, it is helpful to draw an analogy between a honey bee nest and human homes. Mold and fungi are often found in our houses, especially during spring and summer when humidity is higher. The presence of these microorganisms in the air may not always cause a health problem, but some people's immune systems are easily affected by these microorganisms, and the inhalation of molds and fungi can lead to immune activation in more sensitive people. The propolis envelope to bees would be the same as coating the walls of our homes with an antimicrobial material. In that case, the antimicrobial material would effectively decrease the levels of microorganisms growing on the walls of the house and indirectly prevent our immune system from activating an immune response (Simone et al. 2009). Even in the absence of high levels of molds and fungi, the presence of the antimicrobial propolis could directly decrease the need to express immune genes (Borba et al. 2015). Lower immune system activation (the immune system at an efficient "idle") does not imply immune suppression (the immune system turned off; see Sect. 1.2). The lower immune system activation is beneficial because mounting a strong immune response comes with a cost. The immune system needs to use energy to fight off pathogens and when it is always activated, the individual, whether bee or human, is left with less bodily resources and greater immune stress, which may affect overall health and ability to fight off secondary or subsequent infections.

1.2 The Role of the Propolis Envelope for Bees' Natural Defense Against Brood Diseases

In addition to the everyday (constitutive) benefits of the propolis envelope to the bees' immune system (as described in Sect. 1.1), the antimicrobial properties of propolis supports honey bee natural defenses against pathogens. A recent study found that honey bee colonies coated with a propolis extract (experimentally applied envelope) and challenged with *Ascosphaera apis*, a brood fungal pathogen that causes chalkbrood, had less chalkbrood infected brood compared to challenged colonies with no propolis-extract coating (Simone-Finstrom and Spivak 2012). Colonies with a propolis-extract coating had an average of 14.7 ± 7.5 chalkbrood infected larvae per colony, while challenged colonies with no propolis coating had an average of 108.2 ± 49.0 chalkbrood infected larvae per colony. The mode of action by which the propolis decreases clinical signs of chalkbrood in honey bee colonies is not yet understood, but these initial findings were intriguing and led to another study to test the effect of a natural propolis envelope on a different bee disease: American foulbrood.

American foulbrood (AFB) disease is caused by the bacterial pathogen, *Paenibacillus larvae*. American foulbrood is highly infectious to honey bees and can rapidly spread among colonies via drifting (when a forager enters a colony that is not their own) and robbing of contaminated nectar. Young honey bee larvae (1–2 d old) are highly susceptible to this pathogen, while old larvae and adults are considered resistant. A potential reason for this susceptibility is thought to be because young larvae have "less developed" immune defenses compared to older brood and adults (young larvae have lower bee "blood" cell counts and cellular defense mechanisms; Chan et al. 2009; Wilson-Rich et al. 2008).

Previous studies have demonstrated four different mechanisms of colony resistance to AFB: (1) removal of *P. larvae* spores from contaminated honey by the filtering action of the proventricular valve between the bee's crop and ventriculus (stomach; Sturtevant and Revell 1953); (2) detection and rapid removal of AFB-infected brood by adult bees before the pathogen becomes infectious (hygienic behavior; Spivak and Reuter 2001b); (3) genetic ability of larvae to resist AFB infection (Evans 2004; Rothenbuhler and Thompson 1956), and (4) ability of nurse bees to secrete antimicrobial compounds into larval food, which can protect the larvae somewhat from *P. larvae* infection (Rose and Briggs 1969; Thompson and Rothenbuhler 1957). Additionally, numerous laboratory studies have demonstrated that propolis has antimicrobial properties that inhibit the growth of *P. larvae* (Bastos et al. 2008; Bilikova et al. 2013; Wilson et al. 2013, 2015). Therefore, the next experiment explored whether the antimicrobial activity of a natural propolis envelope could support bees' natural mechanism of defense against AFB.

Three questions were posed: (1) After challenging colonies with the bacterium that causes AFB, would the level of immune genes be higher in nurse-age bees in colonies with a propolis envelope compared to nurse-age bees in colonies without the envelope? (2) Would the antimicrobial activity of larval food supplied by nurse

bees to young larvae be higher in challenged colonies with a propolis envelope? And (3) would there be less AFB-infected brood in colonies with a propolis envelope?

In the summer of 2013, ten colonies were stimulated to construct a propolis envelope by stapling propolis traps to the inner walls of standard beekeeping boxes (as explained in Sect. 1.1). Five of the ten colonies were experimentally challenged with *P. larvae* by spraying a sugar solution with a known concentration of *P. larvae* spores on each comb within the colony (propolis + *P. larvae* treatment). The other five colonies with a propolis envelope were left unchallenged (propolis + no *P. larvae* treatment). Another set of ten colonies was not provided with a propolis envelope and the bees deposited propolis in the cracks and crevices within the box where they could. Similarly, five of the ten colonies without a propolis envelope were challenged with *P. larvae* (no propolis + *P. larvae* treatment) and the other five were left unchallenged (no propolis + no *P. larvae* treatment).

Samples of 7-d old bees were collected to test the expression levels of immune genes (as explained in Sect. 1.1), once before and once after challenged colonies showed clinical signs of AFB (August 9 and September 12, respectively). Samples of larval food were collected to test its antimicrobial activity. Larval food from 1- to 2-d old larvae was collected on the same day as the 7-d-old bees were collected (asymptomatic period August 9, and symptomatic period September 12). Prior to larval food collection, an empty frame was introduced into the colony and was marked when eggs were present. Three days after the frames were marked, when 1-2-d old larvae were present, the frames were removed and larval food was collected following Schmitzová et al. (1998). In a temperature-controlled room, each young larva was removed from the cell using a sterile grafting tool, and the larval food from each cell was individually homogenized in 30 μl of phosphate buffer by repeated pipetting and then transferred to individual tubes.

The number of larvae with clinical signs of AFB (sunken wax capping and uncapped cells containing discolored, ropy brood) on each frame of each colony was quantified approximately every 15 days after the appearance of the first clinical sign (August 30, September 16 and October 1).

The antimicrobial activity of larval food was measured in liquid culture. Most bacteria, such as *P. larvae*, can be grown under controlled laboratory conditions, in tubes containing a liquid with the required nutrients for bacterial growth (called broth). Bacterial growth in liquid culture is characterized by the increased turbidity of the culture, and the optical density (OD) of the liquid culture can be measured using a spectrophotometer. This machine produces a light of a preselected wavelength in one end of the chamber that houses the sample, and records the intensity of light detected at the other end of the chamber after it passes through the sample. Samples with greater concentrations of bacteria have a greater optical density and will absorb more light, reducing the intensity of light that reaches the detector. Therefore, the intensity of the light detected decreases as the sample concentration of bacteria, and optical density, increases. The antimicrobial activity assay consisted of allowing a known concentration of a *P. larvae* culture (pre-grown in brain/heart infusion broth for 48 h prior to the assay) to grow in the presence of larval food for

6 h at 37 °C and subsequently evaluating the bacterial growth by measuring the optical density (OD at time 0 h subtracted from time 6 h). Bacterial growth was compared in cultures with added larval food relative to cultures without added larval food (controls).

Immune gene expression analysis of nurse-age bees collected after the appearance of AFB clinical signs showed that bees from challenged colonies with a propolis envelope had a stronger immune response compared to bees in challenged colonies without a propolis envelope, as indicated by significantly higher gene expression levels of two antimicrobial peptides (hymenoptaecin and apidaecin). It is well known that honey bees increase the expression of most antimicrobial peptides, including hymenoptaecin and apidaecin, to fight a *P. larvae* infection (Chan et al. 2009; Evans 2004). However, *P. larvae* spores do not germinate in adult bees and therefore do not cause any harm to nurse-age bees. Thus, the inducible physiological response of nurse-age bees to AFB infection may not be to protect adult bees against this pathogenic infection but to protect young larvae that are fed by them, which are highly susceptible to this disease. These gene expression results indicate that nurse bees from propolis envelope colonies have the ability to synthesize higher levels of antimicrobial peptides and potentially decrease colony-level AFB infection more rapidly and efficiently compared to bees in challenged colonies without a propolis envelope. Importantly, these findings also demonstrate that bees in colonies with a propolis envelope are able to mount a strong immune response after they are challenged. Thus, the lower immune system activation ("quieter" immune system) of bees in apparently healthy colonies with a propolis envelope (see Sect. 1.1) is *not* due to immune suppression (i.e., the inability to mount an immune response), because after challenge these bees are able to quickly activate their immune responses.

Nurse bees perform the behavioral task of feeding the brood by regurgitating larval food into the cells and therefore are in constant direct contact with the susceptible larval stage to AFB. We found that when challenged colonies had a propolis envelope, the bioactivity of the larval food was significantly higher compared to the larval food in unchallenged colonies without a propolis envelope. The higher antimicrobial activity of larval food in challenged colonies with a propolis envelope suggests that antimicrobial compounds from the propolis envelope may contribute directly to the bioactivity of larval food against bee pathogens. Although the propolis envelope may not come into direct contact with larval food, volatile compounds present in propolis can diffuse through the hive and may contribute to the complex way in which bees fight infections. Another hypothesis is that nurse bees in challenged colonies with a propolis envelope that produce more antimicrobial peptides, incorporate these antimicrobial peptides (Bilikova et al. 2001) into larval food fed to 1–2 d old larvae to increase young larvae immune defense mechanism to fight *P. larvae* infection. Either way, these results confirm the existence of a natural defense mechanism in honey bees against AFB by feeding larvae food with a higher antimicrobial activity (Rose and Briggs 1969; Thompson and Rothenbuhler 1957). Importantly, both mechanisms of

defense against AFB (higher immune gene expression and larval food bioactivity) were only observed when challenged colonies had a propolis envelope.

Clinical signs of AFB can be identified by the presence of sunken wax cappings and uncapped cells containing discolored, ropy brood. As a measure of the level of AFB infection, the number of cells containing signs of AFB was counted in each comb (Spivak and Reuter 2001b). A severity score ranging from 0 – 3 was given for each comb (both sides combined) that contained larvae: 0 = 0 cells containing signs of AFB; 1 = 1-5 cells; 2 = 6-25 cells; and 3 = $\geq$ 26 cells per comb (Spivak and Reuter 2001b). An overall AFB severity score for each colony by each month (i.e., August, September and October) was obtained by calculating the median ($\pm$ interquartile range) of the individual comb scores (Table 1). These results indicate that the presence of a propolis envelope inside a colony reduced the number of larvae with clinical signs of AFB over time, but did not eliminate the disease completely. The reduced level of AFB clinical signs in early October in colonies with a propolis envelope compared to colonies without a propolis envelope is likely a result of a combination of the effects of propolis on both the collective and individual behavioral responses (larval food bioactivity and individual bee immune response), as well as the incorporation of antimicrobial peptides by nurse-age bees into larval food. The study by Simone-Finstrom and Spivak (2012) on the effect of a propolis-rich environment on the infection level of chalkbrood disease reported that colonies with a propolis-extract coating inside the nest had a level of infection 86% lower (14.7 $\pm$ 7.5 cells compared to 108.2 $\pm$ 49.0) than observed in colonies without the propolis extract. Similarly, the findings presented here show that colonies with a propolis envelope had 52% fewer cells infected with AFB in October compared to colonies without a propolis envelope (Table 1).

To summarize, the presence of a propolis envelope increased the individual and collective immune responses of bees, possibly by supporting the increased production of antimicrobial peptides in individual nurse bees, and increasing bioactivity of larval food fed collectively by nurse bees. As a result, AFB clinical signs in early October in colonies with a propolis envelope were reduced compared to colonies without a propolis envelope. The propolis envelope served as an external antimicrobial layer around the colony, protecting the brood from *P. larvae* infection and supporting bees' ability to induce a strong and effective immune response with the result of a lower infection load after two months following the challenge.

Table 1 AFB infection level data was measured by counting the number of cells containing signs of AFB in each comb.

Treatment	Number of colonies	AFB clinical sign (median $\pm$ interquartile range)		
		August	September	October
No propolis envelope + *P. larvae*	5	0.875 $\pm$ 1.187	1.5 $\pm$ 1	2.429 $\pm$ 0.863
Propolis envelope + *P. larvae*	5	0.625 $\pm$ 1.125	0.125 $\pm$ 1.875	1.167 $\pm$ 0.733
Statistical significance		$P > 0.05$	$P > 0.05$	$P = 0.036$

The median number of total AFB-infected cells were compared between treatments. A P value lower than 0.05 indicates a statistically significant difference between the two treatment groups

1.3 Do Bees Self-Medicate?

Self-medication is defined as the "defense against pathogens and parasites by one species using substances produced by another species" (Clayton and Wolfe 1993). If bees can truly self-medicate, an individual (or colony) should perform a behavior, such as resin collection, at higher rates when parasitized and at lower rates when healthy. Simone-Finstrom and Spivak (2012) found that honey bee colonies increase resin foraging after exposure to chalkbrood, revealing that bees medicate the colony with resin in response to this particular fungal infection. To extend the knowledge of how honey bees exploit resin to fight pathogen infection, a recent study by R. Borba investigated whether bees also self-medicate in response to a bacterial infection, American foulbrood (AFB).

This study was repeated over 3 years from 2012 to 2014, using new sets of colonies each year. Colonies equalized in population size and food resources were used, and resin foraging activity was monitored when the colony was healthy and after experimentally challenging them with either *P. larvae*, the causative agent of AFB (in 2012, 2013 and 2014), or *Ascosphaera apis,* the causative agent of chalkbrood (CB; in 2014 only). The number of resin foragers was assessed before pathogen challenge by closing the colony entrance once or twice a day (weather depending) for 15 min between 1100 and 1600 h for 12 observation periods (spread over two weeks) and recording the number of foragers returning with a resin load on the hind legs. After 12 observations, one group of colonies was challenged with a *P. larvae* spore solution (using the same methods described in Sect. 1.2), and the second group of colonies served as controls (unchallenged colonies). In 2014, a third group of colonies was provided with a pollen patty containing *A. apis* spores. Resin foragers were again counted over another set of 12 observations periods spanning two weeks. The change in resin foraging between the pre-challenge and post-challenge periods was calculated for each colony by subtracting the total number of foragers before challenge from the total number of foragers after challenge, and this difference was compared among treatment groups (control, AFB- and CB-challenged colonies).

The results showed that colonies challenged with *P. larvae* have a slight numerical increase in resin foraging in 2012, 2013, and 2014 compared to unchallenged colonies (Borba, 2015). In 2014, bees from CB-challenged colonies had a substantial and statistically significant increase in resin foraging, as they did in the study by Simone-Finstrom and Spivak (2012).

Do bees self-medicate with specific plant sources of resin? When people are sick, they can go to the pharmacy and self-medicate by buying an over-the-counter drug that treats the infection they are experiencing (e.g., bacterial or fungal infection). Honey bees self-medicate in a similar way by collecting antimicrobial resins ("drugs") from plants ("pharmacy"), but it is not known if bees choose specific resins that are most able to treat the infection the colony might have.

Chemical composition of resins varies qualitatively and quantitatively within and among plants (Witham 1983). Wilson et al. (2013) conducted a study on the bioactivity of resins from 14 tree species against *P. larvae* growth. The resins were

collected from trees on the St. Paul campus of the University of Minnesota, and the findings revealed a significant difference among botanical sources of resins to inhibit the growth of this bacterium. Likewise, previous research found that propolis samples from different regions had significantly different inhibitory activity against the growth of *P. larvae* (Bastos et al. 2008, Wilson et al. 2015). Because propolis is a mixture of resins collected by individual bees, it is likely that the great diversity in the ability of samples of propolis to inhibit the growth of *P. larvae* is due to the different resins bees collect from various plant species in different regions (Mihai et al. 2012). Therefore, the next step was to explore whether bees change their foraging preference for specific plant resins after challenge with a bacterial or fungal pathogen.

To test if bees alter their selection of resins after colonies are challenged with a bacterial or fungal pathogen (*P. larvae* and *A. apis*, respectively), resin loads were collected from the hind legs of returning resin foragers during each observation (pre-challenge and post-challenge). Individual resin loads were stored in separate glass vials and the botanical source of the resin was further analyzed in the laboratory.

It is difficult to monitor bees foraging for resin on plants because resin foraging is particularly rare, compared to others types of foraging, and bees often collect resin high in the canopy of trees, which makes it difficult to observe resin foraging directly. The plant source of a resin collected by a bee can be identified by chemically comparing the resin loads of returning foragers with resins collected directly from plants. This strategy is very similar to how pollen foraging is tracked. Since the shapes (morphology) of pollen grains are characteristic of specific plants, microscopy is used to match the morphology of bee-collected pollen to the morphology of pollen collected from flowers. Resins have chemistries that are characteristic of specific plants, and these chemical signatures, rather than morphology, are used to identify resin sources.

In collaboration with M. Wilson, J. Cohen and A. Hegeman from the Horticultural Science Department of the University of Minnesota, resin chemistries were examined using two techniques in series, liquid chromatography and then mass spectrometry (LC-MS). Essentially, LC-MS sorts the hundreds of compounds found in resins by water solubility. This information is then condensed into a "fingerprint." If the chemical pattern, or fingerprint, of a bee-collected resin load is the same as the chemical pattern of a resin collected directly from a plant, it can be concluded that the bee visited that specific plant (Fig. 7).

To date, analysis of data from resin loads collected from bee hind legs in 2012 and 2014 revealed that bees collected resin from five botanical sources in St. Paul, Minnesota: *Populus deltoides* (Eastern cottonwood trees), *P. hybrid* (hybrid poplar trees), and three sources that are not yet identified, unknowns 1, 2 and 3 (Borba 2015). The majority of bees in all colonies collected resin from the most abundant resin-producing tree around the St. Paul campus area, Eastern cottonwood (*P. deltoides*), while resin from the other four sources was not collected in great quantities.

For the most part, all colonies continued to collect resin from the same sources after they were challenged with either the bacterial or fungal pathogen, with the

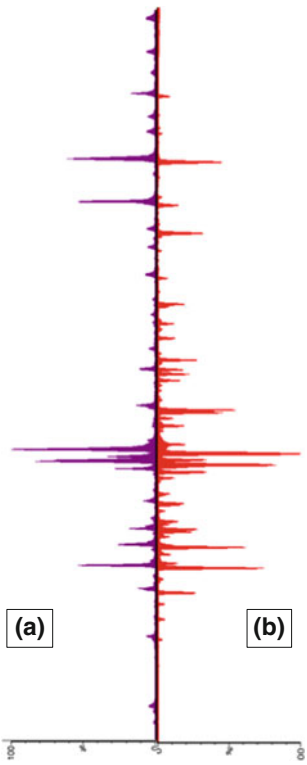

Fig. 7 Resin fingerprint of Eastern cottonwood trees collected from individual tree buds (**a**), and fingerprint of resin collected from the bee's hind leg (**b**). Based on the similarities of the chemical pattern of these two resin fingerprints, we can conclude that the resin collected from this bee is from an Eastern cottonwood tree

exception of colonies in 2012 that did not collect resin from hybrid poplar during the post-challenge period. In general, when colonies increased resin foraging, they simply increased the number of foragers collecting resin from the plants they were already visiting (Borba 2015).

The antimicrobial activity of the resins bees collected was measured in liquid culture using the same assay used to measure the bioactivity of larval food (Sect. 1.2). Of the five different plant sources of resin, the resin from Eastern cottonwood and hybrid poplar had the greatest antimicrobial activity against fungal growth (*A. apis*). Resin from Eastern cottonwood also had the highest antimicrobial activity against bacterial growth (*P. larvae*), but hybrid poplar had relatively low inhibitory activity against this pathogen. Thus, post-challenge colonies did not appear to change their foraging preference to collect resins with higher specific bioactivity (they do not forage for "stronger medicines" for a particular pathogen). Other trees around the St. Paul campus area, such as white spruce (*Picea glauca*), secrete resin with even higher antimicrobial activity against *P. larvae* compared to Eastern cottonwood (Wilson et al. 2013). However, bees apparently do not collect resin from white spruce around the St. Paul campus, as the chemical signatures of the three unknowns did not correspond to white spruce or any other resin-producing plant identified in Wilson et al. (2013).

Bees' decision-making process to collect resin from specific sources after chalkbrood and AFB infection could be driven by the abundance of the plant in the area, the abundance of resin produced by particular plants, the ease of collecting resin from particular plants, distance from the hive, and/or the bioactivity of the resin. Resin collection and choice by bees are unstudied areas that require further investigation.

1.4 Recommendation for Beekeepers

These studies clearly show the benefit of a propolis envelope, particularly an envelope naturally constructed by the bees, to bee health and immune system functioning. The collection of resins to construct a natural propolis envelope is performed by a relatively rare subset of the worker foraging force. The number of resin foragers is probably less than 1% of the total number of foragers in the hive, but this foraging preference may be influenced by the bees' genetics (Butler 1949; Page and Fondrk 1995). Resin collection is partly a genetic tendency and partly a demand-driven process (Martinez and Soares 2012; Nakamura and Seeley 2006). How and what they detect inside the nest to determine need is not clear. When resin foragers encounter rough surfaces and gaps inside the hive, they respond by collecting more resin to seal these cracks in the nest architecture (Simone-Finstrom and Spivak 2010). Therefore, a colony of bees can be encouraged to build a natural propolis envelope within standard beekeeping equipment by modifying the inner walls of bee boxes. Commercial propolis traps can be cut to fit the four inside walls of the hive boxes and stapled with the smooth side of the trap facing the wood and the rough side facing the colony. Using nine frames instead of ten is best when using this method. If the inside of the bee box is built with unfinished, rough lumber, scraped briskly with a wire brush, or if 3 mm grooves are cut in the interior walls of the box, the bees will apply a layer of propolis in the grooves, forming a natural propolis envelope.

A cautionary note for beekeepers. The initial experimental design for the study on the long-term effects of the propolis envelope (see Sect. 1.1) consisted of three treatments: colonies without a propolis envelope (control), colonies with a propolis envelope, and colonies fitted with a propolis trap on top of the frames of the top box, as is done to collect propolis commercially. Bees from colonies with the propolis traps on top of the frames showed inconsistent, and sometimes higher immune-related gene expression, compared to bees in the propolis envelope and control colonies. Moreover, bees from colonies with a propolis trap on top of the frames had significantly higher levels of virus (i.e., DWV) compared to bees in control and propolis envelope treatment colonies in September 2012, May 2013 and May 2014. The presence of high levels of virus has been correlated with colony death and the reduced efficacy of the bee's immune system. It is possible that the presence of the water-resistant propolis trap throughout the year on top of the colony could have altered the microenvironment of the colony (e.g., increasing humidity levels or affecting air circulation within the nest), leading to favorable conditions for the growth of pathogens and maybe viruses. Thus, it appears that

leaving a propolis trap on top of a colony for a long period of time, and especially over the winter, is not beneficial to bee health and is not recommended.

Finally, there is no evidence that bees consume resins or propolis. It is not recommended that beekeepers feed propolis solution to bees. Because of the highly antibacterial and antifungal properties of propolis, it could risk killing the beneficial microbiome in bees' guts that is so critical to their health and survival.

1.5 Summary of Findings

Understanding honey bees' natural defense mechanisms allows us to appreciate how resilient honey bees are and to improve our beekeeping practices to enhance their natural behaviors and defenses. The process of domestication of the *Apis mellifera* species by humans using managed hives has interfered with one very important natural defense mechanism of the honey bee colony, the construction of a propolis envelope. The results of research first by M. Simone-Finstrom and later by R. Borba strongly indicate that the propolis envelope serves as an external antimicrobial layer around the colony, providing fundamental benefits to adult bees' immunity (see Sect. 1.1), greater colony fitness in early spring after the winter (see Sect. 1.1), supports bees' natural defense mechanisms against AFB and chalkbrood disease (see Sect. 1.2) and supports nurse bees' ability to induce a strong and effective immune response after AFB infection, resulting in a lower infection load after two months following bacterial challenge (see Sect. 1.2). Honey bees self-medicate by increasing the number of resin foragers after the colony is infected with the fungal pathogen that causes chalkbrood, but not after infection with the bacterial pathogen that causes AFB (see Sect. 1.3). After bacterial or fungal challenge, colonies do not appear to change their resin foraging preference; instead, it appears that bees simply increase resin foraging for resin sources previously collected by the colony. The decision-making process for the recruitment of specific resin sources after chalkbrood and AFB infection, and whether the decisions are driven by plant resin source abundance or resin bioactivity, requires further investigation.

Given all the evidence provided here, it is important to recognize the significance of the propolis envelope as a crucial component of the nest architecture in honey bee colonies. When searching for an apiary location, beekeepers should take into consideration both flower abundance and diversity, and the presence of resin-producing plants within foraging distance from the apiary.

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Dr. Renata S. Borba is a postdoctoral fellow who has recently joined Dr. Steve Pernal's apiculture research team at Beaverlodge Research Farm, Agriculture Agri-food Canada. Renata received her Ph.D. in Entomology in 2015 from the University of Minnesota studying under Dr. Mara Spivak. Previously, she received her B.Sc. in Animal Science from the Universidade Federal of Ceara, Brazil. Renata's doctoral research focused on evaluating: 1) the seasonal benefits of propolis (a bee-produced resinous material) on the health and immunity of honey bees; 2) the role that types of resins play as a defense against two highly infectious brood pathogens, Ascosphaera apis (a fungus causing chalkbrood disease) and Paenibacillus larvae (a bacterium causing American foulbrood disease); and 3) the effects of the propolis "envelope" within the hive as a natural defense against disease.

Michael B. Wilson is a postdoctoral scientist living and working in Minnesota's Twin Cities. He found his fascination of bees and the people who keep them while training under Marla Spivak and Jerry Cohen at the University of Minnesota. He is currently focused on studying how resinous plants impact honey bee health and understanding the mechanism of benefits bees derive from propolis in their nests. He firmly believes that enhancing and leveraging what bees do naturally to prevent disease will lead to more sustainable beekeeping.

Mike, his wife Fern, and their dog Hannah are enthusiastic gardeners, hikers, and beach bums. When Mike is not doing science or exploring the outdoors, he is getting beat up by his students at Ram's Taekwondo in St. Paul, MN. While his heart is still young, his knees are getting old! He will readily confirm that daily doses of kicking kept him sane throughout graduate school.

Marla Spivak is a Distinguished McKnight Professor in Entomology at the University of Minnesota. She obtained her Ph.D. at the University of Kansas under Dr. Orley Taylor in 1989 on the ecology of Africanized honey bees in Costa Rica. From 1989 to 1992, she was a postdoctoral researcher at the Center for Insect Science at the University of Arizona where she became interested in honey bee hygienic behavior and continued that line of work at the University of Minnesota beginning in 1993. She has bred a line of honey bees, the Minnesota Hygienic line, to defend themselves against diseases and parasitic mites. Current studies include the benefits of propolis to honey bees and the effects of agricultural landscapes and pesticides on honey bee and native bee health.

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