

Final Report
Benefits of Propolis to Honey Bees: Does Propolis Reduce Levels of Viruses in Larvae?
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We initiated a new line of research in my lab in 2006 to explore the benefits of propolis to honey bees. My graduate student, Mike Simone-Finstrom, has reported exciting and novel findings on how propolis helps bees' immune systems (Simone et al., 2009; Simone-Finstrom and Spivak, 2010), and in collaboration with others we have shown that propolis displays good antimicrobial activity in the laboratory setting (Gekker et al., 2005; Bastos et al, 2009). However, in some unpublished trials we found that propolis in field colonies did not appear to actively defend against pathogens and parasites that affect the bee brood, such as American foulbrood, *Varroa destructor*, and with this research, viruses in worker larvae. As we are pioneering this field of research on propolis and bee health, we are exploring questions that have never been asked. To understand how propolis benefits a bee colony, I give a brief explanation of its origin use in natural bee nests (e.g. tree cavities), our line of reasoning in developing this grant proposal, and our ideas for future research.

Background (reviewed in Simone-Finstrom and Spivak 2010). Propolis is the apicultural term for resins that bees collect from the leaf buds of some trees. In temperate regions plants that secrete resin include trees in the genus *Populus* such as poplar, cottonwood, aspen; other trees such as alder, birch, and to a lesser degree coniferous trees such as pine and spruce. The resin acts as a defensive compound for plant wound healing and as a physical barrier around budding leaves to prevent invasion by pathogens and insects. Bees that forage for resins are relatively rare compared to nectar and pollen foragers. Resin foragers collect the plant resins on their hind legs and when they return to the nest, other bees help remove the sticky load and cement the resin in the nest cavity. In a natural nest cavity, such as in a tree bole, bees coat the inside tree surface surrounding the combs with a layer of resin. This coating is called a propolis envelope. They also use resin to seal holes and crevices in the nest cavity, to narrow the nest entrance, and sometimes to entomb intruders. The resins in the nest may be combined with wax but otherwise the bees do not appear to modify the chemistry of the resins.

Propolis collected from bee colonies is highly regarded for its medicinal properties for humans, and the antimicrobial properties of propolis against human pathogens have been known since antiquity (Ghisalberti, 1979). Hippocrates recommended its use for cleaning and healing of wounds, and therapeutic uses of propolis (*torzi* in Hebrew) are mentioned throughout the Old Testament. In general, the antimicrobial properties of propolis are due to the presence of plant compounds (phytochemicals) such as flavonoids, phenolic acids, prenylated *p*-coumaric acids and diterpenes.

Propolis and HIV-1. A number of studies have presented evidence that propolis has strong hepatoprotective, antitumor, antioxidative, antimicrobial and antiinflammatory properties for humans (reviewed in Banskota et al., 2001). My interest in propolis began with a collaborative study with Dr. P. Peterson in the University of Minnesota, Medical School. We found that propolis collected from bee colonies in various locations around the world was active against human HIV-1

infection in CD4+ lymphocyte and microglial cell cultures (Gekker et al., 2005). This research result was exciting but unfortunately Dr. Peterson did not pursue this research because the chemistry of propolis is complex and highly variable, and more background work was needed to determine which compound(s) showed activity against the virus so they could be tested on human cell cultures in controlled doses

I then sought collaboration from Drs. Jerry Cohen and Gary Gardner, two plant chemists and physiologists in the Department of Horticultural Sciences, to help develop high-throughput techniques to test the activity of propolis against bee bacterial and viral pathogens. The long-term goal of our research is to isolate fractions that are active against human HIV-1 by screening the various propolis fractions against bee pathogens to narrow the range of active compounds in an economical and efficient way, while exploring novel compounds beneficial to honey bee health. Because testing activity against HIV-1 is extremely expensive, we will essentially be using the bee as a screen to test the fractions. Our hope is that we find fractions that are effective and thus helpful to both bee and human health.

We obtained funding from the University of Minnesota College of Food, Agriculture, and Natural Resource Sciences to develop high-throughput techniques to test the activity of propolis against the bacterial bee pathogen, *Paenibacillus larvae*, that causes American foulbrood. This bacterial pathogen is readily cultured in the lab and we have had good success in developing techniques that allow us to rapidly test the activity of propolis and various propolis fractions. This research will comprise the PhD thesis of graduate student, Mike Wilson.

Propolis and Bee Viruses: We knew that testing the activity of propolis against bee viruses would be potentially difficult because it is not possible to culture viruses outside the bee, and we do not have a continuous honey bee cell culture in which we could propagate the virus *in vitro*. We decided to take an alternative approach, which formed the basis of this work funded by the National Honey Board. We hypothesized that propolis, applied as an oral treatment in brood food, might reduce the levels of viruses in honey bee larvae. If this technique worked, we could test many propolis samples and fractions relatively quickly.

Graduate student Jessica Burtness initiated this work, after traveling to the USDA-ARS lab in Beltsville to learn techniques from Dr. Judy Chen, an expert on bee viruses (Chen et al., 2006; Chen and Siede, 2007). Dr. Chen advised that we begin with Deformed Wing Virus as it is the most prevalent virus found in bee larvae.

Methods. In the fall of 2009 and summer 2010, we identified colonies that had larvae infected with Deformed Wing Virus (DWV) using Reverse Transcriptase PCR (RT-PCR) to determine viral presence or absence. We then raised larvae *in vitro* from infected colonies to control rearing conditions and propolis treatment. We collected 1st instar larvae from infected colonies and raised them in individual “cells” within a 96-well plate containing 40µl of a brood food mixture in each well. The food mixture consisted of 2 parts royal jelly to 1 part yeast solution (3% Difco yeast extract, 18% Fructose, 18% glucose), plus the propolis or control treatment. We maintained the larvae in the incubator for 72 hours at 34.5°C and 80% humidity then stored them at -80°C for analysis.

The propolis treatments contained either low dose (5-12µg) or high dose (170-187µg) of propolis dissolved in isopropanol added to the food mixture for each larvae. Control treatments contained 10µL of isopropanol solvent (equivalent to the volume of propolis extracts used) in the food mixture, or the food mixture alone. These doses were chosen after preliminary experiments determined that higher concentrations of propolis were toxic to the larvae. We also tested if a volatile treatment; we painted propolis on the lid of the well plate and placed it over the larvae in

untreated food mixture. This treatment would shed light on whether the activity of propolis is in the volatile fraction, rather than in portion the larvae would contact or consume.

We used Quantitative Real Time PCR (qRT-PCR) to compare the *levels* of viruses in individual larvae fed the propolis or control food mixtures. We extracted the viral RNA from the larvae using methods developed by Dr. Jay Evans and previously used in our lab (Simone et al. 2009). We then added the primer pair for Deformed Wing Virus, which was then amplified using the qRT-PCR thermo cycler. We also added the β -actin primer pair as an endogenous control to normalize the virus level relative to actin. The virus levels in each bee was quantified based on the value of the cycle threshold (Ct), which represents the number of cycles needed to generate a fluorescent signal above a predefined threshold.

Results: For analysis, we used data from only those larvae that had detectable levels of Deformed Wing Virus (DWV). When we normalized the virus levels by subtracting the DWV Ct from the actin Ct, we found a curious result: virus levels appeared to be significantly higher in larvae treated with high dose propolis compared to larvae treated with low dose propolis and isopropanol (Table). However, this result stemmed from the fact that actin levels were not consistent as they should have been (Table); actin was significantly higher in larvae treated with high-dose propolis compared to isopropanol control larvae. When we compared the level of DWV (not normalized relative to actin) we found all larvae, irrespective of treatment, had similar virus levels.

Table. The results of various propolis treatments on the level of Deformed Wing Virus (DWV) in larvae reared in an incubator for 72 hours. Only larvae that had detectable levels of DWV were included in the analysis. The mean (\pm s.e.) level of DW is shown normalized to reference gene actin (3rd column). For comparison, the levels of actin, and of DWV are also shown (4th and 5th columns). Means within a column followed by different letters are significantly different based on Tukey's post-hoc comparison of means.

| Treatment | larvae | Actin Ct –DWV Ct | Actin Ct | DWV Ct |
|------------------------------|--------|---------------------|---------------|--------------------|
| Food only (control) | 19 | -5.04 \pm 0.98 ab | 30.93 0.86 ab | 35.97 \pm 0.50 a |
| Food + isopropanol (control) | 24 | -6.35 \pm 0.88 b | 29.42 0.77 b | 35.78 \pm 0.45 a |
| Food + low dose propolis | 37 | -5.47 \pm 0.71 b | 30.87 0.62 ab | 36.35 \pm 0.36 a |
| Food + high dose propolis | 37 | -2.59 \pm 0.71 a | 32.85 0.62 a | 35.44 \pm 0.36 a |
| Volatile | 19 | -5.06 \pm 0.98 ab | 31.02 0.86 ab | 36.08 \pm 0.50 a |

Discussion and Future Research. Based on the DWV-Ct data, which is the actual level of virus found in the larvae (over a certain threshold of detection), we conclude that the propolis treatment, applied to larval food, did not reduce the amount of DWV in treated larvae. While this initial result was disappointing, it is actually informative and allows us to formulate new ideas and methods.

We encountered a systematic problem in obtaining the data: although it was easy to find colonies with larvae that had Deformed Wing Virus (using Reverse Transcriptase PCR), the prevalence of the larvae with that virus was very low. For example, if 96 larvae were reared in a well plate, only 5-10 larvae had detectable levels of virus. (Note that RT-PCR detects presence/absence of the virus, not quantity). It took a large number of trials to obtain the sample size shown in the table, which meant the technique was not high-throughput (rapid).

The fact that actin levels were not consistent was perplexing. Prior studies have shown that actin is generally a good reference gene across different life stages of bees and after treatment with certain chemicals (Lourenço et al. 2008). The variability we found was likely not due to experimental error as actin levels were consistently higher in high-dose propolis treatments across

plates (replicates). It could be that actin is not stable with respect to propolis treatment and other reference genes should be tested.

In retrospect we do not think it is a good idea to give oral treatments of propolis to bees, either in the larval or adult stage. Bees do not normally consume propolis, and it is possible that oral ingestion does more harm than good to bees. As propolis has powerful antimicrobial properties, it likely kills beneficial bacteria and yeasts (probiotics) in bees. In our experiments on the effects of propolis on bee immunity (Simone et al., 2009), we recreated the propolis envelope found naturally in tree cavities by applying a solution of propolis extracted in ethanol to the inner walls of the hive boxes. We did not feed adult bees or larvae propolis; it was used as a contact and volatile treatment in the form of a propolis envelope in the nest cavity. This application was sufficient to positively affect the bees' immune system. The mode of action of propolis may be subtle: we know it benefits the bees immune system, but due to its placement around the nest, it is not clear if it acts directly on adult and larval pathogens. Simone (2010) has intriguing data showing that bees increase collection of resins when infected with the fungal pathogen *Ascosphaera apis* that causes chalkbrood. It is therefore possible that resin-collection and propolis use by honey bees has several direct and indirect effects not only on honey bee health and behavior, but also on various pathogens and parasites. Some of these effects may be quite subtle, and potentially quite important, and thus warrant subsequent study.

Future research: To test the effects of propolis on honey bee viruses it would be best to use a honey bee cell culture that is inoculated with virus, and to then introduce the propolis treatment into the cell culture as was done in the HIV-1 study. One of my graduate students is working with Dr. Tim Kurtti in the Department of Entomology to develop a continuous cell culture with preliminary success, so it may be feasible to use this approach in the future.

It may also be possible to inoculate adult bees with known quantities of virus, using techniques Dr. Chen has recently developed (J. Chen, personal communication), and they to place the bees in a cage environment that has a propolis envelope. We will continue to explore new methods because, although challenging, we feel this is an important area of research and are appreciative of funding such as this that allows us to explore novel ideas.

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