Determining if *Nosema ceranae* and a Previously Unreported Virus are Causal Agents of CCD

Project No.: **09-POLL10-Bromenshenk**

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Objectives:

The primary objective is to greatly strengthen the knowledge of two pathogens that we suspect are associated with Colony Collapse Disorder (CCD). We intend to pursue this objective by analyzing bee colonies from 35 beekeeping operations, which we sampled in three areas of California durring a recent wave of CCD, (fall and winter of 2010). With these samples, we aim to prove or disprove our hypothesis. This being that the interaction of two pathogens, specifically *Nosema ceranae* and a heretofore unreported and unsequenced virus, is the presumed cause of CCD.

From a pragmatic perspective, we have used ABC funding to: (1) investigate the extent and severity of CCD in California just before the 2010 almond pollination, (2) obtain a much broader sample set of bees, including more beekeeping operations with 'good' bees, so that we can truly assess whether healthier bees do or do not have the two pathogens that we have previously found in all CCD colonies, (3) collect fresh material (infected bee tissues) for inoculations to test following Koch's Postulates, (4) provide this fresh material to our virologists in Texas and Mexico, who will try to extract, isolate, and purify the unsequenced virus, and (5) use this material and information in an attempt to produce a PCR primer for fast and inexpensive screening and identification of the virus in bee samples.

In addition, we have labeled a number of beekeeping operations and bee colonies for sequential (longitudinal) testing. Simply stated, we want to find, monitor, and sample colonies that stop growing and then follow them through the sequence of collapse.

Interpretive Summary:

In 2010 Colony Collapse Disorder (CCD) again devastated honey bee colonies in the USA, indicating that the problem is neither diminishing nor has it been resolved. This project was funded by the Almond Board of California (ABC) as an quick response measure to: (1) investigate the extent of the problem in colonies that were staged in California for the 2010 pollination of almonds, and (2) confirm if our own knowledge of the pathogens suspected of causing CCD is actually correct.

As per the problem this year, we published the following note on CCD in the USA in Bee Culture (Bromenshenk, 2010):

"Colony Collapse Disorder (CCD) Is Alive and Well" by Jerry J. Bromenshenk.

Despite published rumors of its demise, this winter CCD again decimated colonies in California and other states. I saw collapsing colonies in Florida, after the American Beekeeping Federation meetings. When I got back to Montana, we had reports of bee problems from several states, along with stories of major collapses in California. I called Kim Flottum and found that we were both getting similar reports. Kim agreed to buy me a plane ticket to go out and see what was going on in California.

What I found was a situation that mirrored that of 2007. Bee losses were widespread. The affected colonies displayed the signs of CCD, with sudden colony collapses, resulting in empty hives or hives with a queen and a fistful of young bees. Some beekeepers lost 80-90 percent of their bees; many more lost 50-60 percent, with the mildest cases reporting 30-40 percent of colonies showing signs of failing to thrive and dwindling.

I inspected and sampled bee colonies from Modesto down to Bakersfield, and saw the same scenario everywhere. Although impossible to quantify, CCD bee losses seemed to be on par or maybe even worse than in 2007. Both intra-state and intra-state migratory beekeeping operations were impacted, as well as stationary beekeepers. Size of beekeeping operation did not appear to be a factor; nor were all beekeepers affected. I saw some exceptionally strong colonies, but in general, strong colonies were only found in operations that did not report CCD.

One 20,000 colony outfit from North Dakota had good looking bees from western North Dakota, 50 percent or higher losses for bees from eastern ND, and about 30 percent losses in Idaho. In addition, additional colonies came out of the wintering shed in Idaho with few or no bees. Twenty thousand colonies dropped to less than ten thousand before almonds, and the beekeeper was scrambling to find sufficient numbers of bees to meet his pollination contracts.

Some of the affected beekeepers had problems with varroa mites, *Nosema ceranae*, or both. Others maintained very tight management, with meticulous treatment records, and

supplemental feeding in the fall with pollen supplements. Obviously, these beekeepers spent the time and money to control bee pests, yet still sustained CCD damage.

Overall, many of the affected colonies had displayed a lack of colony growth and less than expected honey crops the summer before. Most of the sudden collapses appeared to have started as early as mid-December, with the worst losses having been sustained by the time I arrived in California in early February. By mid-month, growers who had not contracted bees, thinking that they were going to get bees at bargain basement prices, were frantically calling beekeepers, offering as much as 150 dollars per colony for any beehive with any bees.

All in all, I was able to sample more than 30 beekeeping operations. The CA Almond Board stepped up to the plate and authorized emergency funding to pay for pathogen analysis, which we intend to subcontract to the U.S. Army Edgewood Chemical and Biological Center. The Cramer laboratory in Bozeman did a quick survey for *Nosema apis* and *N. ceranae*, using microscopy and PCR methods. Preliminary results indicate the *N. ceranae* continues to be a wide-spread organism infecting honeybees in both "strong" and "collapsing" colonies, but its relation to CCD and this current collapse remains unclear and is under intense research.

We suspect that CCD is caused by the interaction of two pathogens. The data set from the sampled beekeeping operations should help us to prove or disprove that hypothesis. Many of the beekeepers that we talked to are now noticing signs that point to a contagious disease. However, there are always alternative hypotheses, and the new and most prevalent one this year is that CCD is associated with cranberries. That might be a factor in the collapse of bees from Washington and Oregon, but it's difficult to argue for bees from North Dakota, Idaho, Texas, and Nebraska. What is clear is that published reports that CCD is on the wane, or that the millions of dollars invested in specific research projects have reduced the incidence of bee loss were premature.

Since this note was published, the Army has released a technical report listing all of the microbes and pathogens that have been detected using Mass Spectrometry based proteomics (MSP) in CCD colonies (Wick *et al.,* 2010), and a major publication is in the final stages of review and re-submission to the journal PLoS ONE.

If our hypothesis is correct, we should be able to provide growers and beekeepers with: (1) identification of the cause of CCD, (2) recommendations for control which initially will focus on control of *N. cernae*, since in can be readily detected and since there are existing management strategies and products that can be used to control and treat this bee pest, and (3) a quick and inexpensive assay for determining whether bees have the virus that we suspect plays a major role in colony collapse. In essence, the latter should make the 'invisible visible'. With an assay that can provide an early warning, beekeepers can focus their management activities on off-setting the problem, and growers will have a means of determining whether colonies that they plan to rent for pollination are indeed free of pathogens that increase the risk of colonies collapsing before or during almond pollination.

Materials and Methods:

The project leader spent most of February in California looking for and sampling colonies. The goal was to find at least 30 beekeeping operations, with approximately one-third having 'good' colonies with strong bee populations, another one-third with colonies that were failing to grow or thrive, and the other final one-third that had colonies that had collapsed or were still collapsing. Identification of these beekeeping operations was made via conversations with both local (California) and migratory beekeepers and with honey bee pollination brokers.

The criteria used to identify the signs of collapsing colonies are based on those of the CCD Working Group (2006) as expanded by Debnam *et al.* (2008). In addition, Dr. Bromenshenk, the project leader is a founding member of the CCD Working Group that first investigated and then later named, agreed upon, and listed the diagnostic signs of CCD.

In February in California, the strongest colonies were two stories (two deep hive bodies) tall, with 20 frames covered by bees. The failing colonies generally had no more than six frames of bees, and in all cases had had almost twice the bee population just a few weeks prior. The collapsed colonies usually were down to four frames or less of bees, many having only a queen and a small retinue of young worker bees, barely able to cover one-half of one side of a comb.

About 100-200 bees were shaken from each sampled colony directly into new, clean one quart Ziploc® or one liter Whirl-Pac® bags. The bags were sealed, placed in a cooler with frozen gel packs, and shipped by overnight express to the U.S. Army Edgewood Chemical and Biological Center (ECBC) laboratory in Maryland, to Bee Alert in Missoula, and to the Cramer Laboratory in Bozeman. Bees were often alive when received and were analyzed immediately. In a few cases, bee samples were frozen and stored in a -80^{o}C freezer until analyzed.

The Cramer laboratory surveyed the bees for *Nosema*, using microscopy and PCR. The Bee Alert laboratory conducted all of the sampling and coordinated sample distribution to the two analytical laboratories. The Army laboratory has contracted to analyze the samples using MSP following methods outlined in Wick (2010) and detailed in Jabbour *et al.* (2010).

In all, more than 30 beekeeping operations were sampled in the areas of Oakdale/Turlock, Madera/Fresno, and Wasco/Bakersfield, California. For each operation, five colonies of bees were sampled at each test location. All hives were marked with bar codes, so that the colonies could be tracked and found again, for later sampling, if warranted.

Some of these colonies are being monitored for sequential or time series sampling. However, to conserve funds, we are only following colonies that continue to show signs of CCD, based on the criteria for following CCD throughout seasonal changes, as listed by Debnam *et al* (2008).

Results and Discussion:

Approximately 135 colonies have been permanently marked with bar codes and sampled. The Cramer laboratory has completed analysis for *N. ceranae*. Not surprisingly, many of the

beekeeping operations had colonies infected by *Nosema*, some with very high levels of this microsporidium.

The Army laboratory is still in the process of analyzing the samples via proteomics in order to search for the suspected virus, as well as to comprehensively profile all microbes in the colonies. That is a very large project, one that takes time for the analysis, database match-ups, and correlations.

The Cramer laboratory has used initial proteomics data to design assays for detection of the virus. As of last week, the laboratory has a primer that shows promise as a means of finding the virus in bee samples.

The bulk of the data for this project revolves around the proteomics results and testing whether there is an interaction between the suspect virus and *Nosema*. A new set of inoculation trials with each pathogen and the two combined is being launched the week of August 2, 2010.

In addition, we are getting reports of and obtaining samples from beekeeping operations that have colonies that are slow in growing, failing to thrive. We believe that these are mid-summer warning signs of CCD. The first of these samples was scheduled to be delivered to the Cramer laboratory on August 5, 2010.

Pending the results of the proteomics work, the *Nosema* data has little relevance and as such was not subjected to statistical analysis at this time. The full data sets for *Nosema* and the virus should be available before the annual meeting of the ABC in December, and the results will be presented at that meeting.

In addition, a major paper on the work leading up to our hypothesis of a dual pathogen interaction has been submitted to PloS ONE, favorably reviewed, revised, and re-submitted. We are awaiting the final decision from the editors.

Research Effort Recent Publications:

Bromenshenk JJ (2010) Colony collapse disorder (CCD) is alive and well. Bee Culture 138: 51.

Wick CH, Stanford MF, Zulich AW, Skowronski E, Bromenshenk JJ, *et al.* (2010) Iridescent virus and *Nosema ceranae* linked to honey bee Colony Collapse Disorder (CCD). Edgewood Chemical Biological Center Technical Report: TR-814, July 2010

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CCD Working Group (2006) Colony collapse disorder preliminary report. Mid-Atlantic Apiculture Research and Extension Consortium (MAAREC). http://maarec.cas.psu.edu/pressReleases/FallDwindleUpdate0107.pdf.

Debnam S, Westervelt D, Bromenshenk J, Oliver R (2008) Colony collapse disorder: symptoms change with seasons and are different with various locations. Bee Culture 137: 30-32.

Jabbour RE, Deshpande SV, Wade MM, Stanford MF, Wick CH, Zulich AW, Skowronski EW, Snyder AP (2010) Double-blind characterization of non-genome-sequenced bacteria by mass spectrometry-based proteomics. Appl. Environ. Microbiol. 76:3637-3644.