Identification of Chemicals Associated with Colony Collapse Disorder

Project No.: **07-POLL8-Bromenshenk**

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Interpretative Summary:

One of the more unusual symptoms of Colony Collapse Disorder (CCD) is an initial lack of robbing of weak or empty hives by other bees and a conspicuous absence of hive pests such as the hive beetle and wax moth. In addition, many beekeepers report, and we also have noticed, a distinctive odor associated with some CCD colonies.

This technical report summarizes our findings with respect to chemicals in bee colonies that may help explain this effect. Phase I of this study was funded by the National Honey Board; Phase II by the Almond Board of California. Phase I funded initial sampling of bee colonies and analysis, resulting in the exclusion of some chemicals as possible causes of CCD, while providing a broad overview of chemicals in bee colonies. Phase II funding allowed additional sampling of bee colonies, along with more directed experimentation with respect to semi-volatile and volatile organic chemicals. It also provided an opportunity to evaluate a far more extensive set of data, especially with respect to the results from Proteomics Mass Spectrometry (Proteomics MS). In addition, Phase II allowed for verification of many of the Phase I results. Phase II builds on the findings of Phase I. As such, we will summarize here Phase I results, as well as present Phase II results. This will put Phase II results into context of the overall study.

Phase I. Based on a cross-section of vigorous, failing, and collapsed colonies that we sampled on both the east and west coasts during 2006-2007, we found that:

- 5-Hydroxymethylfurfural (HMF) did not appear to be a causative factor of CCD.
    - HMF was found at low concentrations in most wax samples. All but one were below <40 ppm, most <15 ppm. HMF concentrations averaged slightly higher in strong colonies (12.3 ppm), compared to failing (6.53 ppm) and collapsed (6.43 ppm) colonies.

- HMF was not present at levels that would be considered to be of concern to human health in any of the honey we sampled. The range of concentrations in honey was <0.5-7.6 ppm, with many values below 3 ppm.
- Occasional bee operations had elevated, potentially toxic (to bees) concentrations of HMF. We discovered 112.9 ppm in syrup from a feed wagon, and >106 ppm in wax from a beehive that was reported as dead.
- High concentrations of HMF may contribute to bee losses, but the presence of HMF did not correlate with CCD.

- Aflatoxins B1, B2, G1, and G2 were detected at low concentrations (< 7 ppb) in beeswax. These toxins were only seen in one honey sample (0.3 ppb).
  - 90% of the vigorous (assumed healthy) bee colonies displayed traces of aflatoxins in wax.
  - 80% of the CCD colonies had no detectable aflatoxins in wax.
  - Aflatoxins do not appear to be a causative factor with respect to CCD, and the absence of these chemicals in CCD colonies was unexpected.
- 144 volatile and semi-volatile organic compounds were found inside bee colonies that we sampled at ambient air temperatures.
  - About half of the volatile and semi-volatile chemicals detected in the air inside of a hive with a *vigorous* population of bees were the same as those seen in Maryland bee hives from 1995-2002, before CCD was known.
  - The atmosphere inside hives containing *collapsed* colonies (CCD) often had less than 8% of the chemicals seen in the Maryland colonies from the period before CCD.
  - Five chemicals were found in CCD colonies that were not usually seen in our previous work.
  - Some of these five chemicals were related to products being used in more recent years to control mites.
  - Paradichlorobenzene was readily evident in many samples, suggesting long-term residues resultant from treatments used to control wax moth.
    - These results indicate that additional care should be exercised by beekeepers using this product in order to reduce the risk of poisoning of bees.
- Preliminary results from high throughput Proteomics MS analysis revealed hundreds of peptides associated with a diversity of biological macromolecules of interest, including viruses, bacteria, bacteriophages, heat shock proteins, and other components of the biological community inside bee hives.
  - The proteomics analysis detected many common bee viruses, including: Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV), Kashmir Bee Virus (KBV), Sacbrood Virus (SC), and Deformed Wing Virus (DWV).
  - The proteomics instrument surveyed for, but did not detect, Chronic Bee Paralysis Virus (CBPV) or Cloudy Wing Virus (CWV).
  - Israeli Acute Paralysis Virus (IAPV) was prevalent in one east-coast bee operation and in Australian bees imported to the east coast of the U.S.
  - IAPV presence was scattered in most western bee colonies and was not correlated with CCD on a national scale.

- Proteomics data also revealed two heretofore unreported bee viruses:
  - A virus tentatively identified as Varroa Destructor Virus1 (VDVI) was found in two bee samples from Florida.
  - Another unreported insect virus occurred in all of the sampled CCD bee yards on both the east and west coasts. The identification of this virus is still pending. As such we will refer to it as Virus? in this report.
- Initial proteomics data yielded significant detections of more peptides and proteins in vigorous colonies than in collapsed colonies; i.e., 610 in bees from vigorous (good) colonies, 605 in failing colonies, and 373 in collapsed colonies.
  - Overall, peptides from viruses of bees, insects, plants, and vertebrate animals were more numerous and diverse in vigorous colonies than in failing or collapsed colonies.
  - In addition, vigorous colonies had a predominance of plant viruses, failing colonies had many more bacteria and bacteriophages, and collapsed colonies displayed a high number of mammalian viruses.

Phase I findings provided a wealth of new insights into the chemistry associated with CCD and the macromolecules associated with bee pathogens. It also provided leads relevant to the epidemiology of CCD and eliminated some chemicals as potential causes of CCD.

Phase II results and outcomes included:

- Confirmation that VDV-1 was in two bee samples from Florida.
  - Issuance on August 29 of press releases announcing our discovery of this virus in U.S. bees.
  - Notification of APHIS and the CCD Steering Committee of the discovery. Heretofore, VDV-1 was only known to be in varroa mites and honey bees in Europe.
- An additional 182 volatile and semi-volatile chemicals were released when combs and hive bodies were heated to 140^{o} F. Heating brought the total of detected compounds up to 326 (i.e., 144 + 182) identified chemicals.
  - The volatile to semi-volatiles chemicals in vigorous and CCD hives were statistically significantly different.
  - However, the difference was mainly due to three terpenes found in wood plus benzoic acid, which suggests a difference due to age of wood (i.e., green wood of controls versus older wood of CCD colonies), rather than something associated with CCD.
- Proteomics MS data were expanded from an initial identification of several hundred peptides and proteins (Phase I) to as many as 14,000 lines of data per bee sample (Phase II).
  - There was an unexpectedly diverse array of plant viruses in bee colonies.
  - Many of these plant pathogens should be of concern to almond growers.
    - Bees have been implicated in the transmission of a few plant diseases and have been suggested as a means of monitoring plant diseases in orchards.

        - Proteomics MS analysis provided a new means of assessing the dynamics of bees and plant viruses.
- We are continuing our work to discover a chemical or chemicals associated with CCD.
- We also are working on four major research articles for submission to peer reviewed journals before the end of the year (2008).
    - These papers are a result of the investigations supported by the National Honey Board and the Almond Board of California.

Objective:
We do not know yet what is causing colony failure, but the symptoms are clear ─ rapid bee disappearance, leaving the queen and young bees behind; few dead bees remaining in the colonies; and ample unused pollen, honey and syrup stores. Also evident and highly unusual, is the absence of any robbing or invasion by typical pests and scavengers after colony failure. This apparent repellant nature of brood nests and food stores is an especially important clue that we at Bee Alert have undertaken to investigate.

We hypothesize that either a highly toxic or a strongly repellent residual compound is produced during collapse, and then persists for days or weeks after colony failure. Whether this is a chemical produced by a pathogen, a consequence of materials used for mite treatment, a pesticide used on crops, or some form of environmental contaminant is unknown. However, if a chemical exists, we hope to find it.

Our first objective was to survey as many affected apiaries as possible and to collect and freeze large samples of bees, wax, comb, pollen, and honey for subsequent analysis. We began with samples from Florida, Pennsylvania, Georgia, and California from the winter and spring of 2006 - 2007.

In August of 2007, another wave of CCD went through the United States. We focused on obtaining additional samples from areas that had not previously reported CCD (i.e., Arizona), or for which we did not have *in situ* samples (e.g., Idaho, Washington). In 2006 - early 2007, we sampled Idaho and Washington colonies that had been taken to California for almond pollination. In the fall of 2007, we were able to sample more bee operations in their home states. We also conducted another round of sampling in California in 2008, immediately after the National Bee Convention in Sacramento. We plan on continuing sampling of colonies throughout 2008.

Initially, we had planned on obtaining bee samples from queen-rearing operations in Louisiana and Alabama, because these states that had not reported CCD. However, after visits to Alabama and Georgia, we learned that many beekeepers had experienced CCD like problems, both recently and in the recent past. We concluded that there was insufficient evidence to conclude that these states were free of CCD, regardless of the absence of reporting. Areas such as Arizona, on the other hand, had seldom reported CCD.

Therefore, we made a concerted effort to inspect and sample bee colonies from Arizona that collapsed in August of 2007. More recently, we sampled bee colonies from a long-established commercial beekeeping operation in Montana. This beekeeping operation engages in limited migratory activities, and it reportedly has never experienced CCD. Because specific and quantitative analyses are time consuming and expensive, we asked the National Honey Board to fund a semi-quantitative, generalized chemical survey for **Phase I**. Analyzing for presence of broad classes for chemical indications of viruses, bacteria, and fungi, biologically produced toxins, as well as some of the more commonly used management materials that could be done at relatively low cost.

We also proposed that if and when, a general class of compounds common to CCD colonies was determined, or if we found bee pathogens that might produce toxic compounds, we would conduct a more focused and quantitative analyses to identify the specific compound and agent responsible for CCD under **Phase II**.

For **Phase I**, we have obtained chemical analysis information for:

- Hydroxymethylfural (HMF) in syrup, honey, and wax. HMF is a contaminant often found in high fructose corn syrup routinely used in bee management as a food supplement. At high levels, it can be toxic to bees.
- Aflatoxins in honey and wax. Aspergillus fungi produce these toxins that at high concentrations are toxic to a broad array of organisms, including humans, and are classified as carcinogens.
- Semi-volatile and volatile organic chemicals inside bee hives (from vapor samples pumped from within hives). We developed the technologies for sampling these materials and have an extensive database for comparison, dating back to 1995.
- Proteins, peptides, and other biological macromolecules related to pathogens. This provided both a means of identifying pathogens that might produce toxic or repellant compounds as well as a new means of surveying a broad array of microbial pathogens found in bee colonies. This new technology was brought to us by the U.S. Army's Edgewood Chemical Biological Center (ECBC).

This two-stage approach of a rapid, semi-qualitative chemical survey, followed by more focused and quantitative research, allowed us to narrow our search for the cause of CCD. By deferring expensive quantitative analyses to **Phase II**, we narrowed the list of possible causes, so that we could then prioritize additional sampling, analyses, and conduct directed experimentation.

This greatly improved our ability to generalize about the causative agent. **Phase I** sample collection was relatively inexpensive, other than travel costs, and sufficient sample quantities were collected and stored in freezers to provide for both the general **Phase I** survey and many of the later **Phase II** specific analyses.

Also, in this report, we present preliminary results of collaborations with the U.S. Army ECBC. The Army brought to Phase II of this study a new technology to examining materials in bee hives – the ability to look at biological macromolecules by proteomics mass spectrometry.

The proteomics results can be correlated with the results of another new technology from the Army, the Integrated Virus Detection System (IVDS). IVDS analyses were initially performed by ECBC, and later by BVS, Inc. BVS, Inc. has co-located with Bee Alert technology to facilitate close research collaborations. The two companies are side by side in the same building, which leverages the funding of the Almond Board of California to BVS to help set up a laboratory to survey viruses in honey bees. BVS is conducting virus surveys, while Bee Alert coordinates all sampling, coordinates chemical research, and provides screening for *Nosema ceranae* along with other services to beekeepers and growers.

MATERIALS AND METHODS:

Honey bees were shaken from frames into new Whirl-Pac® or Zip-Loc® bags, sealed, placed in a cooler under frozen gel packs or dry ice, depending on the subsequent analyses to be performed. Most bee samples consisted of 250 or more bees, although for some testing 60-100 bees were used. Wax samples consisted of entire frames, removed from colonies, wrapped in aluminum foil, and then bagged in a sealed, plastic freezer bag. Syrup samples were taken and stored in new, clean glass or plastic bottles. Honey samples were obtained from the sampled frames. Hive air samples were collected using vapor sampling tubes inserted into the hive body. Digital pumps that controlled and documented flow rates were used to pull vapor samples onto the tubes.

All samples were kept cool during collection and shipping, and stored in laboratory freezers. No chemical preservatives were used for any sample. Sample delivery to analytical laboratories was by overnight courier.

Procedures used to investigate 5-HMF and aflatoxins are detailed in the Phase I report to the National Honey Board.

University of Montana Air Sampling and Analysis Methods for Volatiles (Smith et al., 2002).

Air samples are collected on 11.5 cm x 6 mm OD x 4 mm ID three-phase Carbotrap 300 thermal desorption tubes (Supelco) or four-phase Carbotrap molecular sieves of increasing activity that sorb volatile and semi-volatile organic compounds over a molecular size range from C_1 to C_{30}.

Desorption tubes are connected to constant flow pumps, generally set at rates between 0.080 and 0.150 dm^3/min. The distal end of the sorption tube is attached to copper tubing (2 mm ID x 3 mm OD) with a brass compression fitting and a vespel/graphite ferrule. The copper tube is inserted directly into the hive interior between the wooden frames that support the wax combs. The outlet end of the sorbent tube is connected to a constant flow pump (SKC, Inc.) with a 1-m section of a 5 mm ID x 8 mm OD Tygon tubing. Pumping periods ranged from 30 minutes, to as long as 8 to 12 hours. Sample

tubes are sealed in individual vials and stored in a dedicated 4° sample refrigerator until analyzed.

Thermal desorption analysis
Sample tubes are desorbed in a direction opposite to sampling flow. After a 4-min helium purge to remove incidental moisture, tubes are subjected to a 10-min desorption cycle at 250°C. Each tube is then given a 6-min cooling flush. A helium flow rate of 0.025 dm^3/min is used in the desorption tube. Make-up helium flow from other paths on the multi-station desorber (Tekmar LSC2000) yields a total flow of 0.040 dm^3/ min) and was split 1:20 thereafter.

Chromatographic separations are accomplished on a Hewlett Packard GCD or comparable instrument containing a 60m x 0.32 mm ID Restek RTX-502.2 capillary column (phenylmethyl polysiloxane, 1.8 µm coating). The helium flow is 0.001 dm^3/min, and the total time for an analysis is 50 min (5 min at initial temperature 40°C, ramp 5°C/min to 220°C, 9 min hold time at 220°C). Mass spectra are collected over a range of 35 to 450 amu.

Computer matches with the National Institute of Science and Technology (NIST) database initially identifies compounds. Many, though not all, are subsequently confirmed using commercial mixtures of analytical standards. The concentrations of all compounds are computed on a relative scale (ion abundance/ dm^3 air sampled) but are not reported here. Compounds of interest to regulatory agencies can be rigorously quantified.

<u>Proteomics Mass Spectrometry</u>
Several grams of bees in pure water are homogenized in an industrial blender. The resultant sample is pre-filtered to remove course media (e.g., bits and pieces of bees, any debris, etc.) Samples are centrifuged to remove remaining cellular debris then ran through an ultrafilter to concentrate the viruses and proteins of interest. The resultant solution was directly analyzed for viruses using IVDS.

For proteomics MS, the ultrafiltered samples are digested with trypsin @37^oC for overnight. The sample was then diluted with mobile phase (90/10/0.1% H2O/ACN/FA). A 10 uL aliquot was injected by the auto sample onto the Mass Spectrometer (MS). The MS conditions are as follows: The top five ms spectra were acquired for every full MS scan, which means that there are five MS/MS scans per one full MS scan. This is done to increase the information about the protein sequence. The MS range is from 350-1800. The voltage is 2.0kV, heated transfer capillary is 180^oC. Convectron was at 0.64*10^-5 torr, and the ion gauge is at 0.96 torr. The sample analyzed was then processed using sequest to match the data with a database generated from all sequenced materials available at the NCBI website.

RESULTS AND DISCUSSION:

The results for 5-HMF, aflatoxin and fumonisin in syrup, honey, and wax are detailed in our Phase I report to the National Honey Board.

Volatile and Semi-Volatile Organic Chemicals

Samples Collected at Ambient Air Temperatures
Initial analyses for volatile and semi-volatile chemicals revealed that 144 compounds commonly appeared in the more than 100 bee hives sampled over the past year. The results for these 144 chemicals are presented and discussed in our Phase I report.

In 2002, we published a list of 212 volatile and semi-volatile chemicals commonly seen in bee hives. In all cases, the air inside the beehives was sampled at ambient air temperatures. The 2002 sampling was conducted over several years and often at high, summer, air temperatures. Most of the initial CCD colony testing in 2006 and 2007 was conducted over a period of nine months, during fall, winter, and spring, when air temperatures tended to be cool. Similarly, 2008 samples were also taken during winter and spring months. In addition, CCD sampling consisted of 30 minute to 1 hour sample intervals, whereas much of the work reported in 2002 consisted of 8-10 hour samples.

We suspect what we would have found more volatile chemicals in beehives from beekeeping operations that evidenced CCD if the temperatures inside the colony were higher. In other words, we hypothesized that if we heated the boxes in a sealed chamber, we might drive off more volatile and semi-volatile chemicals.

Samples Collected from Heated Bee Boxes
We designed and constructed a test chamber consisting of a 55 gallon drum with a band heater, sealable lid, internal thermostats, and air sampling probes. We placed individual bee boxes into the drum, sealed and heated it up, then drew off samples of the atmosphere inside the barrel. This procedure yielded measurable concentrations of an additional 182 organic compounds, bringing the total detected to approximately 232.

Figure 1 (from our Phase I report) displays those compounds that exceeded the average ion abundance for all detected compounds. This set of chemicals represents the top 10% based on relative abundance.

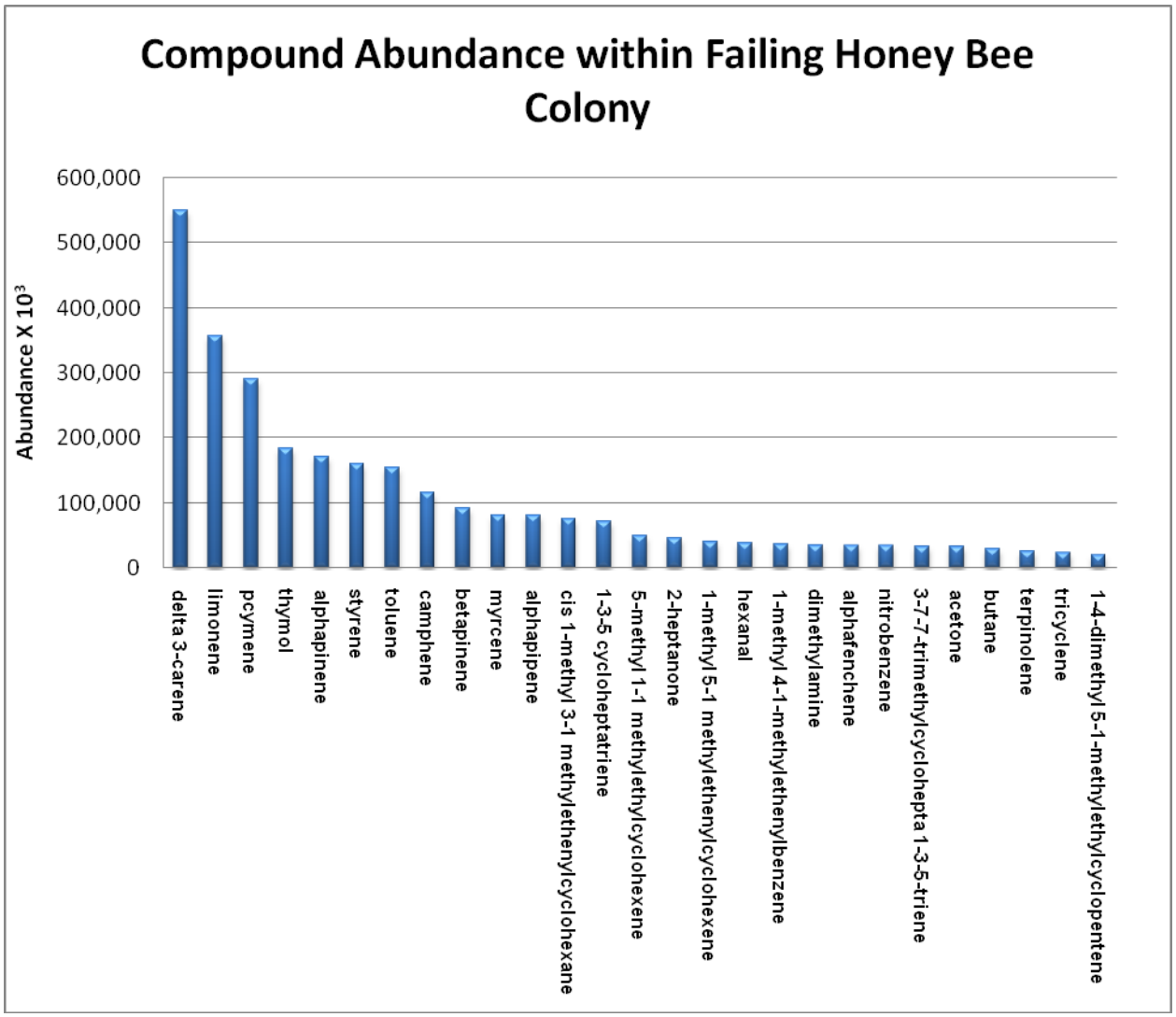

Figure 1. Top ranked chemicals based on relative abundance in heated bee boxes.

We also compared those chemicals detected at the top of the box versus those in the bottom of the box. Four chemicals displayed a gradient for top compared to the bottom of the box (Table 1). Acetic acid and thymol concentrations were about two-fold higher at the top of the hive body. There was no measurable concentration of undecane at the bottom of the box. On the other hand, sulfur dioxide was somewhat higher at the bottom of the box.

Table 1. Chemical compounds showing sample location differences – box top versus bottom.

Location		thymol	acetic acid	sulfur dioxide	undecane
Top	Mean	17605.71	461.43	167.14	871.43
	N	7	7	7	7
	Std. Deviation	31196.456	238.078	242.674	1433.776
Bottom	Mean	8650.00	252.86	238.57	.00
	N	7	7	7	7
	Std. Deviation	22643.487	308.043	219.274	.000

Overall Statistical Results of Volatile Extraction Analysis

For a statistical comparison, frames from eleven colonies, seven controls and four diseased were evaluated. The top and bottom samples for each frame were analyzed separately, then the counts for identified compounds were summed for comparison between diseased and control colonies. In all, for this data set, the GCMS identified 251 unique compounds that were extracted from all frames.

Because of the large number of compounds that were extracted relative to the number of colonies sampled, it was not possible to perform a simultaneous analysis for differences between the control and diseased groups. Instead stepwise discriminate analysis was used to reduce the large number of compounds to the few that were significant and also significantly discriminated between the groups.

Discriminate analysis based differences on Wilk's lambda, with minimum criteria for compound inclusion set at $P \leq 0.05$. Because discriminate analysis is analytically related to multivariate analysis of variance (MANOVA), the final model that was produced was similar to a MANOVA for differences between frames from control and diseased colonies, but with a reduced number of compounds.

The discriminate analysis was significant, indicating that control frames and diseased frames could be statistically differentiated based on volatile chemistry (Table 2).

Table 2. Significance of Discriminate Analysis for differences in volatile compound in control and diseases frames. Test statistic was Wilks' Lambda.

Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
I	.000	55.705	4	000

However, inspection of the compounds that differed significantly, indicated that only four – sabinene, beta fenchene, 1,4 pentadiene, and benzoic acid - of the 251 were different

between frame groups (Table 2). All four compounds had higher concentrations in control frames. Beta fenchene and 3 Ethenyl 1,4 Pentadiene occurred only in control frames.

Peptides and Proteins in Bees

In Phase I, we reported that bee samples from colonies that we rated as vigorous had 610 peptides that could be identified from the genomics database, failing colonies had 605, and collapsed colonies averaged 373 peptides. The types of peptides and proteins identified covered a wide range of pathogens and pathogen-related substances

These initial results provided a unique catalog of biological macromolecules in bees and beehives, ranging from keratin from the fingers of a beekeeper who collected bees with Deformed Wind Virus by catching them between his thumb and forefinger, to heat shock proteins, bacteriophages, and viruses of bees, other insects, plants, wildlife, and domestic livestock to name a few.

For Phase II, we worked closely with the Army. We identified VDV-1, found a suspect virus that seems to be correlated with CCD (which we call Virus ?), and discovered a wealth of information about plant pathogens in bee colonies.

Bee Viruses

Results of proteomics MS surveys for viruses are presented in the Phase I report. During Phase II, we confirmed our discovery of VDV-1 in two samples of bees from the east coast. We informed APHIS and the CCD Steering Committee of its presence and released a press release in late August announcing the discovery.

This is the first reported finding of this virus in North America. It was first identified and described in Europe around 2006. Virus ? continues to be investigated. It appears in all of the CCD bee operations that we have sampled to date. We are working with the Army ECBC laboratory and with the DeRisi genomics laboratory at UCSF to identify this virus.

Plant Viruses

Proteomics MS identified 209 plant pathogens in sampled honey bee colonies (Table 3). Eight pathogens, that are known to affect almonds, were identified to species (Table 4), and two others belonged to genera known to include species that affect almonds. Another 35 viruses that are known to infect citrus and other fruits were named at the species level (Table 5).

This was a very diverse, species rich assortment of pathogens. The list of 209 pathogens covered a wide array of plants including bee pollinated fruits, nuts, and crops, as well as wind pollinated plants such as maize (Table 3). Pathogens of concern to almond growers included several viral and bacterial diseases, as well as two fungal diseases.

Table 3. A list of 209 plant pathogens found in bee colonies by proteomics MS.

Plant Pathogens Found in Honey Bee Colonies

Agrobacterium
American plum line
Apple mosaic
Apple stem grooving virus
Apple stem pitting
Banana streak GF virus
Banana streak Mys virus
Banana streak OL virus
Barley stripe mosaic virus
Barley yellow dwarf virus - MAV
Bean common mosaic necrosis virus
Bean common mosaic virus
Bean golden yellow
Beet mild curly top virus
Beet necrotic yellow vein virus
Beet soil-borne virus
Blackberry yellow vein-associated virus
Blackcurrant reversion virus
Blueberry red ringspot virus
Botrytis virus X
Bradyrhizobium japonicum USDA 110
Brome mosaic virus
Brome streak mosaic virus
Burkholderia cenocepacia
Burkholderia cenocepacia AU 1054
Burkholderia cepacia
Cactus virus X
Cardamine chlorotic fleck virus
Carnation mottle virus
Carnation ringspot virus
Cassava vein mosaic
Cestrum yellow leaf curling virus
Chaetoceros salsugineum nuclear inclusion virus
Chayote mosaic virus
Chenopodium mosaic virus X
Cherry necrotic rusty mottle virus
Cherry rasp leaf virus
Cherry virus A
Chickpea chlorotic stunt virus
Chino del tomate virus
Citrus leaf blotch virus
Citrus leaf rugose

Citrus psorosis virus
Citrus tristeza virus
Coconut foliar decay virus
Commelina yellow mottle virus
Corchorus yellow spot virus
Corchorus yellow vein virus -
Cowpea chlorotic mottle virus
Cowpea mottle virus
Cowpea severe mosaic virus
Cryphonectria hypovirus 2
Cucumber necrosis virus
Cucumber yellows virus
Cucurbit yellow stunting disorder virus
Cytoplasmic citrus leprosis virus
Daphne virus Y
Discula destructiva virus 1
Dracaena mottle virus
Dulcamara mottle virus
East Asian Passiflora virus
Erwinia carotov
Faba bean necrotic yellows virus
Fiji disease virus
Fireblight - Erwinia amylovora
Garlic virus C
Grapevine fanleaf virus
Groundnut bud necrosis virus
Helminthosporium victor
Hibiscus chlorotic ringspot virus
Hop latent virus
Horseradish curly top virus
Indian citrus ringspot virus
Johnsongrass chlorotic stripe mosaic
Kalanchoe top-spotting virus
Konjak mosaic virus
Kyuri green mottle mosaic virus
Lettuce infectious yellows virus
Lettuce necrotic yellows virus
Lily mottle virus
Little cherry virus 1
Little cherry virus 2
Loofa yellow mosaic virus
Lucerne transient streak virus
Maize chlorotic dwarf virus
Maize chlorotic mottle virus
Maize fine streak virus

Maize necrotic streak virus
Maize rayado fino virus
Maize streak virus
Mal de Rio Cuarto virus
Maracuja mosaic virus
Melon chlorotic leaf curl virus
Melon yellow spot virus
Mesorhizobium loti MAFF303099
Mesorhizobium sp. BNC1
Mint virus 1
Mint virus X
Mirabilis mosaic virus
Miscanthus streak virus
Narcissus degeneration virus
Nootka lupine vein-cle
Oat chlorotic stunt virus
Oat golden stripe virus
Oat mosaic virus
Obuda pepper virus
Olive latent virus
Tomato yellow leaf curl Indonesia virus-[Lembang]
Onion yellow dwarf virus
Orgyia pseudotsugata MNPV
Oryza rufipogon endornavirus
Oryza sativa endornavirus
Paprika mild mottle virus
Passiflora latent carlavirus
Patchouli mild mosaic virus
Pea early browning virus
Pea enation mosaic virus-1
Pea stem necrosis virus
Peanut clump virus
Peanut mottle virus
Peanut stunt virus
Pectobacterium atrosepticum SCRI1043
Pelargonium chlorotic ring pattern virus
Pelargonium zonate spot virus
Pennisetum mosaic virus
Pepino mosaic virus
Pepper huasteco yellow vein virus
Pepper mottle virus
Pepper ringspot virus
Petunia vein clearing virus
Phytophthora endornavirus 1
Plantago asiatica mosaic virus

Poinsettia mosaic virus
Poplar mosaic virus
Potato leaf roll virus
Potato mop-top virus
Potato virus M
Potato virus V
Potato virus Y
Potato yellow mosaic virus
Prune Dwarf virus
Prunus necrotic ringspot virus
Pseudomonas syringae pv. phaseolicola 1448A
Pseudomonas syringae pv. tomato str. DC3000
Raspberry mottle virus
Raspberry ringspot virus
Rhizobium etli CFN 42
Rice black streaked dwarf virus
Rice grassy stunt virus
Rice ragged stunt virus
Rice stripe virus
Rice tungro bacilliform virus
Rice yellow stunt virus
Rupestris stem pitting-associated virus
Ryegrass mosaic virus
Shallot yellow stripe virus
Sida golden mosaic Florida virus
Sida golden mosaic virus
Siegesbeckia yellow vein virus
Soil-borne cereal mosaic
Soil-borne wheat mosaic virus
Sonchus yellow net virus
Sorghum chlorotic spot virus
Southern bean mosaic virus
Soybean chlorotic mottle virus
Soybean dwarf virus
Squash mosaic virus
Strawberry latent ringspot virus
Strawberry necrotic shock virus
Strawberry pallidosis associated virus
Subterranean clover mottle virus
Sugarcane bacilliform IM virus
Sugarcane bacilliform Mor virus
Sugarcane mosaic virus
Sugarcane streak Egypt virus
Sugarcane streak Reunion virus
Sweet potato chlorotic fleck virus

Sweet potato chlorotic stunt virus
Sweet potato feathery mottle virus
Sweet potato mild mottle virus
Taro bacilliform virus
Taro vein chlorosis virus
Tobacco etch virus
Tobacco rattle virus
Tobacco streak virus
Tobacco vein-clearing virus
Tomato aspermy virus
Tomato black ring virus
Tomato chiao La Paz virus
Tomato chlorosis virus
Tomato leaf curl Iran virus
Tomato leaf curl Mali virus
Tomato leaf curl Taiwan virus
Tomato mosaic virus
Tomato mottle Taino virus
Tomato spotted wilt virus
Tomato torrado virus
Tomato yellow leaf curl Indonesia virus-[Lembang]
Tomato yellow leaf curl Thailand virus
Tomato yellow spot virus
Turnip mosaic virus
Turnip yellows virus
Watermelon silver mottle virus
Wild potato mosaic virus
Wisteria vein mosaic virus
Xanthomonas axonopodis pv. citri str. 306
Yam mosaic virus
Zucchini yellow mosaic virus

Many pathogens of stone fruits can infect more than one species of plant. Table 4 lists viruses found in bee colonies that are known to be agents of disease for citrus and other fruits. These 35 are in addition to those listed as being diseases of almonds in the previous Table.

Table 4. Pathogens relevant to almond diseases. + indicates identification, - indicates no detection. A few diseases were identified to genus (i.e., fungi and one bacteria), whereas most viruses and bacteria were keyed to the species level.

Diseases of Almond (*Prunis dulcis*)		
Viral Diseases	***Genus***	***Species***
Calico (genus *Ilarvirus*, Prunus necrotic ring spot virus)	+	+
Enation (genus *Neposvirus*, Tomato black ring virus)	+	+
Infectious bud failure (genus *Ilarvirus*, Prunus necrotic ring spot virus)	+	+
Peach mosaic (Cherry mottle leaf virus)	+	+
Peach yellow bud mosaic (genus *Nepovirus*, Tomato ringspot virus)	-	-
Bacterial Diseases		
Almond leaf scorch (*Xyella fastidiosa*)	+	+
Bacterial canker (*Pseudomonas syringae* pv. *Syringae*)	+	+
Bacterial hyperplastic canker (*Pseudomonas amygdali*)	+	-
Bacterial spot (*Xanthomonas campestris* pv. *pruni*)	+	+
Crown gall (*Agrobacterium tumefaciens*)	+	+
Kernel decay (*Aspergillus niger* , *A. flavus*, *A. parasiticus*)	+	-
Fungal Diseases		
Alternaria leaf spot (*Alternaria alternata*)	+	-
Anthracnose (*Colletotrichum acutatum*)	-	-
Armillaria root rot (*Armillaria mellea, Rhizomorpha subcorticalis*)	-	-
Band or Dothiorella canker (*Botryosphaeria dothidea*, *Dothioralla* sp.)	-	-
Brown rot (*Monilinia fructicola*, *M. laxa*, *Monilia* sp.)	-	-
Ceratocystis canker (*Ceratocystis fimbriata*)	-	-
Green fruit rot (B*otrytis cinerea*, *B. fuckeliana*, *Monilinia fructicola*, *Sclerotinia sclerotiorum*)	-	-
Hull rot (*Monilinia* sp, *Rhizopus arrhizus*, *R. circinans*, *R. stolonifer*)	-	-
Leaf blight (*Discostroma corticola, Seimatosporium lichenicola)*	-	-
Leaf curl (*Taphrina deformans*)	-	-
Leucostoma cander (*Leucostoma cincta*, *L. persoonii*, *L. leucostoma)*	-	-

Phomopsis canker and fruit rot (*Phomopsis* or *Fusicoccum amygdali*)	-	-
Phytophthora root and crown rot (*Phytophthora* spp.)	+	-
Powdery mildew (*Podsphaera tridactyla*, *Oidium passerinii*, *O. leucoconium*, *Sphaerotheca pannosa*)	-	-
Red leaf blotch (*Polystigma ocraceum*)	-	-
Rust or Stone fruit rust (*Tranzschelia discolor*)	-	-
Scab (*Venturia carphophila*, *Cladosporium carpophilum)*	-	-
Shot hole (*Wilsonomyces* or *Stigmina carpophilus*)	-	-
Silver leaf (*Chondrostereum purpureum*)	-	-
Verticillium wilt (*Verticillium dahliae*)	-	-

Table 5. Viruses of citrus and other fruits found in bee colonies by proteomics MS.

Viruses of Citrus and Other Fruits Found in Bee Colonies

Apple mosaic virus
Apple stem grooving virus
Apple stem pitting virus
Blackberry yellow vein-assoicated virus
Blackcurrant reversion virus
Blueberry ringspot virus
Botrytis virus X
Cherry rasp leaf virus
Cherry virus a
Citrus psorosis
virus
Citrus tristeza virus
Cytoplasmic citrus leprosis virus
Fireblight - *Erwinia amylovora*
Little cherry virus
Olive latent virus
Potato leaf roll virus
Potato mop-top virus
Potato virus M, Y, V
Prune Dwarf virus
Strawberry latent ringspot virus
Strawberry necrotic shock virus
Strawberry pallidosis associated virus
Tomato aspermy virus
Tomato black ring virus
Tomato chiao La Paz virus
Tomato chlorosis virus
Tomato leaf curl virus
Tomato mosaic
virus
Tomato mottle virus
Tomato spotted wilt virus
Tomato torrado virus
Tomato yellow leaf curl virus
Tomato yellow spot virus

Discussion:

Volatile and Semi-Volatile Chemicals in Hive AtmospheresPreviously, we published an extensive review (Smith *et al.,* 2002) of semi-volatile and volatile chemicals seen over eight years of sampling of hive atmospheres inside bee hives from Maryland. Not surprisingly, many of the chemicals inside bee hives in the 1990's were also seen in the 2006-2007 samples.

One chemical of concern that was reported in Phase I warrant repeating here. The insecticide paradichlorobenzene was found in many of these samples. Paradichlorobenzene is used by beekeepers to protect stored equipment from wax moth damage. We found this chemical in all categories of colony strength, including vigorous, failing, and dead. Because of its nearly ubiquitous presence, this chemical does not appear to be the cause of CCD.

However, it does indicate that this chemical has a long residence time in bee hives, presumably in the wax. Whether the observed residues were from the previous season's treatment during storage or the consequence of build up over several seasons is unknown. Nor do we know whether beekeepers used this chemical according to label directions regarding application and dosage. We also do not know whether this chemical, at the levels observed, would be hazardous to bees, but its widespread prevalence warrants a caution about usage and argues for periodically changing out wax comb to reduce exposure to toxic chemicals that can accumulate in wax. Although, our initial Phase I surveys did not reveal any specific chemicals known to be associated with pathogens such as fungi, we did find six chemicals across all categories of colony strength that were also reported by Strobel *et al.*, 2001 as volatiles that they found to be associated with *Mucor* fungi.

After reviewing the 144 chemicals initially found in bee hives that were sampled in bee yards at ambient air temperature, we speculated that we might need to better isolate the equipment being sampled from contaminants in the ambient air. We constructed a sealed barrel heating system that we could use in the laboratory. We expected heating would drive off more volatiles, and that the sealed drum would contain them so that they could be more easily sampled. We hoped to increase our ability to discover trace amounts of these chemicals in bee equipment.

As expected, heating the boxes and combs yielded an additional 182 compounds. The concentrations of some compounds, such as terpenes from wood, were very high. This is consistent with our previous published work from 2002 (Smith et al., 2002). Sampling for long periods on hot days also yielded many terpenes.

We were concerned that some volatile compounds would be heavier and some lighter than air. As such, we sampled from both the top and bottom of each box. For Phase I, we reported that only acetic acid showed a significant difference in concentrations based on where the sample was taken.

We also found that 27 compounds that were consistently at levels above the average concentration for all 182 chemicals. In general, taking a sample from the top or the bottom of the hive body did not make a difference in detectable concentrations. Only four chemicals displayed a position difference. Acetic acid and thymol were higher at the top of the box. Undecane was at the bottom of the box and was not detectable at the top of the box. Sulfur dioxide was somewhat more concentrated at the box bottom. With respect to the statistical analysis of the chemicals yielded by heating, benzoic acid is a common constituent of gums, and presumably of propolis, while the remaining three compounds are most likely products evolving from the wood materials in the frames.

Sabinene and beta fenchene are monoterpenes, and 1,4 pentadiene is derived from wood resins. It appears that the principal differences between the two sets of frames was primarily due to variation in the wood used in their construction, with a contribution due to the quantity of propolis present on the frames. Since we buy all of our frames from Western Bee in Polson, we did not expect to see a difference based on wood.

However, in retrospect, the CCD frames came from colonies that collapsed last winter. The control frames came from healthy colonies established this summer. We speculate that the controls had newer (i.e. greener wood) than the CCD colonies with wood frames at least a year old.

The reason for a difference in the amount of benzoic acid is not readily apparent. It may be an indication of different types of propolis. The CCD colonies should have a predominance of propolis collected in the fall, whereas the controls would have been more likely to have propolis collected in the spring. So, the difference could be due to the type of plant from which propolis was collected. It might be indicative of some role of this chemical in suppressing whatever causes CCD, but that seems to be taking the data farther than is warranted.

Discovery of a difference based on the age of the wood suggests that we have a very sensitive analysis method. If a significant difference can be seen for wood of different ages, we should have been able to detect a repellant or toxic chemical difference, if it was present.

Note however, that we are making this conclusion only with respect to the molecular weight chemicals that we could examine by GC/MS/TD. It is possible that such a chemical exists, but that it is a different category of chemical from what our instrument could detect.

Peptides and Proteins in Bees

The proteomics results from Phase I provided a preliminary look at a unique catalog of biological macromolecules in bees and beehives, ranging from keratin from the fingers of a beekeeper who collected bees with Deformed Wind Virus by catching them between his thumb and forefinger you said this earlier, you might reword it, to heat shock proteins, bacteriophages, and viruses, to name a few.

For Phase II, we worked closely with the Army, spending ten days with them, going through the data, and discussing the implications. In all, the Army was able to provide us with listings of thousands of detectable biological molecules (i.e., peptides). Many were associated with viruses. However, the proteins and peptides detected included not only bee viruses, but also other insect viruses, plant viruses, and even mammalian viruses.

As mentioned in our Phase I report, vigorous bee colonies contained many plant viruses. Failing colonies had stronger detections for a variety of bacteria and viruses of bacteria. Collapsed colonies had a greater diversity of mammalian derived materials. Higher amounts of plant-derived materials in strong bee colonies probably reflect active foraging for pollen and nectar. Lower numbers of plant viruses in collapsed colonies may be indicative of little or no foraging for plant resources, since these colonies have few forager bees left in the population.

An increase in animal viruses in collapsed colonies may be a reflection of breaching of beehives by mice moving into weak or empty hives or of feeding on the weakened bee population by animals such as skunks. Although this is hypothesis is speculative, we have received anecdotal reports of unusual numbers dead mice in some CCD hives. In general, virtually any and all of the identified bee viruses appear in healthy bees. As the colonies dwindle, some of these viruses disappear. Eventually, we see one or two viruses remaining in collapsed colonies, especially the un-named virus that we refer to as Virus ?. This virus often occurs with Kashmir or Deformed Wing Virus.

Our initial data eliminated Cloudy Wing Virus and Chronic Paralysis Virus as biomarkers or potential causes of CCD, since neither was detected in any bee sample. Similarly, our Phase II research shows that IAPV virus may be a marker of CCD in its later stages, but overall, it did not correlate with CCD.

We started the proteomics work as a means of identifying microbial pathogens that might produce chemicals that could drive bees out of the box or that are toxic to bees. We now have a large list; one that has provided many candidates for which there is no information concerning any chemicals that may be associated with them.

However, one aspect of this work should be of immediate interest to the almond industry, as well as to growers of other fruits. As evident from the Tables of results for plant diseases, bees bring back to the hive a wide assortment of plant diseases. Whether these are viable and able to infect plants is not known nor do we know whether bees carry some of these agents back out to the plants that they pollinate. Some of the microbes were from wind pollinated plants.

Some investigators have suggested that bees might be used to provide an early warning of a plant disease outbreak. The diversity of pathogens found in sampled bee colonies suggest that the hive does function as a collector of biological materials from its surroundings. We have long capitalized on the chemicals bees bring back to the

hive for environmental monitoring of chemicals from industrial, urban, and military sources (Bromenshenk et al., 1985).

In Italy, Porrini et al. (2002) demonstrated for the first time that bees could be used as a bioindicator to detect the presence of phytopathogenic microorganisms. They studied *Erwinia amylovora*, the causal agent for a severe disease of *Rosaceae* known as Fire Blight. *E. amylovara* was also found in our surveys. The Italian scientists were part of a team investigating an epidemic that started in 1994 and affected fruit trees including pears and apple, as well as being capable of damaging over 200 species belonging to the family *Rosaceae*.

The Italians founded their investigation on the assumptions that if bees can spread the disease by carrying the bacteria within each colony's foraging range, then it should also be possible to use bees to detect the presence of the bacteria by the presence of it on bees or in other materials carried back to the hive by bees. After two years of study across four to six monitoring stations, Porrini et al. concluded that they had proven that bees can "detect the presence of the bacterium before it manifests itself in visible symptoms on affected plants".

Their overall objective was to use bees to improve monitoring of hard-to-inspect areas, to monitor the spread of the disease, to help guide disease-prevention teams, and to try to predict where it would next occur. They also hoped to transform bees from 'alleged plaque-spreaders' into an ally in the battle to control Fire Blight.

Worldwide, the lack of a satisfactory means of controlling Fire Blight has lead to the abandonment of the cultivation of susceptible fruit trees in some areas. The only effective way of dealing with the disease is prevention by means of direct, early detection of disease symptoms.

In the U.S., not only is Fire Blight a problem, but two new phytopathogenic microorganisms have recently appeared. These are plum pox and citrus greening disease. Fortunately, neither of these diseases was seen in sampled bee colonies. Greening has recently been seen in California, and plum pox is just beginning to spread. Our data verify the finding of the Italian scientists that bees bring Fire Blight bacteria back to the hive where it can be monitored. Our findings also indicate that bees bring back to the hive a wide array of plant diseases. Thus, it would appear that bees might be used to monitor any number of hard to control plant pathogens.

An immediate question that we would like to address is whether bee colonies might detect Greening before its symptoms show up in trees. This bacterial pathogen reportedly has a two year latency period. We did not see Greening in bee samples from Florida. However, we sampled Florida bees in the winter. It is our understanding that Greening is most likely to be spread as trees are leafing out. In addition, growers of crops other than almonds have voiced concerns about what bees might pick up from almonds and re-distribute to their orchards and crops. The proteomics MS provides a means to answering this question.

Conclusions and Recommendations:

The suite of semi-volatile and volatile organic compounds found in bee colonies appears to be changing over time, probably as a result of the use of new products in bee hives, particularly essential oils for mite control like thymol.

Preliminary results warrant closer inspection of a few chemicals, ones that we haven't seen before, as well as some that appear in bee hives and have been reported as products of fungal metabolism. The sealable test chamber into which we could place whole hive bodies with frames, then heat them to better drive off the volatiles, approximately doubled the number of chemical compounds that we could detect inside bee hives. We saw some differences in results by position of the sampling probe for top versus bottom of the box, but for most chemicals, position of the sampling tube did not make much of a difference. Additional investigations of volatile compounds in bee colonies are ongoing. We intend to look at other groups of compounds such as bee pheromones, which are too large to be detected by the sampling procedure that we have been using.

The proteomics work showed amazing sensitivity as evidenced by a strong detection of human skin keratin from the fingers of a beekeeper third time you mentioned this. The results show a diversity of phytopathogenic microorganisms, including not only bee viruses, but also viruses, bacteria, and fungi from other insects, plants, wildlife, and livestock.

With respect to bee viruses, in Phase I we detected Israeli Acute Paralysis virus (IAPV) in some bee operations with CCD, but not to the degree reported by others (Cox-Foster *et al.*, 2007). Our data do not support IAPV as a biomarker of CCD other than possibly in the last stages of the disease in some geographical regions. Our data does indicate that some bee operations have a higher than usual incidence of IAPV.

The absence of any detection of Cloudy Wing Virus or Chronic Paralysis Virus makes these two viruses unlikely as markers or causes of CCD. Similarly, the discovery of Varroa Destructor Virus 1 (found in Phase I, confirmed in Phase II) appears to be the first report of this virus in North America. There is no indication that this virus is associated with CCD, which occurs nationwide.

We have discovered another unreported virus that does appear across the nation in bee operations with collapsed bee colonies. The proteomics database provides a strong match for this virus to a known family of insect viruses, both in terms of the degree of correlation and the number of peptides on which the identification is based.

Although there is a known species of Virus? from a family of viruses that is known to infest another species of *Apis* bees, our initial genomic sequence work did not provide a match or identification with the known bee virus.

Why the Army's proteomics analysis detected this virus, while the genomic analysis did not, is unknown. We speculate that we may have found a variant of the *Apis* bee virus, or that the proteomics instrument is detecting a species of this virus that is known to infect other insects, but that has not yet been reported in bees.

The prevalence of this virus in collapsed colonies has lead to additional testing. We have gone back to the freezers and have pulled more samples to screen for this virus, which we refer to as Virus ? To the best of our knowledge, Virus ? has not previously been reported in *Apis mellifera*.

Our preliminary data indicate a correlation with CCD. Whether this virus is a biomarker, a causal agent, or simply a consequence of the colony decline is unknown at this time. However, the novelty of this virus, which has not been reported by other investigators, plus the documented effects of this group of viruses on other insects, warrants additional investigation. Since we are unsure of the identification of the virus, we have chosen to call it Virus ?, until the proteomics and genomics laboratories can provide a more specific identification. We will keep the Almond Board of California informed about our progress as we continue our quest to name and understand the significance of this virus in honey bee operations that have had CCD.

Acknowledgements:

We would like to thank the National Honey Board and the Almond Board of California for funding Phase I and Phase II of this work respectively. We also thank all of the beekeepers who helped us sample their colonies. David Westervelt and Jerry Hayes of the Florida Apiary Division were especially helpful in terms of the field sampling support, both in Florida and in California. Dave Wick of BVS, Inc. has worked closely with us. The US Army's Edgewood Chemical and Biological Center has made it possible to investigate aspects of Colony Collapse Disorder in ways that we never imagined. The uniqueness of their approach provides new insights, as evidenced by the first discovery of VDV-1 in North America and the inventory of plant pathogens found in bee colonies.

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