2.7 Quantitative analytical tools for bee health (*Apis mellifera*) assessment

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Abstract

Background: The number of honeybee (*Apis mellifera*) colony losses has grown significantly in the past decade, endangering pollination of agricultural crops. Research indicates that no single factor is sufficient to explain colony losses and that a combination of stressors appears to impact hive health. Accurate evaluation of the different factors such as pathogen load, environmental conditions, nutrition and foraging is important to understanding colony loss. Commonly used colony assessment methods are subjective and imprecise making it difficult to compare bee hive parameters between studies. Finding robust, validated methods to assess bees and hive health has become a key area of focus for bee health and bee risk assessment.

Results: Our study focused on developing and implementing quantitative analytical tools that allowed us to investigate different factors contribution to colony loss. These validated methods include: adult bee and brood cell imaging and automated counting (IndiCounter, WSC Regexperts), cellular transmitting scales and weather monitoring (Phytech, ILS) and pathogen detection (QuantiGene® Plex 2.0 RNA assay platform from Affymetrix). These techniques enable accurate assessment of colony state.

Conclusion: A major challenge to date for bee health is to identify the events leading to colony loss. Our study describes validated molecular and computational tools to assess colony health that can prospectively describe the etiology of potential diseases and in some cases identify the cause leading to colony collapse.

Key words: Colony loss, colony assessment methods, cellular transmitting scales, weather monitoring, QuantiGene® Plex 2.0.

Introduction

Colony losses have been monitored across the USA since 2007 and found to average around 30% (1). However, higher losses, ranging from 30% to 90% (2), have been reported by beekeepers. Recent research indicates that the decline of managed hives during winter months is influenced by a combination of several factors, including pests, parasites, bacteria, fungi, viruses, pesticide exposure, nutrition, management practices and environmental factors (3-7). Accurate risk assessment and measurement of colony health based on equalized, validated and objective measurements are needed to accurately predict the reasons for colony decline. Our goal was to develop and deploy quantitative analytical tools to assess the contribution of different factors to colony health. Commonly used colony assessment methods have several drawbacks such as subjectivity, high variability and sensitivity to environmental differences. In addition, different methods are used by various scientists making comparison between studies difficult.

Commercial beekeepers evaluate colony strength by assessing adult bees and capped brood frame coverage (8). The 'frame-coverage' method, employed by beekeepers and almond inspectors (COLOSS Beebook, www.coloss.org/beebook) when assessing hive strength, is subjective, not accurate and shows high variability within and among inspectors (9, 10).

We have implemented a QuantiGene method (QuantiGene® Plex 2.0 RNA assay platform from Affymetrix) that enables us to simultaneously detect the presence of all known viruses and other pathogens, and also to learn at what levels they become relevant for disease. With QuantiGene it can be determined whether pathogens are actively replicating and thus causing acute disease, or whether they are present but benign. By employing remote sensing hive scales that sample

weight periodically, foraging activity during the day can be indirectly assessed. This allows predictions to be made of when a hive is on the verge of collapse and possible reasons for the collapse to be identified.

Quantitative tests along with environmental monitoring conditions will allow researchers to achieve more accurate colony assessments and obtain a better understanding of the root causes of colony losses. Finally, using a tool kit to assess the total bee and brood cells numbers, along with accurate data collection, reduces concerns due to inspector subjectivity.

Experimental methods

Hive Equalization

All study hives were equalized by re-queening with queens of the same age and similar genetic background. Acceptance was verified two weeks following replacement. Colonies were equalized to have a set number of frames covered with bees and frames containing capped brood.

Almond Grower Assessment Method (AGM)

Almond Grower assessment Method (AGM) was performed as used by beekeepers across the US prior to almond pollination. In general, hives were graded by the number of covered bee frames assessed after looking at the top and bottom of each hive. In most studies capped brood area is not measured, and if done, the number of brood frames was stated (8).

Sampling

Bees

Bees intended for Quantigene® Plex 2.0 assay were collected from the outer frame in a 50mL tube. Immediately following collection, samples were placed on dry ice and kept at -80°C until analysis. For *Varroa* counts, half a cup of bees was sampled from the inner frame (~500 bees) into Wide-Mouth HDPE Packaging Bottles with PP closure (Thermo Scientific cat 03-313-15D). Bottles were brought to the lab, weighed and bees were shaken for 5 minutes in 200ml EtOH, and then filtered through a sieve that collects the mites. The sieve is then turned over a white paper in order to count mite numbers. The number of mites per 100 bees is calculated using average bee weight.

Weather Data Collection

A weather collection station monitoring temperature, humidity, and precipitation was placed at each site (Phytech, ILS). The data were transmitted in real-time over a cellular network and collected on our computers.

Adult bee and brood cell counts

All frames from each colony were taken gently one at a time to minimize disruption. Each frame was placed on a designated frame holder onto which the camera is fixed, and its photo was taken. The frame holder allows a fixed and steady positioning of the frame with bees in front of the camera, thus improving the image quality and reproducibility. The camera support is mounted to the frame holder. Photos are taken from both sides of the frame, with all bees on them. Then, frames containing capped brood were gently shaken into the hive, making sure not to harm the queen if present, and a second set of photos was taken for capped brood count. Total number of bees on each frame and the number of capped brood cells was determined using image recognition software adjusted for this purpose (IndiCounter, WSC Regexperts). The software was validated in different locations and different times of the day in order to analyze the effect of time and location.

Pathogen prevalence

Quantigene®, a quantitative, non-amplification-based nucleic acid detection analysis, is performed on total lysate from frozen honey bee or *Varroa* mite samples. The oligonucleotide probes used for the QuantiGene® Plex 2.0 assay were designed and supplied by Affymetrix, using the sense strand of bee virus sequences as template or negative strand for replicating virus. The probe, designed to detect the sense strand, reflects the presence of virus (viral load) and the probe designed to detect the anti-sense strand reflects levels of viral replication. Housekeeping gene probes were designed from sequences of *Apis mellifera* Actin, Ribosomal protein subunit 5 (RPS5), and Ribosomal protein 49 (RP49). For *Varroa* mites, actin and α tubulin were used as housekeeping gene references.

The QuantiGene® assay was performed according to the manufacturer's instructions (Affymetrix, Inc., User Manual, 2010) with the addition of a heat denaturation step prior to hybridization of the sample with the oligonucleotide probes. Samples in a 20 µL volume were mixed with 5 µl of the supplied probe set in the well of a PCR microplate, followed by heating for 5 minutes at 95°C using a thermocycler. Heat-treated samples were maintained at 46°C until use. The 25 µl samples were transferred to an Affymetrix hybridization plate for overnight hybridization. Before removing the plate from the thermocycler, 75 µl of the hybridization buffer containing the remaining components were added to each sample well. The PCR microplate was then removed from the thermocycler and the content of each well (~100 µl) transferred to the corresponding well of a Hybridization Plate (Affymetrix) for overnight hybridization. After signal amplification, median fluorescence intensity (MFI) for each sample was captured on a Luminex 200 machine (Luminex Corporation).

Statistical analysis

BoxPlot analysis was used to compare AGM assessment to IndiCounter bee counts ($P<0.05$). Parallel Regression and Anova were used for validation of the counting software and comparisons between time and location of imaging.

Results and Discussion

AGM vs Bee Counting Software

Two methods were used to assess hive strength: the Almond Grower Method (AGM), used by beekeepers to assess hive strength as number of bee covered frames before almond pollination; and imaging software, counting bees from frame images. The number of bees in the hive provides a reliable proxy to the comparative strength of the hive. Figure 1 shows results of the two methods. While the AGM assessment showed equal hive strength at the start point for all three sites, the frame imaging software indicated that Site 3 had significantly ($P<0.05$) fewer bees than the other two sites at the same time point.

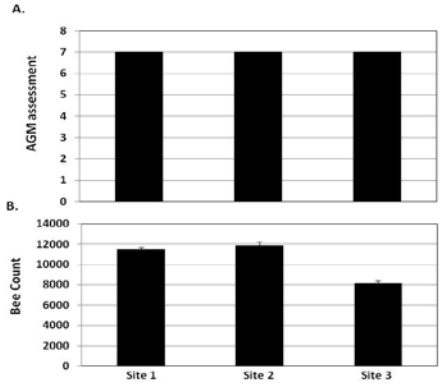

Figure 1 AGM assessment and bee counts following colony equalization. A. hives were assessed using Almond Grower Method. B. Bee number as calculated by imaging software (IndiCounter, WSC Regexperts) at each site.

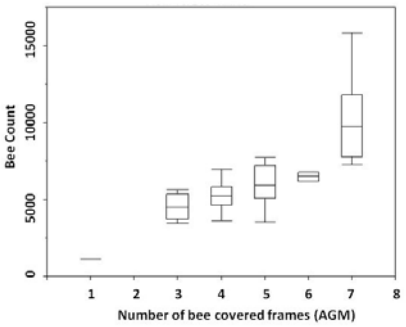

Figure 2 Comparision between AGM and Indicounter Bee counts. Hives were assessed using AGM (X Axis). Photos of bee frame were analyzed for bee counts (Y Axis). The two parameters were compared using BoxPlot analysis ($P<0.05$).

The differences became even larger and more significant when frame coverage by AGM was correlated to total bee numbers as calculated by IndiCounter software when hives are not equalized during winter changes. Seven fully covered frames, as assessed by the AGM inspectors, showed a range from ~7000 bees to over 15,000 bees as counted by the software (figure 2). 95% of these counts were between 7500 to about 12,000 bees with a median of 9500 bees. It is also common to have hives that are rated anywhere from 4 to 7 frames and upon counting turn out to have the same number of bees (~7000) because of human's eye inability to assess the bees' distribution over the frame. While the AGM may be sufficient for beekeepers to assess hive strength prior to pollination, it is inadequate for a risk assessment study to determine colony strength. Moreover, from the perspective of the beekeeper, there is a large commercially relevant difference between 7000 or 15,000 bees in a hive that will be reflected in foraging activity. We have discovered that the total number of bees can be very different among hives that were assessed as having similar "covered frame count". The bee counting software, IndiCounter, is now fully functional and validated (IndiCounter, WSC Regexperts), and the software was proven to provide accurate data. Human counts were compared to the bee counting software in order to test the effect of location and time of performing the frames imaging (Figure 3.). Within the range of counts given, a straight line model appears to be sufficient. Most of the intercepts are close to zero, indicating an almost 1:1 relationship between actual count and auto recognized count. Accurate adult bee and capped brood numbers will give reliable and comparable indication of colony general state.

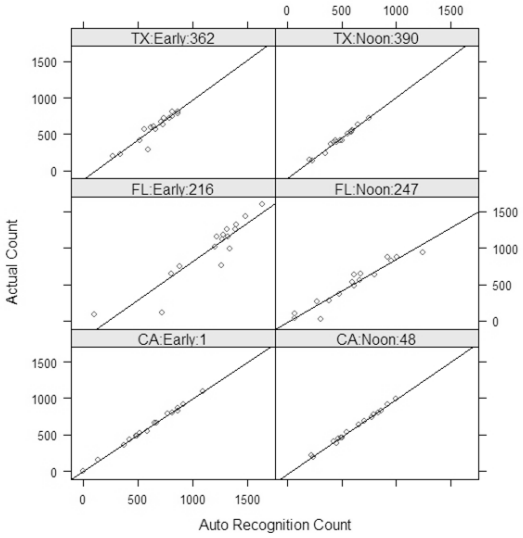

Figure 3 Count validation data showing the effect of time and location. Bee numbers were assessed using actual count (Y Axis). Photos of bee frames were analyzed for bee counts (X Axis). The two parameters were compared, revealing that within the range of counts given, a straight line model appears to be adequate. Most of the intercepts appear close to zero, indicating an almost 1:1 relationship between actual count and auto recognized count.

Scales and Environmental Control Units

Cellular transmitting hive scales and weather monitoring units sample the weight on a programmed schedule of your choice (e.g., every minute, every hour or once a day) and transfers the data automatically to your computer (Phytech, ILS). Figure 4. reflects the daily amplitude in weight resulting from foraging and nectar accumulation during the day and water evaporation from collected nectar at night.

Weather monitoring, along with cellular scales during the trial, can help monitor and explain differences in colony losses. Figure 5 shows two different colony loss scenarios as captured by the cellular transmitting monitor systems. The first (Figure 5A.) occurred shortly after queen replacement. While the hive gradually lost weight, daily amplitude was still observed indicating that the hive was still active but loosing bee numbers and the forging force was decreasing, resulting in colony weight loss. This may suggest a queen loss scenario, where the adult bees are still active, but in the absence of an egg laying queen and newly emerged bees, the population will slowly deteriorate. The second scenario (Figure 5C.) occurred immediately following a cold snap, as measured by our environment monitoring system (Table 1). The dark gray amplitude indicated water pulses, while the light gray graph amplitude illustrates hive weight. Around the time of colony collapse there were several days of cold snap along with heavy rain (the pick of high water pulses illustrated in the figure 5C. and Table 1 shows the low temperatures), followed by hive collapse (Figure 5C.). Collapse was verified by human inspection. Figure 5B. indicates normal winter weight loss. At the beginning of spring, colony weight increased rapidly and the colony swarmed. Remote monitoring could allow identification of the exact time when a super box is needed to prevent swarming.

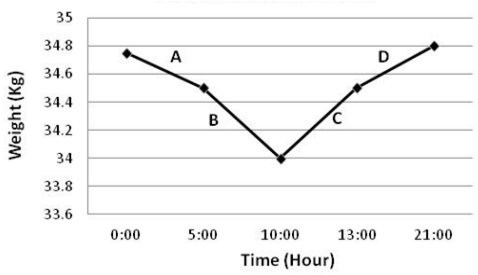

Figure 4 Daily hive weight amplitude as measured by a cellular transmitting unit. X Axis represents daily time (Hour). Y Axis represents weight (Kg). A. Water evaporation from collected nectar. B. Foragers exiting the hive. C. Nectar accumulation in the cells. D. Returning foraging bees.

Date	Temperature Min (°C / °F)
12/16/2012	17 /62.6
12/17/2012	7.5 / 45.5
12/18/2012	6.5 / 43.7
12/19/2012	17 /62.6
12/20/2012	**4.5 / 40.1**
12/21/2012	**3 / 37.4**
12/22/2012	**4.5 / 40.1**
12/23/2012	17 /62.6
12/24/2012	16 /60.8

Table 1 Minimum temperatures (0C/OF) at trial site location prior to hive collapse, as measured by an environmental control unit (Phytech, ILS).

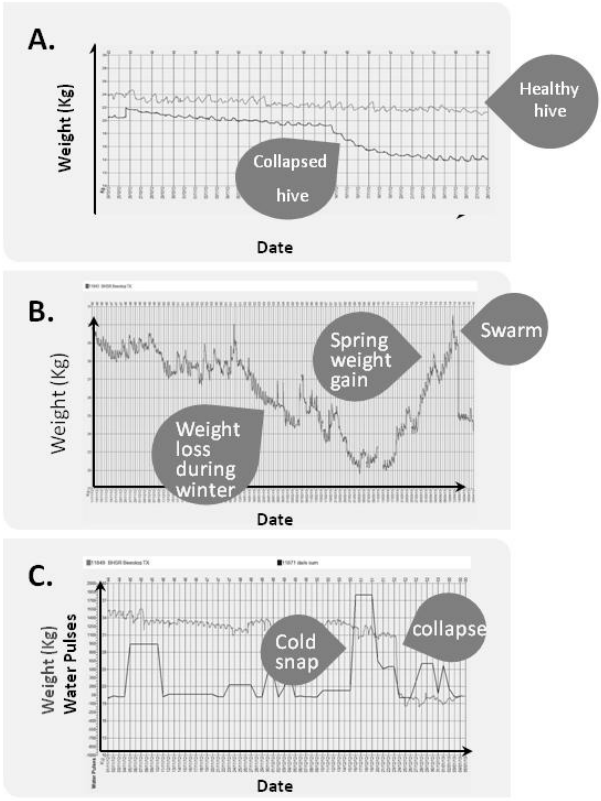

Figure 5 Different colony behaviors as indicated by cellular transmitting scales and environmental control unit. Cellular transmitting scales were placed under selected hives. Environmental transmitting unit was placed in the field. A. Weight loss due to queen loss. B. Colony behavior during winter C. Square diagram represents daily water pulses. Other diagram represents hive weight.

Cellular transmitting scales and weather monitoring units allow constant colony weight monitoring, shows foraging activity, can provide the beekeeper with potential prediction of

swarming or the need for a super. Monitoring both weight and weather will allow linkages to be made between weather conditions, colony loss and hive health (11).

Molecular analysis of viruses' detection (QuantiGene® Plex 2.0 RNA assay platform from Affymetrix)

Bees, like other organisms, carry viruses asymptomatically and, under stress, the viral pathogen might be activated and cause acute disease leading to premature bee death (12, 13).

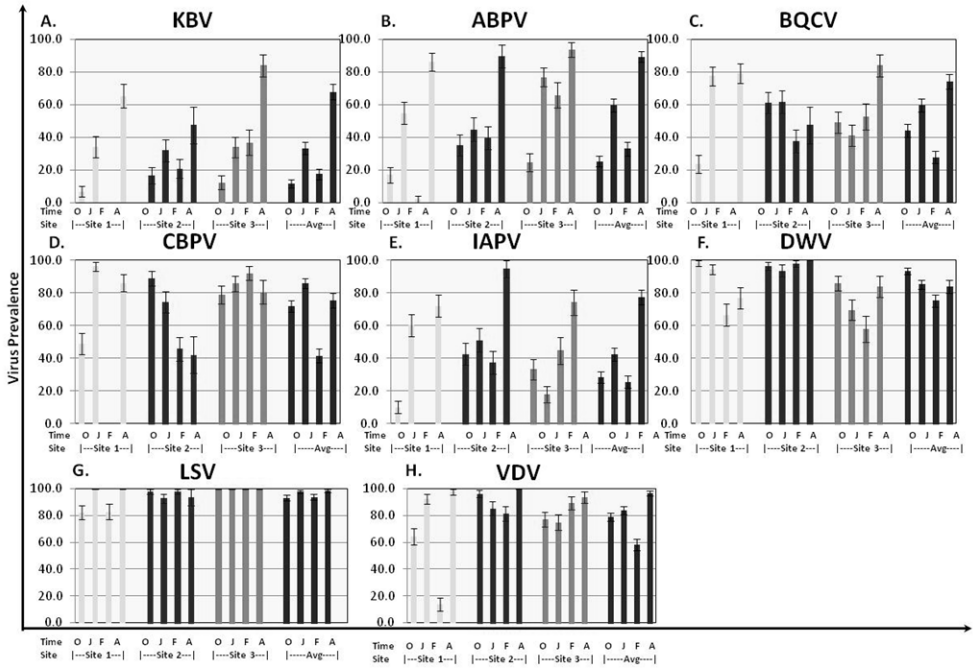

Figure 6 QuantiGene analysis of eight bee viruses. X axis shows sampling times: October, January, February and April as well as the three sites separately (site #1 - #3) and their average (Avg). Y axis represents Virus Prevalence. A – H are the charts, each for each tested virus.

We developed an analytical method to indicate virus presence and absolute and relative levels. Importantly, this method reveals whether the virus is actively replicating and causing an acute disease that may lead to colony loss. Figure 6 shows prevalence of eight bee viruses (defined as percentage of hives where the virus was detected) detected using QuantiGene® Plex 2.0 platform in an eight month field testing period. Bee virus prevalence reported here is a snapshot of the prevalence for those hives that were classified as live at the time of sampling. As the study progressed, the number of sampled hives decreased due to colony loss. The paralysis viruses (Kashmir Bee Virus, Acute Bee Paralysis Virus and Israeli Acute Paralysis Virus) exhibited similar patterns (Figure 6A, 6B, 6E) and their levels increased by trial end to >65% across sites. DWV (Figure 6F) was found at high prevalence (75%-95%) throughout the trial with no significant difference among sites. Using the QuantiGene® analysis method allows one to detect most hive pathogens in one plate reaction as well as the reverse strand probes for the detection of replicating viruses. In conclusion, QuantiGene® analysis is faster and simpler because it can use cell lysate without the need to purify RNA. Since the method detects molecular markers, we can use the same sample to quantify levels of all known honeybee viruses as well as *nosema* and thus create over time 'the full pathogen picture of the hive': viruses, *nosema* and even *varroa*, if present.

In the last seven years, mean annual colony losses across the USA increased to approximately 30% (1). Extensive research has been performed to characterize reasons for these increased losses (5, 7, 14-19). These losses highlight the need for accurate methods for colony and general bee health

assessments. We describe quantitative analytical tests that allow more accurate assessments of bee and hive health to be conducted to get at the root causes of colony collapse. Using these methods to assess the total bee and brood cells numbers, along with accurate data collection, removes inspector subjectivity and variability that complicate hive health assessments. We have also utilized a high-tech tool kit with extensive molecular analysis to assess colony health. This includes an IndiCounter that provides accurate measures of hive population, QuantiGene® analysis to detect replication of most bee pathogens, and scales and weather monitoring units to monitor foraging activity, weight gain. Taken together these tools allow the factors determining colony losses to be identified.

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