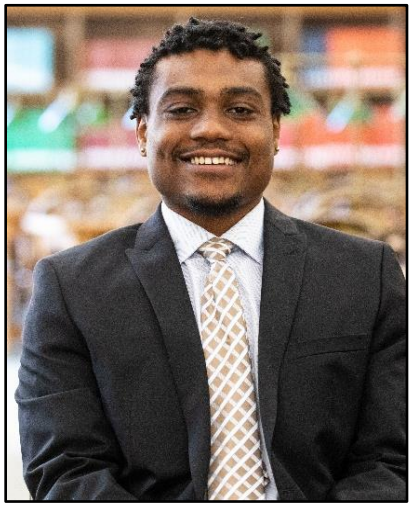

Assessing the Effects of Deformed Wing Virus Infections on Biomarkers of Honeybee (Apis mellifera) Immune Function and Stress

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Abstract

US beekeepers lose 40% of their colonies annually, and viral infections are significant contributors to these losses. One of the most well-characterized and problematic honey bee viruses is Deformed Wing Virus (DWV). DWV infections result in reduced activity and shortened lifespans in honey bee workers. DWV is transmitted by the parasitic *Varroa destructor* mite. Mite infestations and DWV are both positively correlated with colony mortality. DWV exhibits high genetic diversity with multiple "master variant" strains (mainly DWV-A and DWV-B) which have different infection dynamics and health implications. Here, we examine how honey bees respond to high and low DWV-A and DWV-B infection levels. Quantitative real-time PCR was used to monitor DWV levels and expression of two genes that are potential markers of an immune response (*Dicer-like*) and bee health (*Vitellogenin*). This study will help determine if different expression levels of these genes can serve as robust indicators of viral infections and bee health.

Introduction

Maintaining large and healthy honey bee population levels is crucial for food production as honey bees are the world's most important food crop insect pollinator for a vast majority of domestic and foreign produce. Approximately three quarters of the major global crops are dependent on honey bees for pollination.[1] However, in recent years there has been an alarming decline in honey bee colonies. Between 2018 and 2019, U.S. beekeepers lost 40.7% of their colonies, which is a slightly higher mortality rate than the previous year.[17] These losses can be attributed to stressors such as pathogens, parasites, pesticides, and poor nutrition. It is critical to develop sustainable approaches that mitigate the impacts of stressors to support food security and improve beekeepers' economic outcomes.

Recent studies have begun to identify the severe contribution of viruses to these colony losses.[4] Thus, maintaining and expanding honey bee populations requires improving our understanding of virus and host interactions in this system.

One of the foremost studied viruses of honey bees is Deformed Wing Virus (DWV), which has well-characterized infection dynamics and a significant negative impact on developing pupae.[9] Honey bee pupae inoculated with DWV develop more slowly, produce nonfunctional wings, and die soon after emergence as adults. DWV is a positive single-stranded RNA virus in the Iflaviridae family. DWV exists as multiple "master variants", with most focus on master variants DWV-A and DWV-B (also known as Varroa Destructor Virus-1 or VDV-1). DWV-A and DWV-B are similar, sharing 84% of the same nucleotide sequence and 95% of amino acids.[11] Despite these relatively small sequence differences, DWV-B has different infection dynamics and has been shown to have a higher virulence compared to DWV-A in adults.[13]

Both DWV variants can be transmitted by the ectoparasitic *Varroa destructor* mite, another worldwide stressor of bees associated with colony declines, DWV-B was isolated and sequenced from *Varroa*.[9] The *Varroa* mite was introduced into the U.S. in 1987, and has rapidly spread to become a ubiquitous parasite of colonies independent of beekeeper management or operation.[18] While feeding on developing bee hemolymph (insect equivalent of blood), the *Varroa* mite vectors, or transmits, multiple honey bee viruses including DWV. This virus and vector association is the primary cause of colony decline and mortality.[4] By providing a new transmission route for viruses like DWV, the *Varroa* mite serves as an influential factor on viral genotypes, by selecting for genotypes that transmit better through a vector transmission route compared to other routes such as fecal-oral or vertical transmission. Vector transmission of DWV by the *Varroa* mite increases the DWV's pathogenicity by inducing higher viral titers and selecting for reduced viral genotype diversity compared to oral transmission [4,13]. These effects may be due in part to *Varroa's* ability to suppress the honey bee immune system.[15]

The primary immune responses in honey bees include the Imd, JNK, JAK/STAT, Toll and RNA interference (RNAi) pathways. The RNAi pathway, in particular, is the main antiviral defense mechanism for insects.[19] RNAi is a post-translational, sequence-specific, gene regulation mechanism.[5] In honey bees, the RNAi pathway component *Dicer-like* is the predicted endoribonuclease which cleaves the viral dsRNA into shorter segments which allows for viral transcript degradation by the RNAi pathway and, therefore, suppressed virus replication.[5] Expression of *Dicer-like* increases with higher Israeli Acute Paralysis Virus titers in honey bees.[6] Expression of *Dicer-like* may therefore be a possible marker for an active immune response against viral stressors. Another critical immune gene is *Vitellogenin*, a yolk precursor gene in insects which also serves a special function by regulating the onset of foraging behavior and priming bees for specialized foraging tasks.[7] Hence, a decreased expression of *Vitellogenin* is a signal of accelerated maturation from nursing to foraging behavior. Becoming a forager is stressful, its when mature bees are vulnerable factors outside of the hive. The differential expression of these genes during viral infection can provide insight into how the honey bees' stress and health levels differ according to viral titer and genotype. This study aims to evaluate the different effects of the DWV variants DWV-A and DWV-B in developing honey bee pupae by measuring the transcription levels of *Dicer-like* and *Vitellogenin* after infection by high and low concentration of DWV-A and DWV-B.

Methods

Virus Propagation

DWV-A and DWV-B was first propagated to generate sufficient quantity of viral inoculum for the experimental injections. We obtained pure isolates of DWV-A and DWV-B from Dr. Robert Paxton (Martin Luther University, Halle, Germany), under USDA APHIS Permit P526P-18-03011. Pupae were injected with 2µL of either DWV-A or DWV-B viral inoculum. The pupae were left to develop for four days and then were collected and stored at -80ºC for later use. The pupae were homogenized in phosphate buffer saline (PBS) in a FastPrep homogenizer for 45 seconds at 6.5 m/s, centrifuged for 3 minutes at maximum speed, and the supernatant was passed through a 0.2µM filter to separate virus particles (smaller than 0.2µM) from animal cells (larger than 0.2µM). This crude virus purification became the "High" DWV-A and DWV-B inoculums. "Low" DWV-A and DWV-B were created by making a 1:10 dilution of the "High" inoculum (see Table 1).

Field Preparation

To obtain age-matched bees for viral infections, healthy and productive queens were caged on an empty honeycomb frame within their colony for 24 hours to lay eggs. After 24 hours, the queen was removed and the cage remained around the comb to prevent the queen from laying additional eggs. After fourteen days (white-eyed pupae stage) the pupae were collected for inoculation. Healthy and productive queens and colonies were assessed through weekly colony inspections. The colonies health was confirmed (for example, large amounts of food and multiple ages of brood) to ensure a favorable status of the colony and queen. This experiment utilized three different colonies for each different Trial. Two colonies were head by a queen (on average 75% related sister bees due to honey bee haplodiploid genetics) (Trials 1 and 2) and one colony was headed by a naturally-mated queen (Trial 3).

Collection and Infection

White-eyed pupae (14 days old) were collected by removing the frame from the colony and uncapping the cells. The frame was then inverted, and pupae could be removed from the comb through gravity, requiring little manipulation and injury. Properly aged pupae were sorted into experimental treatment groups (n=5 per group). Bees were injected with virus or PBS buffer to mimic viral transmission by the *Varroa* mite. To perform the injections, capillary needles containing the virus or buffer were inserted into the pupae abdomen between its integuments. To avoid contamination, the micropipette injector capillary tube was changed between virus groups.

Pupae were injected with 2µL of inoculum, buffer (PBS), or left as a no treatment control. Virus concentration per µL for all treatments in Trial 3 can be found in Table 1, where the virus quantities were normalized between the DWV-A and DWV-B inocula. Trials 1 and 2 were injected with inoculum without normalizing the differing concentrations of DWV-A and DWV-B (ranging from 1.5E8/µl- 2.5E8/µl) within the inoculums. Thus, the description of the results will focus on the Trial 3 data.

After infections, the pupae developed in an incubator at 34ºC and 50% relative humidity within sterile Petri dishes. Five days post-infection, the five pupae from each viral group were collected and stored at -80ºC until screened for immune gene expression and DWV-A and DWV-B quantification.

Inoculum	Quantity/µL
DWV-A Low	1.5×10^7
DWV-A High	1.5×10^8
DWV-B Low	1.5×10^7
DWV-B High	1.5×10^8
PBS	1×PBS
Control	n/a

Table 1: DWV-A and DWV-B were propagated in pupae and then extracted. The viral titers of the high and low concentration groups were normalized to each other. There is a 1 to 10 dilution factor between the high and low quantities.

RNA Purification and Extraction

Abdomens from five-day-post-infected pupae underwent an RNA extraction protocol using a Qiagen RNeasy Plus Mini Kit (Hilden, Germany) following the manufacturer's protocol, including a DNase 1 incubation. RNA was eluted in 50ul of molecular grade water and the concentration was assessed via NanoDrop (Thermo-Fisher Scientific, Waltham, MA, USA).

cDNA Synthesis

A High Captivity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to produce complementary DNA (cDNA) from the extracted RNA following the manufacturer's protocol, including RNase inhibitor, starting with 200 ng of RNA.

Real-Time Polymerase Chain Reaction (RT-qPCR)

After cDNA synthesis, qPCR (Sybr Green PCR master mix, Applied Biosystems) for *Dicer-like, Vitellogenin, eIF-S8,* DWV-A and DWV-B (for primer sequences, see Table 2) was conducted. For viral quantification, a dilution series of oligonucleotides for DWV-A and DWV-B PCR target sequences, ranging from 10^2- 10^6 copy numbers, were included. To quantify the immune gene expression, a dilution series ranging from 10^{-1}- 10^{-5} was made from a control sample to assess plate and primer efficiency. Each sample was analyzed in triplicate, and the results were averaged to generate an accurate quantification.

Target Gene	Primer	Reference
eIF-S8	F: 5'- TGA GTG TCT GCT ATG GAT TGC AA-3' R: 5'- TCG CGG CTC GTG GTA AA- 3'	Galbraith, Yang, Niño, Yi, & Grozinger, 2015
Vitellogenin	F: 5'- TTG ACC AAG ACA AGC GGA ACT -3' R: 5'- AAG GTT CGA ATT AAC GAT GAA -3'	Kocher et al. 2008
Dicer	F: 5'- CCA ACA GGA GCT GGA AAA AC -3' R: 5'- TCT CCA CTA AGT GCT GCA CAA -3'	Galbraith, Yang, Niño, Yi, & Grozinger, 2015
Deformed Wing Virus -A (DWV-A NS)	F: 5'- TTC ATT AAA GCC ACC TGG AAC A -3' R: 5'- CAA GTT CGG GAC GCA TTC CAC G -3'	Ryabov et al., 2014
Deformed Wing Virus -B (Varroa destructor Virus (VWV-1 NS))	F: 5'- TTC ATT AAA ACC GCC AGG CTC T -3' R: 5'- CAA GTT CAG GTC TCA TCC CTC T -3'	Ryabov et al., 2014

Table 2: RT-qPCR Specific Forward and Reverse Primer Sets

Data Analysis and Statistics

Each primer expression value was calculated based on the standard curve. Within triplicates, outliers with a >0.5 threshold cycle (CT) from the average were removed. *Dicer-like* and *Vitellogenin* absolute quantities were normalized to *eIF-S8* (control gene). The fold difference was then calculated by normalizing the values to the control sample. Analysis of Variance (ANOVA) test was utilized to assess target gene expression differences ($p \leq 0.05$) between treatment and control groups. Statistics were conducted using the aov() function in R.[21] Figures were generated in Excel.

Results

Since the concentrations of DWV-A and -B used to inoculate bees in Trials 1 and 2 were not equivalent, we focused our analysis on Trial 3 . PBS- and non-injected samples were virus-free. DWV-B High and Low groups showed higher DWV levels than both DWV-A High and Low groups despite the inoculums starting at the same concentration. Samples injected with low concentration inocula had similar final viral levels as samples injected with high concentrations. DWV-A Low and High group had significantly higher DWV-A levels compared to DWV-B High (One way ANOVA, p=1.0E-3, 0.025) and Low (One way ANOVA, p=1.0E-3, 0.025) groups (Figure 1). Similarly, the DWV-B Low and High group had higher DWV-B levels compared to DWV-A High (One way ANOVA, p=7.8E-3, 2.8E-5) and Low (One way ANOVA, p=7.8E-3, 2.8E-5) groups (Figure 2). Thus, the incocula seemed to contain only DWV-A and DWV-B, with no evidence for cross-contamination.

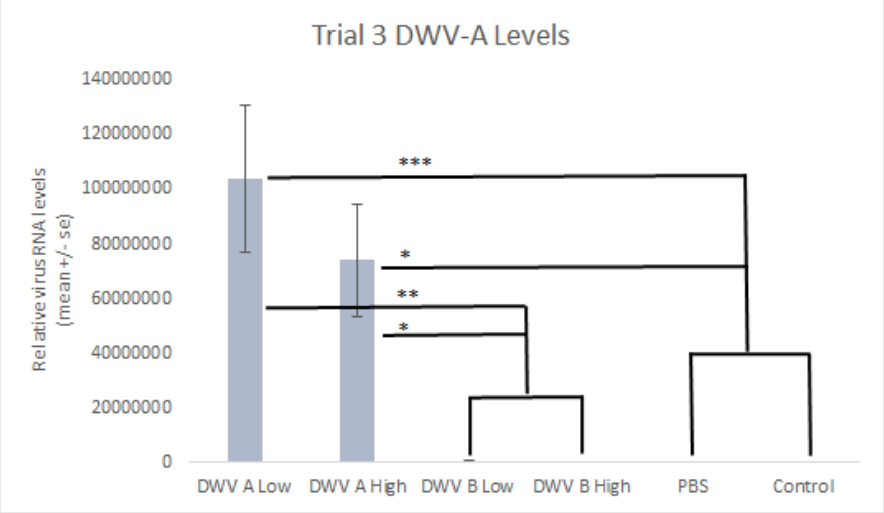

Figure 1: Trial #3 DWV-A levels in experimental groups

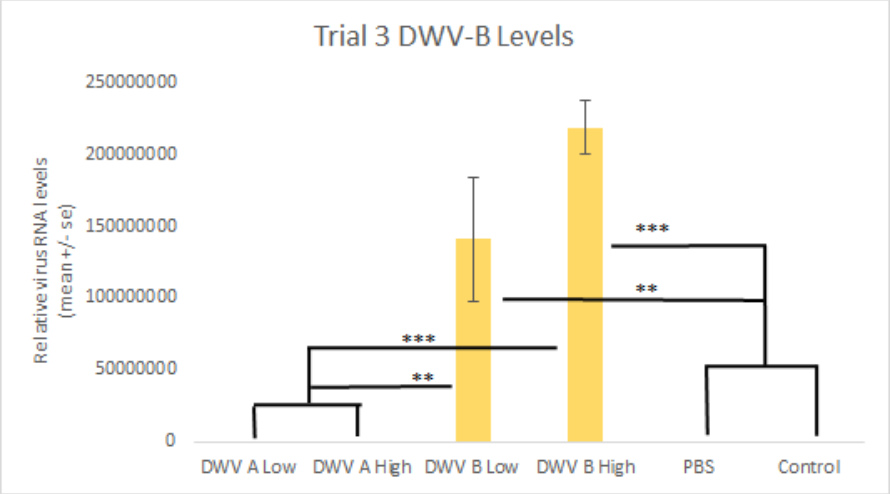

Figure 2: Trial #3 DWV-B levels in experimental groups

In Trial 3, the DWV-A High infected group exhibited significantly higher elevated levels of *Dicer-like* expression compared to the DWV-B Low and High groups (One way ANOVA, p=0.04), see Figure 3. All other groups were not statistically different from the control group (One way ANOVA, p>0.05). The RNA levels of *Vitellogenin* were not significantly different between the inoculated and control groups in Trial 3 (data not shown).

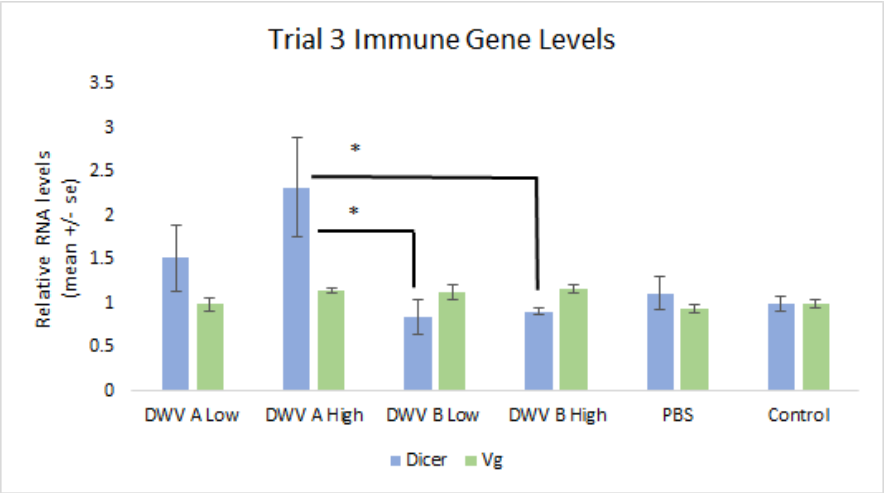

Figure 3: Trial #3 *Dicer- like* and *Vitellogenin* levels in experimental groups

In Trials 1 and 2, the amount of DWV-B injected was lower than the amount of DWV-A injected, and thus the overall levels of DWV-B in the sample was lower (data not shown). However, interestingly, when data from all three trials are combined, levels of *Dicer-like* expression were significantly higher in the DWV-A High group compared to the Control and PBS groups, and levels were significantly higher in the DWV-A Low group compared to the DWV-B High and Low groups (Two way ANOVA, p<0.05, data not shown). When the data from all three trials are combined, there was still no notable difference in expression levels of *Vitellogenin* across the inoculated and control groups.

Discussion

This study aimed to determine if *Dicer-like* and *Vitellogenin* are suitable bio-markers for stress and health in DWV infected pupae. The expression results of *Dicer-like* and *Vitellogenin* from the DWV infections suggest they are not valuable bio-markers for health and stress. *Vitellogenin* RNA levels did not fluctuate between the inoculated and control groups. *Dicer-like* RNA levels were significantly higher in the DWV-A infected groups but not DWV-B groups, and thus *Dicer-like* does not serve as a consistent marker of viral infection. Intriguing, the *Dicer-like* expression data suggests that the pupal immune system responds differently to the two DWV variants.

The different *Dicer-like* levels resulting from DWV-A and DWV-B infection indicates that the pupae had different immune responses to the variants in Trial 3. Only the High DWV-A inoculum was able to induce a significant increase in expression of *Dicer-like*. The low DWV-A concentration inoculum did not display any significant changes in *Dicer-like* transcription compared to other treatments. Also, the *Dicer-like* levels in the PBS group did not significantly vary from the control group. These results also indicate that DWV-A stimulated an alteration to the immune gene levels, which was not dependent on *Varroa* mite feeding, which was mimicked by the PBS injection. Neither the injection nor DWV infection appeared to affect *Vitellogenin* transcription levels compared to the control groups.

The lack of observed differences in expression of *Vitellogenin* among the DWV infected and control groups is perhaps due to age-dependent expression differences. Changes in *Vitellogenin* expression have previously been described in adults, where levels are associated with behavioral maturation and the transition from nursing to foraging behavior.[7] In the current study, honey bee pupae were examined. In this developmental stage, it appears that viral infection has no effect on *Vitellogenin* RNA levels.

Viral infections have previously been shown to lead to increased activity in the RNAi pathway and increased expression of *Dicer-like*,[6] which is what was observed for the DWV-A injected group. However, DWV-B infection had no effect on *Dicer-like* RNA levels. It is possible that the DWV-B evades the RNAi pathway or slightly suppresses it. Viruses can develop mechanisms to suppress the RNAi immune pathway. For example, the Flock house virus produces the B2 protein that binds to dsRNA and prevents Dicer dsRNA cleavage and siRNA loading into the RNA-induced silencing complex (RISC).[5] Since it lacks these characterized viral suppressors of RNAi target protein machinery, DWV-B may be using a previously unobserved method of RNAi suppression through modulating gene expression.

Future studies are needed to determine if DWV-B is suppressing gene expression of other components of the RNAi pathway, such as Argonaute (AGO2). Additionally, to determine if the DWV-B genotype is modulating only the RNAi pathway, expression of genes in other immune pathways could be measured. For example, the expression of *dorsal-1A* in the Imd (immune deficiency) pathway can be analyzed. In the response to a DWV infection, *dorsal-1A* is expected to be down regulated and have no expression differences due to *Varroa mite* transmission route.[12] This can also determine if DWV-B is causing an overall down regulation in immune pathway expressions.

To further validate these results, this experiment should be repeated utilizing a wider range of viral inoculum concentrations, as well as co-infections of DWV-A and -B. Injecting virus concentrations of 10^3, 10^5 and 10^7 can provide a more comprehensive evaluation of how viral titers can influence *Dicer-like* expression. Based on the current study's results, one would expect a stronger positive correlation between the DWV-A titers and *Dicer* transcription levels and a consistent or potentially decreased *Dicer* expression with increasing DWV-B titers. A co-infection with both variants of DWV could determine if DWV-B can suppress the RNAi response even in the presence of DWV-A.

Immune responses in honey bees vary across tissues and life stages.[16] This variation could lead to differences in *Dicer-like* expression levels. In this study, white eyed pupae were evaluated. The pupal development stage was chosen because *Varroa mites* feed, reproduce and transmit the viruses while the honey bee is pupating in a closed cell. However, mites continue to feed throughout pupation and there is evidence they may feed on adults. Newly emerged adults instead of pupae can be analyzed for *Dicer-like* levels after viral inoculation during pupation.

Conclusion

This study demonstrated that expression levels of *Dicer- like* and *Vitellogenin* are not effective biomarkers for health and stress in DWV infected honey bees. In fact, two genotypes of the one virus resulted in significantly different expression of *Dicer- like*. Interestingly, infection with DWV-A triggered an increase in *Dicer- like*, indicative of an active RNAi anti-viral response, while infection with DWV-B did not result in an increase in *Dicer- like* expression, despite equivalent levels of viruses in Trial 3. Thus, the DWV-B may be able to evade or suppress the host's RNAi response. Meanwhile, *Vitellogenin* had no expression changes due to the viral infection. More genes need to be assessed as potential biomarkers. This study's results also suggest that no single target gene can be effectively used assess bee health. Analyzing the honey bee's immune function and stress requires holistic monitoring and maintenance from the cellular level to the environment to support honey bee populations.

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