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Localization of Deformed Wing Virus (DWV) in the Brains of Apis mellifera (European Honey Bees)

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LOCALIZATION OF DEFORMED WING VIRUS (DWV) IN THE BRAINS OF *APIS MELLIFERA* (EUROPEAN HONEY BEES)

by

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ABSTRACT

Honeybees, *Apis mellifera*, are important for agriculture because they pollinate crops. Bees play a very important role in pollination because they maintain flower constancy. There are at least eighteen different viruses that can infect honeybees. Deformed Wing Virus (DWV) is one of the most common viruses and it is positive-strand RNA virus. DWV has been isolated from the brains of aggressive bees in Japan and shares a high sequence homology to Kakugo Virus (KV). Currently, there is an ongoing debate of whether DWV and KV are two different viruses or simply regional isolates. DWV can be transmitted both vertically and horizontally between bees in a colony and can lead to both symptomatic and asymptomatic infections in bees. In environmentally stressful conditions, DWV has the potential to cause the demise of a honeybee colony. In 2006, Colony Collapse Disorder (CCD) was defined as the disappearance of worker bees leading to the total collapse of a bee colony. Because DWV infection contributes to bee illness, there is a possibility that DWV causes CCD. The purpose of the current research project is to design a successful in-situ hybridization to identify regions within the brains of honeybees where DWV replicates. The localization of the virus in the brains of the bees can draw a connection between CCD and DWV.

In-situ hybridizations were carried out with both sense and anti-sense probes on the brains of honeybees that were positive for DWV as measured by real-time RT-PCR. The result of the in-situ hybridizations demonstrated localization of the DWV positive-

strand genome in the visual neuropils. The virus genome was also found in the mushroom bodies and antenna lobe neuropils. Weaker staining with the sense probe found in the same regions shows that the antigenome of the virus is also present and that the virus is actively replicating in these regions of the brain. Real time RT-PCR results showed that variable levels of viral genome can be found in both symptomatic and asymptomatic honeybees.

In conclusion, these results demonstrate that in bees infected with DWV the virus replicates actively in very important regions of the brain, including neuropils that are responsible for vision and olfaction. This means that the virus could adversely affect the vision and olfaction of the honeybees making it difficult for bees to behave normally. Further behavioral studies need to be conducted to confirm this assumption that viral infection modifies normal bee behavior. However, this honors thesis suggests that DWV is one of the contributing factors for CCD.

INTRODUCTION

Ecological Effects of Honeybees

European honeybees (*Apis mellifera L.*) are very important components in agricultural ecosystems because they help pollinate crops (Kremen et al. 2002; Sabbahi et al. 2005). Bees are responsible for pollinating about fifteen to thirty percent of crops used in worldwide food production (McGregor 1976). In greenhouse grown tomato crops, honeybees have shown to boost overall crop quality (Sabara and Winston 2003). Besides enhancing crop quality, honeybees have also been shown to increase overall crop yield. In a canola plant seed study, the presence of honeybees impacted the overall crop yield by a forty-six percent increase in seed yield compared to a similar crop raised without the bees (Sabbahi et al. 2005).

There are two specific advantages of using honeybees as pollinators in agriculture. The first advantage is that, unlike other Hymenoptera, they are not species-specific pollinators, which means that they are not restricted to pollinating only one type of plant. Thus, honeybees can be used to pollinate a wide variety of different crops making them economically feasible to be used as commercial pollinators (Sabara and Winston 2003). The second advantage of using honeybees as pollinators is that, unlike bumblees, they maintain flower constancy. This means that bees tend to limit their visits to the same species of plants while on a single foraging trip and therefore passing over other valuable food sources (Chittka et al. 1999; Gegear 2005). This behavior allows them to cross-pollinate a single crop very efficiently.

Honeybee Colony Structure and Communication

To ensure continued success within a honeybee colony, honeybees have evolved age-dependent behavioral roles. Young adult bees, less than 14 days old, are responsible for nursing the broods by feeding them the worker jelly, which is food made by the mandibular glands of worker bees. Older bees, more than 14 days old, complete foraging trips for pollen and process nectar into honey (Sasagawa et al. 1989). The roles taken on by the bees can change based on the needs of the colony (Rosch 1930). The age dependent-roles of the bees can be correlated to a physiological change that takes place in the hypopharyngeal gland of the honeybees (Sasagawa et al. 1989; Simpson et al. 1968). Studies have also demonstrated hormonal changes that can be correlated to the needs of the colony. Usually, adult worker bees work in the hive for the first three weeks and then spend their final one to three weeks foraging (Robinson 1992). An increase of JH helps with early development; upon exposure to the hormone, worker bees begin foraging two weeks pre-maturely (Huang and Robinson 1992).

Bees are extremely social insects because they are dependent upon group living; they are constantly communicating within their hives. Bees are interdependent on each other for the normal functioning of the colony. Bees share information with each other through tactile and chemical signals shared within a hive (Seeley 1997). Forager bees are known to perform the waggle dance to disclose the location of a food source to other foragers within their colony (von Frisch 1967). This interdependency and communication among the bees is a hallmark of the social living

Colony Collapse Disease (CCD)

In 2006, a phenomenon known as colony collapse disease (CCD) was identified and defined as the dramatic and rapid disappearance of adult honeybees from particular colonies. Adult worker bees were taking foraging trips but did not return to their hives. The mysterious disappearance of the adult bees caused massive die-offs of individual colonies (vanEngelsdrop et al. 2009). Currently, there are no single known causes of colony collapse disorder (vanEngelsdrop et al. 2009); however, the disorder is speculated to be caused by or related to viral infections (Johnson et al. 2009; Cox-Foster et al. 2007).

Honeybee Viruses

Of the 18 viruses that have the potential to infect honeybees, there are six viruses that are of utmost importance because they cause the most common infections. These include Deformed wing virus (DWV), Black queen cell virus (BQCV), Sacbrood virus (SBV), Kashmir bee virus (KBV), Acute paralysis virus (ABPV), and Chronic bee paralysis virus (CPBV) (Chen and Siede 2007). These six viruses that can infect bees belong to the picorna-like group of positive-strand RNA viruses. Positive strand RNA viruses package their genomes as mRNAs that are ready to be translated by cellular ribosomes into functional proteins as soon as they infect the cells (Allen and Ball 1996). Of all the honeybee viruses, complete genome sequences of only six bee viruses including ABPV (Govan et al. 2000), BQCV (Leat et al. 2000), DWV (Lanzi et al. 2006), KBV (De Miranda et al. 2004), Kakugo virus (KV) (Fujiyuki et al. 2004), and SBV (Ghosh et al. 1999), and partial genome sequence of CBPV have been reported. Based on the genomic organization, the honeybee viruses are divided into two groups. The first

group (*Iflavirus*), which includes SBV, DWV, and KV, has genomes that contain one large open reading frame (ORF) with the structural proteins encoded on the 5'-end and nonstructural proteins encoded on the 3'end. These viral genomes also contain a 5' unstranslated region (UTR) that contains the internal ribosomal entry site (IRES) to initiate translation (Figure 1). In order to replicate their genomes, these viruses attract the host cellular ribosomes to the IRES and produce one long polyprotein that autocatalytically cleaves itself into functional proteins. The second group (*Dicistrovirus*), which includes ABPV, BQCV, and KBV, has genomes that contain two nonoverlapping ORFs with the nonstructural proteins encoded on the 5'-end and the structural proteins encoded on the 3'end. These viral genomes also contain a 5' UTR and the untranslated intergentic region (IGR) between the two ORFs that can initiate efficient translation (Figure 1). The reason for this is that both the 5' UTR and the IGR can function as an IRES for translation. The viruses that belong to this group produce two different poly-proteins, which are then cleaved into functional structural and non-structural proteins, at any given time to replicate their genomes (Chen and Siede 2007).

Sacbrood Virus (SBV)

SBV was first identified in 1913 in the United States (White 1913). Ever since its identification, this virus is very common and is found to be on every continent where honeybees are present (Allen and Ball 1996). SBV has the potential of attacking both brood and adult stages of bees, but larvae, which are 2 days old, are the most susceptible to the viral infection. Adult bees infected with SBV do not show any symptoms but have a decreased life span (Bailey 1969; Bailey and Fernando 1972).

Black queen cell virus (BQCV)

Initially, BQCV was isolated from dead queen larvae and pre-pupae sealed in their cells. The cells that contained the dead queen larvae had turned dark brown to black along with the walls of the cell (Bailey and Woods 1977). BQCV mainly affects developing queen larvae and pupae in the capped-cell stage. The virus is found in high amounts when colonies are in the queen rearing stage during early summer and spring (Laidlaw 1979). The virus is found in North America, Central America, Europe, Oceania, Asia, Africa, and the Middle East (Allen and Ball 1996).

Kashmir Bee Virus (KBV)

The origin of KBV is not very clear. The virus was first isolated from adult honeybees that were experimentally inoculated from a diseased bee in Kashmir (Bailey and Wood 1977). KBV is known to infect all stages of the bee life cycle (Hornitzky 1981 and 1982). There are no clear disease symptoms for the virus. KBV is considered to be the most virulent of honeybee viruses because once a few viral particles are introduced into honeybee hemolymph the infected bee dies within three days. The virus cannot be transmitted via food. The virus probably infects bees through their cuticle when an infected bee comes in contact with an uninfected bee (Bailey et al. 1979).

Acute Bee Paralysis Virus (ABPV*)*

ABPV was first isolated during laboratory infectivity tests with CBPV (Bailey et al. 1963). The virus can be detected in both brood and adult stages of bee development. ABPV causes acute infections does not cause disease or death among infected bees, hence the name (Bailey et al. 1981). The virus is spread via salivary gland secretion of

infected adult bees (Bailey and Ball 1991). ABPV does use *Varroa destructor* (VD) mites as vectors to infect healthy bees (Bakonyi et al. 2002). The mites are not the sole factor contributing of the disease because there is a previous study that shows the presence of the virus even in the absence of the mites (Tentcheva et al. 2004), indicating an alternative transmission route.

Chronic bee paralysis virus (CBPV)

CBPV causes the adult honeybees to become paralyzed (Bailey et al 1963). CPBV is present in all the parts of the world except for South America (Allen and Ball 1996). The virus causes two forms of paralysis symptoms in infected bees. The first and the most common form of paralysis is trembling of the body and wings, crawling on the ground due to flight inability, bloated abdomens, and dislocated wings. The other form of paralysis is characterized by the presence of hairless, shiny, and black appearing bees that are attacked and rejected from the colonies by guard bees (Kulincevic and Rothenbuhler 1975; Rinderer et al. 1975). The virus is spread among bees through infected food sources and through surface contact with an infect bees (Bailey and Ball 1991).

Deformed Wing Virus (DWV)

DWV is a very commonly detected honeybee virus, which belongs to the *Iflavirus* genus, a group of viruses that is distantly related to the human picornaviruses, like polio and rhinovirus (Lanzi et al. 2006; Baker et al. 2008). In a microarray study comparing RNA levels between CCD and non-CCD colonies, there were several picrona-like viral RNA transcripts identified; however, DWV viral transcripts had the highest

level of expression compared to other picorna-like viruses (Johnson et al. 2009). DWV was first isolated from honeybees in the 1980s in Japan. Currently, the virus is found to infect bees in all parts of the world where *Varroa mites* are found (Allen and Ball 1996; Calderon et al. 2003; Berenyi et al. 2007). *Varroa destructor* is a parasitic mite that feeds on immature and adult honeybees and can serve as a vector to transmit the virus horizontally (Bailey and Ball 1991; Morse and Flottum 1997; Bowen-Walker et al. 1999). The virus can be transmitted horizontally when the mite feeds on an uninfected honeybee after feeding on an infected one (Bowen-Walker et al. 1999). The death of more than one third of the managed honeybee population in the United States of America can be attributed to virus that is transmitted by *Varroa* mites (Allen and Ball 1996). The second mode of viral transmission is vertical which takes place when an infected queen bee lays infected eggs. The virus can also be transmitted vertically by an infected queen to its young by direct contact with larvae (Chen et al. 2006; De Miranda and Fries 2008).

In honeybees, DWV is known to cause both symptomatic and asymptomatic infection. An increased density of mites can cause the normal immune system of the bees to be suppressed leading to symptomatic infections (Yang and Cox-Foster 2005). Typical symptoms exhibited by an infected bee include crumpled wings, bloated abdomens, paralysis, and drastically shortened life span (Bailey and Ball 1991). In some cases, symptomatic bees also show impaired associative learning and cannot form memories. This is evident when bees that are DWV positive show an increased responsiveness to water and low sucrose concentrations; in non-infected bees increased sucrose responsiveness is linked to improved associative learning. One would expect

bees to response to sucrose and not to water because sucrose serves as a potential food source (Iqbal and Mueller 2007). On the other hand, asymptomatic bees tend to have lower viral titers and do not display any morphological or behavior deformities (Bailey and Ball 1991). An asymptomatic colony can be transformed into a symptomatic one under environmentally stressful conditions, such as a lack of food during winter months. The lack of food causes more and more bees to become symptomatic leading to a decrease in performance by worker bees that causes the entire colony to collapse (Berenyi et al. 2006; Chen et al. 2006; Norstrom 1999; Tentcheva et al. 2004).

DWV is a positive-strand RNA virus that produces a 30-nm icosahedral particle that is made up of three major structural proteins. The virus has a single open reading frame meaning that the entire genome is translated as one long polyprotein that auto-catalytically cleaves itself into smaller functional proteins (Lanzi et al. 2006). On either side of the open reading frame there are 5' and 3' nontranslated regions that contain replication and translation control elements. The 5' end contains an internal ribosome entry site (IRES) that recruits the cellular translational machinery (Ongus et al. 2006). The DWV RNA is polyadenylated, which means that it contains a poly A tail at the 3' end to protect the genome from being degraded by nucleases, and the sequence encoding the structural proteins of the virus are located on the 5' end (Lanzi et al. 2006).

DWV/KV Connection

The DWV genome is highly conserved in most parts of the world (Berenyi et al. 2007) and is closely related to two other honeybee viruses, Kakugo virus (KV) and *Varroa destructor* virus 1 (VDV-1) (Fujiyuki et al. 2004; Fujiyuki et al. 2006; Lanzi et al.

2006). At this time, experts disagree on whether DWV and KV are regional isolates of the same virus (Lanzi et al. 2006) or two distinct species (Fujiyuki et al. 2006). KV was first isolated from aggressive bees in Japan (Fujiyuki et al. 2004), but there is no clear evidence that links DWV or KV to increased aggressiveness among honeybees (Rortais et al. 2006). Specific molecular techniques have been developed to identify both KV and DWV genomes; however, the high sequence homology between the viruses (97%) makes it difficult to differentiate between them (Fujiyuki et al. 2006; Lanzi et al, 2006; Yue et al. 2005). Further studies need to be conducted to see if KV and DWV are different variants of the same species or whether KV is a species separate from DWV. One will need to look at different biological properties of both viruses, such as antigenicity, natural cell and tissue tropism and disease processes (Chen and Siede 2007).

Goal

In this honors thesis, I have developed a successful in-situ hybridization technique (Velarde et al. 2005) that has identified the presence and replication of DWV viral RNA in several areas of the brains of infected honeybees. The data collected from the work demonstrates that the virus localizes in the olfactory and visual regions that may cause the bees to have alter behavior causing the collapse of the colony.

METHODS

Collection of Bees

Four hives used in these studies were located in Bucknell University's apiary and labeled Hives A – D. Hive D was obtained from D. Cox-Foster, Penn State University, because the hive was known to be infected by DWV. Bees were collected at random from combs as they walked with the brood area. Upon collection, the honeybees were placed on ice and then later killed by freezing at -80^{o}C Celsius. For some experiments, both asymptomatic and young bees with crumpled wings were chosen from Hive D. Bees with known virus infection were identified by their crumpled and deformed wings.

Isolation of RNA

RNA was only isolated from the abdomen and thoraxes of adult bees, whole pupae, and larvae using Trizol (Invitrogen). First the bees' tissues were homogenized using forceps in 800 µl of Trizol. Then the RNA was extracted using 200 µl of chloroform. Following the extraction step, the RNA was precipitated from an aqueous solution with 400 µl of isopropyl alcohol. The RNA pellet was then washed using 75% ethanol, air dried, and then re-suspended in RNAse free water. The quality and quantity of isolated RNA was measured using a Nanodrop spectrophotometer.

cDNA Synthesis

Two µg of total isolated RNA was used to synthesize cDNA. The 2ug of RNA was combined with random decamers and the procedure from RETROscript cDNA synthesis kit (Ambion) was followed.

PCR Primer Sequences and Condition

A specific region of the DWV genome was inserted into plasmid DNA to serve as a probe for in-situ hybridization. The DWV genome between nucleotide 8371 and 8748 was obtained using PCR and the following primer sequences: DWV1-F (5'-GACTGAACCAAATCCGATGTCATCACG-3') and DWV1-R (5'-TCTCAAGTTCGGGACGCATTC-3'). Failsafe PCR kit (Epicentre) was used to conduct PCR. The PCR mix contained 1 µl of cDNA, 1 µM of forward and reverse primers, 0.5 µl of Failsafe PCR polymerase and 25 µl of the appropriate 2x PCR buffer.

PCR Product Cloning

The 378bp PCR product was identified using gel electrophoresis. The PCR product was cloned into the pCR2.1 dual promoter vector using the TA cloning kit (Invitrogen) according to manufacturer's instructions. The identity of the cloned insert was confirmed using DNA sequencing (Penn State University). Two plasmids were generated, each containing the PCR insert in opposite direction in relation to the T7 promoter. When the plasmids were transcribed using the T7 RNA polymerase, the forward plasmid (pDWV-F) gives rise to a RNA sequence that is sense to the viral genome. On the other hand, the reverse plasmid (pDWV-R) upon transcription gives rise to a RNA sequence that is antisense to the DWV viral genome.

Real-Time PCR

The best way to quantify the amount of DWV RNA was to carry out real time RT- PCR. In order to measure cellular and viral RNA, two sets of primers were used, that recognize actin and DWV. The Primer3 program was used to design primers and the

specificity of the primers was confirmed using the BLAST program (NCBI). The designed primer sequences were as follows: primer for actin right was (5'-GGGATTCGGGGAATGAGTAT-3'), primer for actin left was (5'-GACGAGTCTGGACCATCCAT-3'), the DWV left primer sequence was (5'-AGCATGGGTGGAAATGTC-3'), and the DWV right primer sequence was (5'-ATATGAATGTGCCGCAAACA-3'). The DWV primers amplified the region between base 5288 and 5390 on the DWV genome. Each real-time PCR reaction contained 12.5 µl of 2x SYBR super mix (Bio-Rad), 1 µl of a 1:5 dilution of cDNA, and forward and reverse primers at a concentration of 0.1 µM. The iCycler program (Bio-Rad) was used to conduct a two-step real time PCR on a 96 well plate. All reactions were run in triplicates. The iCycler program was used to determine the Ct number of each reaction. In all reactions, the average Ct number calculated from the triplicate was used. Ct numbers from the actin reactions were used to standardize the Ct numbers for DWV from the same samples. Melt curves produced at the end of the amplification confirmed that each primer pair produced a single amplicon with a single melting temperature (Tm). To obtain a standard curve, the 100 bp DWV amplicon was ligated into the pCR2.1 vector using a TA cloning kit (Invitrogen) and the identity of the insert was confirmed using DNA sequencing. Then the plasmid was purified from bacterial cells, quantified, and diluted from 10^2 to 10^{10} copies per reaction. The standard curve reactions were run in the same manner as the unknown samples and on the same plate each time the real time PCR was carried out. The standard curve helped form a relationship between Ct number and copy number. The standard curve was linear from 10^3 to 10^{10} copies per reaction (R^2 =

0.99). The linear equation obtained from the standard curve was used to measure the number of viral RNA molecules per microgram of total honeybee RNA.

Production of the Probe for in-situ Hybridization

Either the pDWV-F or pDWV-R plasmid DNA was subjected to a restriction digestion. The plasmids were digested using *Hind*III and phenol/chloroform extracted followed by ethanol precipitation. Upon linearization of the plasmid DNA, 1 μg of the linear plasmid DNA was added to the Digoxiginen Labeling Mix (Roche) in-vitro transcription reaction with the T7 RNA polymerase according to manufacturer's instructions. Then the dot blot method was used to quantify the resulting single-stranded riboprobes, which were about 400 nucleotides in length.

Fixation/Sectioning of Bee Brain

Bee brains were dissected from the heads of the honeybees in DEPC treated bee saline solution. Then the brains were placed in 4% paraformaldehyde/1x PBS solution for 2 hours. The honeybee brains were incubated in 18% sucrose/1xPBS solution for 24 hours at 4^{o} C before being embedded into the OCT embedding medium. After being embedded, the brains were crysosectioned at 10 μm and mounted on FisherPlus slides. The sections were left at 27^{o} C to be dried for 24 hours and then stored at -20^{o} C until the in-situ hybridization was carried out.

In-situ Hybridization/Detection

The entire protocol for this section was obtained by referring to Velarde et al. paper (2005). The sections were first fixed in 4% paraformaldehyde for 15 minutes at room temperature. Brain sections were incubated with 10mg/ml Proteinase K in 10mM

Tris-HCl, pH 8.0, 1 mM EDTA. Then the sections were fixed again in freshly prepared 4% paraformaldehyde for 10 minutes and then rinsed for 1 minute in 1xPBS solution. Upon completion of rinsing step, the sections were dehydrated in increasing concentrations of ethanol (70%, 80%, 95%, 100%). Finally, the sections were hybridized to sense or anti-sense digonegenin-labeled riboprobes (1000 ng/ml) in a solution of 50% formamide, 10 mM Tris-HCl, pH 7.6, 200 mg/ml tRNA, 1x Denhart's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA overnight at 50^{o} C. The following day, the sections were washed in following buffers at 50^{o} C: 0.2x SSC, 2x SSC, 2x SSC/50% formamide, 5x SSC). The increase of salt concentration was attributed to increase probe binding efficiency. Digoxigenin-labeled probes were detected using a sheep anti-digoxigenin-alkaline phosphatase antibody according to manufacturer's instructions (Roche). In order to develop color, a solution with NBT/BCIP was made in a buffer containing lavimisole (1 μM). The purpose of adding lavimisole was to inhibit endsomous phosphataes enzymes in the tissue sample and reduce background staining. Sections without any probe served as the negative controls. The sections were left in NBT/BCIP solution for about 2 to 3 hours or until optimum staining was reached. Upon reaching optimum staining, the sections were subjected to a stop solution for 10 minutes. The stop solution contained 10 mM Tris-HCl and 1 mM EDTA. After the sections were incubated in stop solution, the sections were rinsed briefly, approximately 5 minutes, in ultra pure water. In order to protect the brain tissue, a coverslip was mounted on the sample using the permanent mounting agent Fluormount G. The pictures of the staining were captured using a Nikon E800 microscope and a Hamamatsu digital camera using the

Simple PCI software. Adjustments of brightness and contrast of each image was done using Photoshop.

RESULTS

Real Time RT-PCR Results

Real time RT-PCR was used to quantify DWV viral RNA and to identify bees that carried relatively high viral loads (Table 1). The RT-PCR was carried out on bees that belonged to different hives to determine the level of DWV RNA in the bees that were going to be used in the in-situ hybridization. Real time RT-PCR demonstrated that the there was a high degree of variability among bees, whether the honeybees were morphologically symptomatic or not for DWV infection (Table 1). The level of DWV RNA increased in bees as the season progressed and weather conditions cause food sources to dwindle. The data also suggested that almost all of the bees in hive D carried some levels of DWV RNA (data not shown). There were some asymptomatic bees that showed high amounts of viral RNA and brains from theses bees showed a strong hybridization with the virus probe. Also, there were asymptomatic bees that had very low amounts of DWV RNA and brains showed a very weak hybridization with the viral probe. Most of the symptomatic bees had some varying levels of DWV RNA present in their abdomen and thorax.

In-situ Hybridization Results

The in-situ hybridizations were conducted to detect the precise localization of the DWV RNA in the honeybee brains. The positive-strand viral RNA was detected in the visual neuropils, including the crescent shaped medulla, as shown from the staining pattern observed with the antisense probe (Figure 2 A, C, D). The staining pattern with

both the antisense and sense probes is localized in a punctuate pattern throughout the medulla region of the optic lobe of the bee brain (Figure 2A, B, C, D). In-situ hybridizations were conducted on bees that were collected early in summer from hive D (Figure 2C) that showed moderate level of viral RNA in a RT-PCR test (Table 1). Whereas, darker staining was observed (Figure 2A) from Bee #5 that had high levels of viral RNA (Table 1). In order to see if the virus was actively replicating in the brains of the bees, a second section from Bee#5 was taken and hybridized to the sense probe (Figure 2B). The hybridization of the sense probe revealed a lighter staining pattern and detected the antigeome; thus, confirming that the DWV viral RNA is actively replicating in the brains of the honeybees. In addition, the viral genome was detected in the subesophageal ganglion found near the back of the brain (Figure 3). A different section from the brain of Bee#5 was subjected to in-situ hybridization to see if the virus replicates in the antenna lobes. The in-situ hybridization showed both the antigenome and viral RNA in the antenna lobes suggesting that the virus infects and replicates within the antenna lobes of the brain (Figure 4A, B, C). The antenna lobes are responsible for receiving and processing olfactory signals from the antenna.

The corpora pedunculata neuropil (mushroom bodies) of the honeybee brains is crucial for the detection and integration of external stimuli. Therefore, in-situ hybridizations were conducted to see if the viral RNA was present in the mushroom bodies of Bee#6 and Bee#2. In both asymptomatic Bee#2 and symptomatic Bee#6, which carried high level of viral RNA (Table 1), the mushroom bodies contained viral RNA (Figure 5A, B, C). The staining pattern of Bee#2 (Figure 5C) is lighter than that of

Bee#6 (Figure 5A). The reason for this is that Bee#2 contains less viral RNA compared to Bee#6 (Table 1). Bee #6 also showed the presence of antigenomic viral RNA (Figure 5B). To ensure that the in-situ hybridization probes do not detect cellular RNA and only hybridize to viral genome, in-situ hybridizations were conducted on Bee#1 that contains very low levels of viral RNA (Table 1). The in-situ hybridization results of Bee#1 (Figure 5D) showed that probe does not hybridize and produce a signal when viral levels are very low; thus, confirming the legitimacy of the probes.

DISCUSSION

DWV/KV Relationship

Deformed wing virus (DWV) is a positive-strand RNA virus that shares a close sequence homology with Kakugo virus (KV). The genome sequences of the two viruses are ninety-seven percent identical (Lanzi et al. 2006). The close similarity between the two viruses has made it difficult to differentiate between them using molecular methods. In fact, some scientists even argue that KV and DWV are regional isolates of the same virus that contain minor sequence differences (Yue et al. 2005; Lanzi et al, 2006). This finding is to be expected since RNA viruses mutate rapidly (Holland et al. 1982). However, there are others that argue that KV is a different virus from DWV, and since KV was first identified in attacker bees in Japan, there has been some interest in determining if DWV/KV can cause aggressive behavior in the honeybees (Fujiyuki et al. 2006). There are some reports that suggest that KV/DWV infection does not cause aggression (Rortais et al. 2006), while other studies suggest that there might be a link between virus infection and either aggression or learning deficits in bees (Fujiyuki et al. 2004; Fujiyuki et al. 2006; Iqbal and Mueller 2007). Because of the link between DWV/KV infection and changes in the behavior of the bees, I sought to develop an in-situ hybridization technique that would help determine the precise localization of the DWV/KV RNA in the brains of the honeybees.

Real Time RT-PCR

Real time RT-PCR was conducted to quantify the DWV viral RNA loads in honeybees and to identify bees that had high viral titers (Table 1). The results shown in Table 1 suggest that there is no way to positively identify a DWV/KV infected bee without conducting real time RT-PCR. The reason for this is that asymptomatic bees subjected to real time RT-PCR showed high levels of viral RNA; this discovery suggests that even morphologically normal bees could possibly have some behavioral modifications because of the virus infection. The real time RT-PCR data also identified morphologically normal individuals in hive D, which was known to be positive for DWV, with low levels of viral RNA. The low amounts of viral RNA found in bees is important because in previous studies stressful environmental conditions, increase in mite infestation, or lack of food sources have shown to increase viral titers among previously identified bees that had low amounts of viral RNA (Tentcheva et al. 2004). This finding indicates that stressful conditions can activate the previously dormant viral infection and even cause behavior modifications among physiologically normal bees.

In humans, a similar behavioral modification pattern is observed among patients suffering from herpes simplex virus (HSV) encephalitis. Among ninety percent of the human population, HSV infection is dormant and the virus exists in a latent form in sensory neurons and ganglia. However, in some cases, the virus comes out of dormancy due to environmental reasons and can lead to encephalitis. Patients that suffer from HSV encephalitis and survive do show neurological impairments such as cognitive deficits, behavioral abnormalities, disinhibition, inappropriate hypersexuality, and compulsive grasping and exploring (Whitley and Lakeman 1995).

In-situ Hybridization

The in-situ hybridization results demonstrated that the DWV genomic RNA was present in high concentrations in the optic and antennal neuropils in the brains of bees. The DWV RNA also localized in high concentrations in the mushroom bodies in the bees' brains. The conclusion that can be made from these results is that the virus is present in the brain regions that are responsible for processing the bees' sensory information. The results also indicated lighter staining in the same regions when the brain sections were hybridized in the sense probe, suggesting the presence of the antigenomic viral RNA. The antigenomic strand of RNA is used as the template to make more genomic RNA copies during viral infection. Therefore the presence of the antigenomic RNA in optic and antennal neuropils of the bee brain and mushroom bodies demonstrates that the virus is actively replicating in these areas of the infected bee brains. The lighter staining with the sense probe was expected because picorna-like viruses produce about ten percent of the antigenomic RNA to act as a template strand to replicate their viral genomes (Verheyden et al. 2003). Lastly, most of the viral RNA also localized in punctuate pattern, which may be the cell bodies of the neurons. This finding is consistent with known subcellular location for replication of other picornaviruses, such as polio (Caliguiri and Tamm 1970). However, there is a possibility that the punctuate staining can represent viral RNA replication in other non-neuronal cells, such as glial cells. Further staining with neuronal and glial markers need to be done to reach to a conclusive answer. In Drosophila glial cells, expression of a glial-specific homeo domain protein has been associated with the reversed polarity (repo) gene. Perhaps, one could

use an antibody that recognizes the glial-specific homeo domain protein to stain the honeybee glial cells and identify which cells of the brain are supporting virus replication (Xiong et al. 1994).

In previous research, the DWV viral genome was detected using in-situ hybridization in other tissues, such as the reproductive organs, in queens and drone bees (Fievet et al. 2006), but was failed to be detected in the brains of infected bees. The reason the in-situ hybridization was successful in my case is because I used a longer riboprobe and cryosectioned the tissue, which produced higher sensitivity. I also looked at worker bees compared to queen or drone bees, which could explain the difference in my results. In another study, the KV viral genome was broadly detected in the brains of naturally infected worker bees (Fujiyuki et al 2009). Among experimentally KV inoculated bees, the virus was detected in restricted parts, such as the ocellar nerve and some neuronal cell clusters, of the brain during early stages of infection. Later, the KV viral genome was found in various brain regions, including mushroom bodies, optic lobes, and ocellar nerves. The experimenters also detected the viral RNA in hypopharyngeal glands and fat bodies, indicating a systematic infection. Finally, the expression of a novel gene was also detected among naturally and inoculated KV positive bees. The paper supports my work indicating that the KV/DWV genome localizes to the brain and can alter brain and physiological functions by turning on genes and causing the expression of novel proteins (Fujiyuki et al. 2009).

Because the virus replicates in the optic neuropils, there is a possibility that the infected bees can have impaired vision. Similarly, the presence of viral RNA in the

antennal lobes could be correlated to a deficit in olfactory processing since antennal lobes process olfactory information. Therefore, there is a good possibility that the presence of the virus in the bee brain could lead to altered bee behavior. For example, infected bees could have alteration in flight behavior, homing performance, and perception of odorants; however, to positively say that infected honeybees have altered behavior further behavior assays need to be conducted.

Future Studies

In order to positively attribute behavioral impairment to DWV infection, behavioral assay needs to be conducted. Previously, in my honors proposal, I had proposed to carry out some behavioral work; however, due to time constrains and lack of proper equipment I was unable to conduct the behavioral assay. However, to draw a stronger correlation between DWV and behavioral modifications, one does need to carry out the following behavioral assay. The behavioral assay would be based on the bees' ability to distinguish color and to learn visual information by correlating it to a negative stimulus, which would be an electric shock. The electric shock would be applied to one color and not the other and the bees should be trained three times. After the training period, the bees would be exposed to the same two colors without an electric current. The proposed outcome of this behavior assay would be that uninfected bees would avoid the color, where the electric current was previously present, but infected bees would fail to learn and associate a color with electric shock. Thus, the infected bees would not discriminate between the two colors and would be more evenly distributed compared to

the uninfected bees. This would support the hypothesis that DWV replication in the brain is altering optical perceptions and behavior.

DWV/CCD Connection

While future behavioral assays will undoubtedly correlate behavior impairments to DWV, it is also possible that the virus infection contributes to disappearances associated with colony collapse disease (Cox-Foster et al. 2007; Johnson et al. 2009). Since the virus replicates in nueropils that control vision and olfaction in honeybees, there is a possibility that the localization of the virus alters the normal behavior pattern of the bees. Bees infected with DWV can be disorientated since they lack proper neuronal functioning; thus, there is a possibility that bees may be embarking on foraging trips but never returning to their hives. Since bees are highly interdependent on each other for normal functioning of the colony (Seeley 1997), one can speculate that losing foragers can have a devastating effect on the colony because it leads to a decrease in food storage. When the weather is favorable, bees can handle the lack of stored food. However, under environmentally stressful conditions honeybees cannot sustain their colony causing the entire colony to collapse and leading to CCD. Additional evidence is also available linking DWV to CCD. In a recently conducted study, it was suggested that viral infections, most likely DWV since it is a common infection in many US hives, might lead to the fragmentation of ribosomal RNA (rRNA) within cells, affecting cellular metabolic function and leading to CCD (Johnson et al. 2009).

Viral infections leading to behavioral modifications have been identified among humans and other primates too. There are two known viruses that infect humans and

alter normal behavior. Borna disease is caused by the negative RNA borna disease virus (BDV) and is known to infect a wide range of warm blooded animals from birds to primates to humans and cause behavioral disturbances, which are reminiscent of some neuropsychiatric syndromes (Hatalski et al. 1997). The other virus that infects humans and alters behavior is the rabies virus. Rabies virus is a negative strand RNA virus that infects human neuronal cells and causes the cells to die via an apoptosis pathway (Fu and Jackson 2005). There are viruses that have the potential to infect the brains and alter behavior in other organisms. Thus, there is a good possibility that DWV replication in the brains of honeybees causes the bees to exhibit altered behavior patterns.

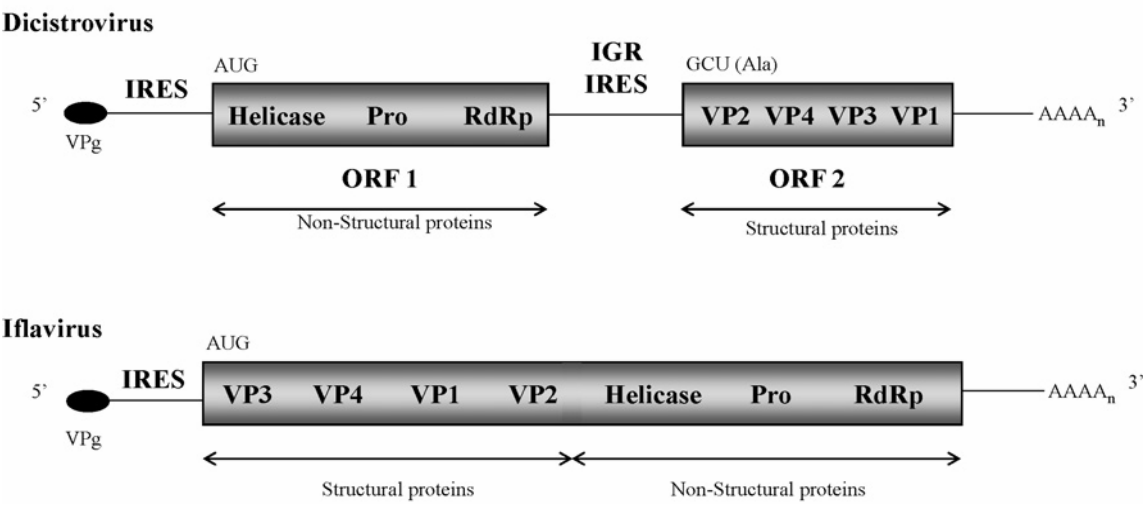
Table 1: Real Time RT-PCR used to get the number of copies of DWV genome in both symptomatic and asymptomatic honeybees

Bee Number	Hive	Date Sampled	Symptomatic	DWV copies/□g total
1	B	Jul-07	-	$<10^3$
2	D	Jul-07	-	1×10^5
3	D	Jul-07	-	5×10^6
4	D	Sep-07	-	6×10^6
5	D	Sep-07	+	4×10^7
6	D	Sep-07	+	5×10^7

The above table shows the number of copies of DWV viral RNA per □g of total RNA calculated for honeybees shown in Figures 1 and 2 using Real Time RT-PCR. The standard curve containing the DWV amplicon was used to calculate the copy number. In the Real Time RT-PCR actin was used as internal control and the Ct numbers were found to be within 2.0 of each other.

Figure 1: Two groups of Honeybee viruses divided based on their genome structure

The figure shows the genome structure of *Iflavirus* that contains only one open reading frame and one IRES sequence located on the 5'end. *Iflaviruses*, such as DWV, KV, and SBV, have their structural protein located on the 5'end and their non-structural protein on the 3'end. The figure also shows the genome structure of *Dicistrovirus* that contains two non-overlapping open reading frames and two IRES sequences located at 5'untranslated region and the intergenic region. *Dicistroviruses*, such as ABPV, BQCV, and KBV, have their non-structural proteins located on the 5' end and their structural proteins on the 3'end.



(Figure from Roberts and Groppelli 2009)

Figure 2: Detection of the genome and anti-genome of DWV in optic regions of honeybee brains

In-situ hybridization of DWV positive bee brains with sense (B) and anti-sense probe (A, C, D). A, anti-sense probe from Bee#5, 4x. B, sense probe from Bee#5 with sense probe, 4x. C, anti-sense probe from Bee#2, 4x. D, anti-sense probe from Bee#5, 10x. The Me notion in figures (A, B, C, D) represent the crescent shaped medulla region of the brain.

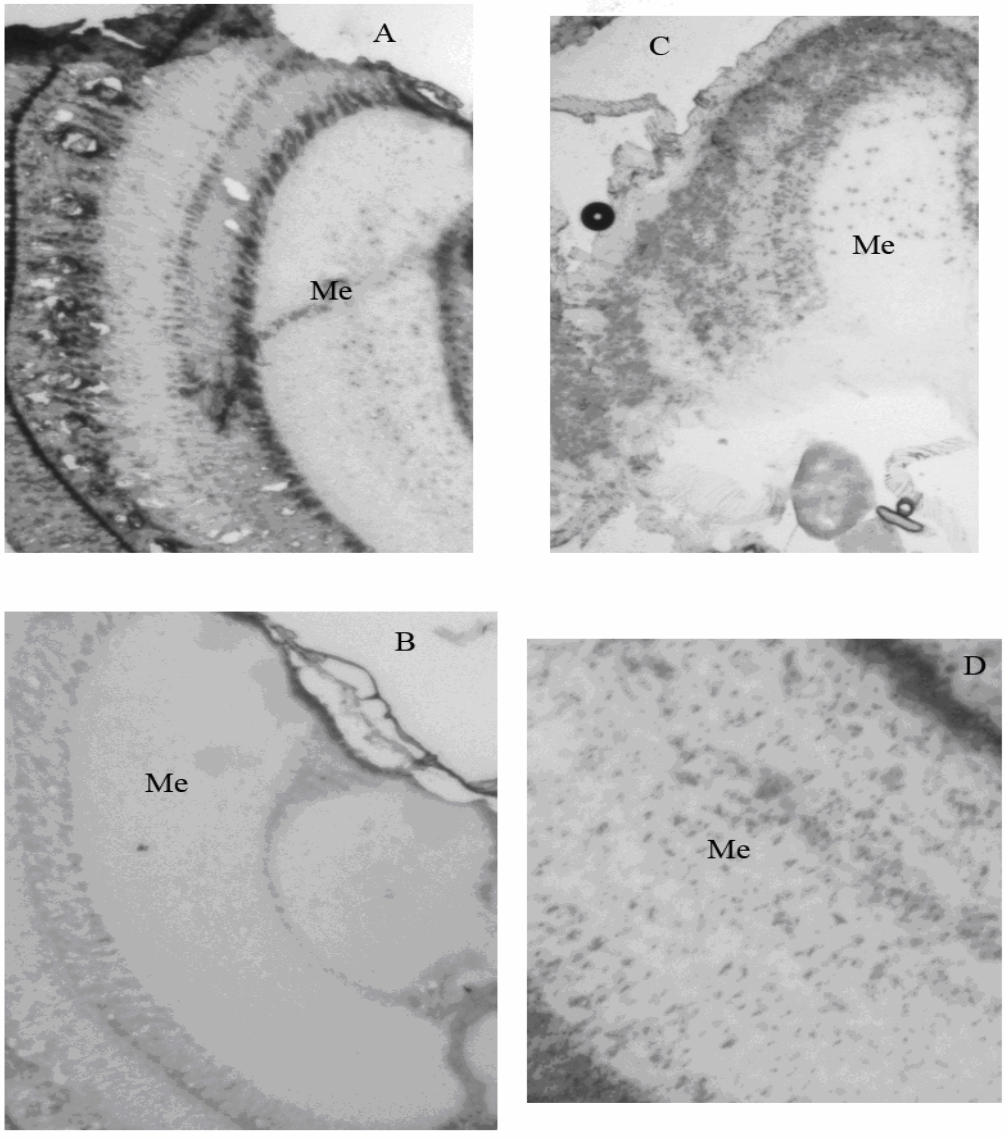


Figure 3: Detection of the genome of DWV in the subesophageal ganglion of the honeybee brains

In-situ hybridization of DWV positive bee#3 brain with anti-sense probe showing the localization of the virus in the subesophageal ganglion, 4x.

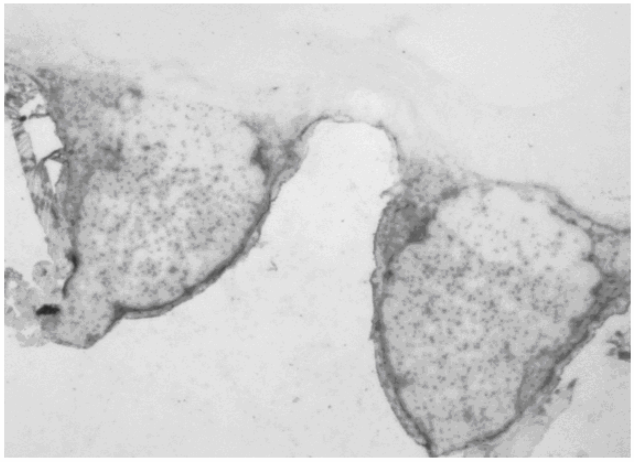
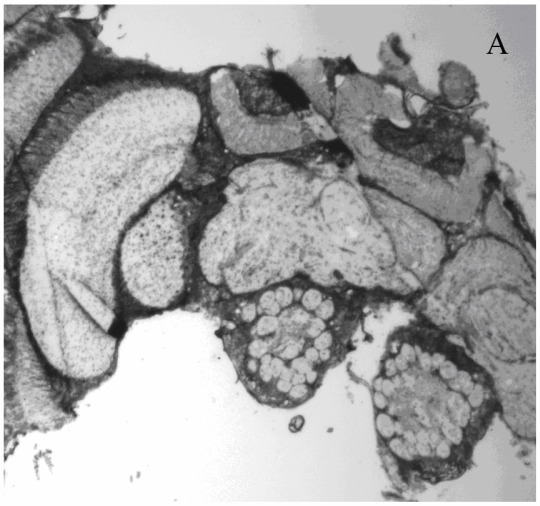
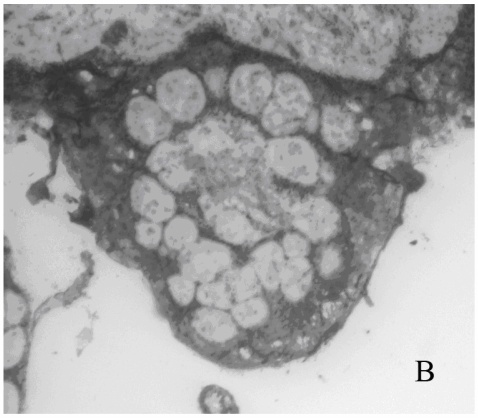


Figure 4: Detection of the genome and anti-genome of DWV in the antenna lobe of honeybee brains

In-situ hybridization of DWV bee brains with sense (C) and anti-sense (A and B). A, antenna lobe from Bee#5 with anti-sense probe, 4x. B, antenna lobe from Bee#5 with anti-sense probe of Bee#5, 10x. C, antenna lobe from Bee#5 with sense probe, 10x.





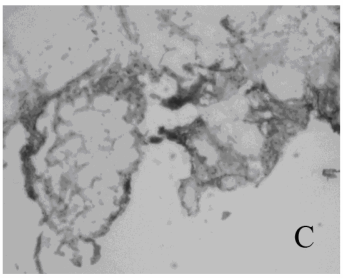
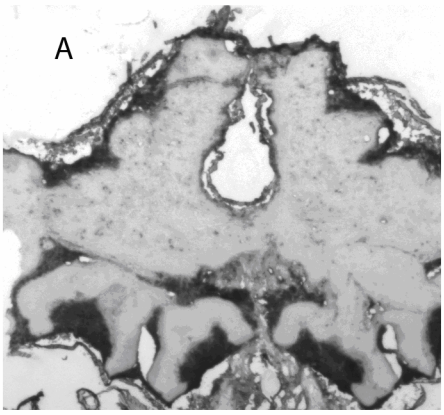
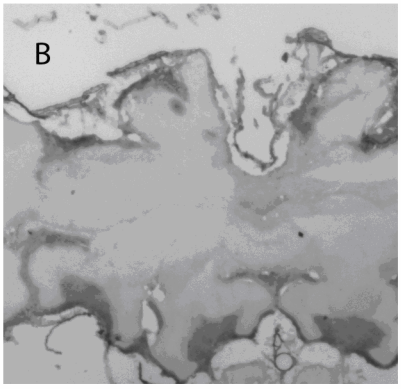
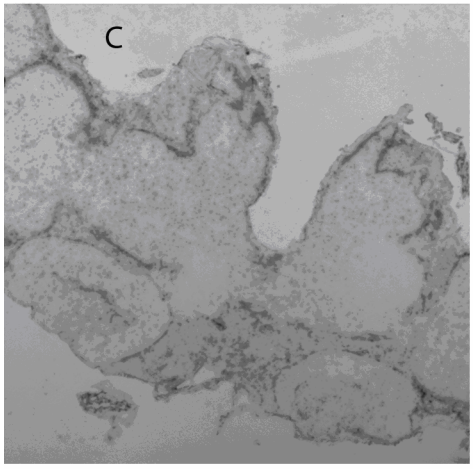


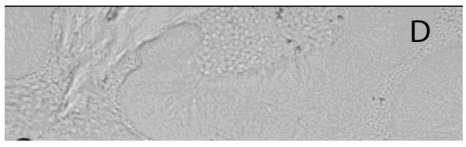
Figure 5: **Detection of the genome and antigenome of DWV in the mushroom bodies of honeybee brains**

In-situ hybridization of mushroom body with DWV positive (A, B, C) and DWV negative (D) bee brains. A, anti-sense probe from Bee#6, 4x. B, sense probe from Bee#6 with sense probe, 4x. C, anti-sense probe from Bee#2, 4x. D, anti-sense probe from Bee#1,4x.









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