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The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees

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Abstract

To determine the roles of varroa mites in activating or vectoring viral infections, we performed quantitative comparison of viral infections between bees with and without mites by dot blot analysis and enzyme-linked immunosorbent assay (ELISA). Under natural and artificial mite infestations, bee pupae contained significantly higher levels of Kashmir bee virus (KBV) and deformed wing virus (DWV) RNAs and KBV structural proteins than mite-free pupae. Moreover, in mite-infested bee pupae, DWV had amplified to extremely high titers with viral genomic RNA being clearly visible after separation of total bee RNA in agarose gels. Linear regression analysis has shown a positive correlation between the number of mites introduced and the levels of viral RNAs. The detection of viral RNAs in the nymph and adult mites underline the possible role of varroa in virus transmission. However, most groups of virus-free adult mites (9/12) were associated with bee pupae heavily infected by viruses, suggesting that the elevated viral titers in mite-infested pupae more likely resulted from activated viral replication. Based on these observations and our concurrent research demonstrating suppressed immune responses in bees infested with mites, we propose that parasitization by varroa suppresses the immunity of honey bees, leading to activation of persistent, latent viral infection.

Keywords: Honey bee; Immune response; Persistent infection; RNA viruses; Varroa mite

Introduction

The varroa mite (*Varroa destructor*, Anderson and Trueman) is considered a major pest of honey bees *Apis mellifera* (Crane, 1978). Varroa females initiate reproduction by entering the brood cells of last-stage worker or drone larvae, normally within 20–40 h before the cells are sealed (Boot et al., 1992). The mites feed upon the hemolymph of the prepupae, the pupae, and the adults. About 60 h after the bee cell is capped, the adult female mite deposits her first egg and can produce over 10 progeny (Sammataro et al., 2000). The adult female mite and progeny feed on the hemolymph of pupae from a single feeding site (Kanbar and Engels, 2003). All reproduction of varroa occurs in the brood cells, and only the adult females survive after the bee emerges. Some immature females, eggs (rarely), and males are left and removed by the nurse bees when the bee emerges. Varroa mites suck the hemolymph from adults and developing pupae of honey bees, thereby weakening the bees and shortening their life span. The rapid spread of varroa mites among bee colonies may be due to several factors, including drifting of infested bees, movement of bee swarms, and robbing of weakened colonies (de Jong, 1997). In addition, migratory beekeeping practices and the importation of infested stocks of bees have aided the rapid distribution of varroa mites through large geographical regions (Sammataro et al., 2000).

Varroa mites have been blamed for the outbreaks of so-called parasitic mite syndrome (Shimanuki and Knox, 1997), a condition with complicated and highly variable symptoms. Despite this variability, all varroa-infested colonies have an unusual presence of diseased brood, which is often infected with one or more bee viruses. Although it is

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not clear how mites kill bee colonies, a general presumption is that varroa mites have a significant influence on virus infections of bees with the possibility of mites serving as vectors or activators of the viruses (Bowen-Walker et al., 1999; Brodsgaard et al., 2000). There are several lines of evidence supporting this assumption. First, the appearance of severe viral diseases throughout the beekeeping world has coincided with the emergence in 1970s of varroa mite as a pest of honey bees. When varroa mites invaded the US in 1987, the incidence of viral infections rapidly increased, accompanied by widespread loss of bee colonies (Hung et al., 1996). Second, recent research has suggested that some of the viruses are normally relatively harmless to honey bees. In Britain where varroa mites did not occur, acute bee paralysis virus (ABPV) never caused bee mortality (Allen et al., 1986). However, the viruses can cause lethal infections in colonies infected with varroa (Hung et al., 1995, 1996; Bowen-Walker et al., 1999). Third, the increased levels of bee viruses in the case of varroa infestations led to the suggestion that varroa mites activate viral replication. For example, by comparing the prevalence of bee viruses in colonies with different levels of varroa, Ball and Allen (1988) showed that ABPV titers were significantly higher in bee samples with varroa mites. Fourth, the detection of bee viruses in varroa mites further suggests that varroa mites may actively transmit the viruses to honey bees (Hung and Hachiro, 1999; Hung et al., 2000; Ongus et al., 2004; Tentcheva et al., 2004; Shen, 2003; Shen et al., 2005). These findings led to the speculation that viruses and mites acting together had a synergistic negative effect on the honey bee colonies, which may underlie honey bee mortality and colony collapse (de Jong et al., 1982; Allen et al., 1986; Glinsky and Jarosz, 1992; Korpela et al., 1992; Brodsgaard et al., 2000).

The role of viruses in honey bee pathogenesis is of increasing concern, especially in combination with varroa mites. So far, 18 viruses have been identified from honey bees (Allen and Ball, 1996). In this study, we have focused upon three picorna-like viruses, Kashmir bee virus (KBV), deformed wing virus (DWV), and sacbrood virus (SBV). All these three viruses infect different stages of honey bees. The complete genome sequences of KBV, DWV, and SBV (9525, 10,167, and 8833 bp, respectively) have been obtained (Ghosh et al., 1999; de Miranda et al., 2004; de Miranda et al., unpublished, GenBank accession No. AY292384). DWV and SBV are similar to mammalian picornaviruses and belong to a "floating genus", *Iflavirus*, with capsid proteins being encoded in the 5′ region of the genome. KBV is a member of the genus *Cripavirus* in the family of Dicistroviridae (Liljas et al., 2002). The *Cripavirus* genomes have two major open reading frames (ORFs), with the replicase protein encoded in the 5′ region of the genome and capsid proteins encoded by the 3′-proximal ORF (de Miranda et al., 2004; Shen et al., 2005). DWV is known to infect both honey bees (*A. mellifera*) and varroa mites (*V. destructor*) (Ongus et al., 2004). KBV and SBV can be detected in mites and may also infect varroa mites (Shen et al., 2005).

Previous research revealed the association of honey bee viruses and varroa mites with reduced honey bee colony fitness, but the interactions between the varroa mites and bee viruses are poorly understood. In this report, we provide quantitative measures of the effect of varroa mites on honey bee viruses by comparing the viral titers in mite-free bees and bees naturally or experimentally infested with mites. Our results strongly support the hypothesis that varroa mites can activate the replication of KBV and DWV.

Results

Impact of natural mite infestations on honey bee viral infections

To determine if there was a positive correlation between viral titers and a mite infestation within a single colony, we chose a bee colony with a high level of mite infestation and individually sampled pupae with known numbers of varroa mites. Among the samples, 36 pupae lacked mites and 46 pupae had different numbers of varroa mites. Dot blot analysis revealed that 80.4% (37/46) of mite-infested pupae were positive for KBV (Fig. 1A). In contrast, the RNA from mite-free pupae did not hybridize to the KBV probe. Quantification of the hybridization signals also indicated that pupae with mites had significantly higher KBV viral RNA levels than pupae without mites ($P \leq 0.0001$) (Fig. 1B). Similarly, the RNA from 78% (36/46) of mite-infested pupae hybridized strongly to the DWV probe, while 15% (7/46) of the pupae hybridized weakly to the DWV probe. This was in sharp contrast to the lack of hybridization of RNA from pupae without mites to the DWV probe (Fig. 1C). Quantification of the hybridization signals showed that pupae with mites had significantly higher DWV RNA titers than pupae without mites ($P = 0.002$) (Fig. 1D). It is also interesting to note that RNA from 32/46 (70%) bee samples with mites hybridized strongly to both probes (Figs. 1A and C). Linear regression analysis showed that KBV and DWV infections were highly correlated with each other ($R^2 = 0.413$, $P < 0.0001$). However, given that the dot blot hybridizations with the two probes were performed under the same conditions, the shorter exposure time for DWV (4.5 h) versus the longer time for KBV (15 h) shown in Fig. 1 suggested that the RNA levels of DWV were much higher than those of KBV in the mite-infested bees. The hybridization also demonstrated that 10/46 (21.7%) of bee pupae had only KBV or DWV infections, indicating that not all bee pupae had coinfections of KBV and DWV. Hybridization to the SBV probe was also evident in bee pupae (data not shown). However, there was no significant difference between the groups with and without mites (ANOVA, $P = 0.94$).

In the mite-infested samples, the number of mites for each pupa varied. To test whether the number of mites was

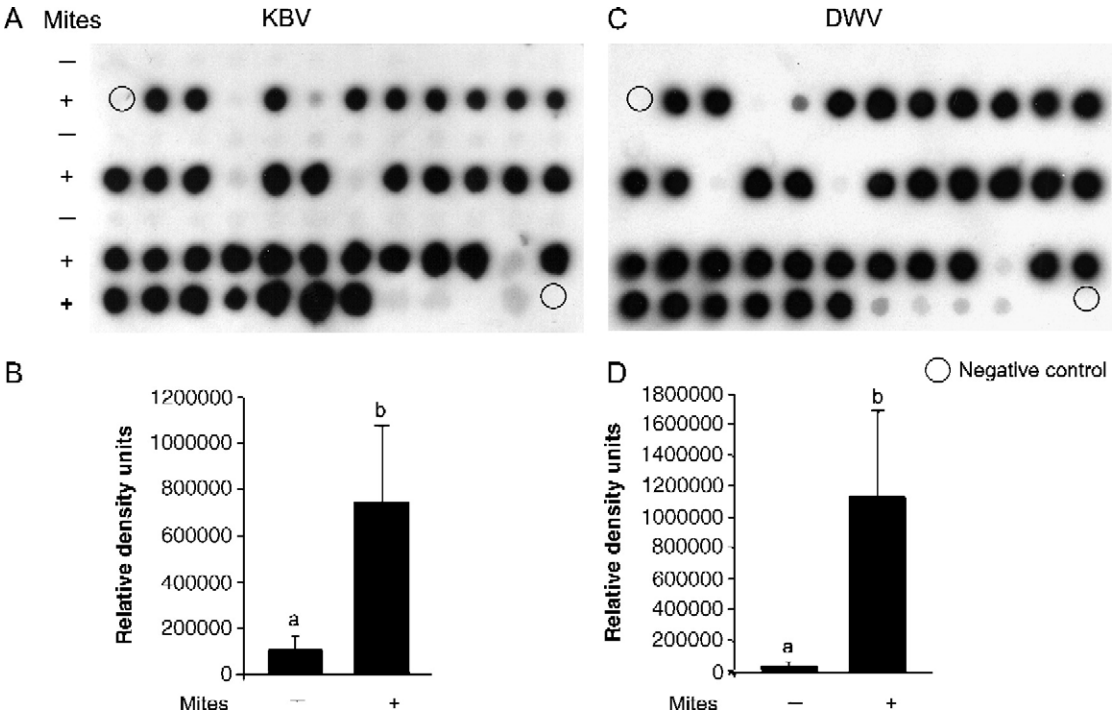

Fig. 1. Detection of KBV and DWV RNAs in pupae naturally infested with varroa mites and mite-free pupae by dot blot. Upper panel (A, C) shows the results of duplicate membranes hybridized to the KBV and DWV probes, respectively. In each membrane, 15 μg of total RNA was spotted from 82 bee pupae with (+) or without (−) mites during natural mite infestation from the same bee colony. Circles indicate negative controls (15 μg of total RNA, *H. zea*) that were used to determine background hybridization. Lower panel (B, D) indicates the quantification of the hybridization signals of the respective dot blots by densitometry. The relative densities of hybridization signals between the bee samples with mites (+) and without mites (−) were analyzed by ANOVA (StatView). The different letters indicate significance at the levels of $P < 0.0001$ (B) and $P = 0.002$ (D).

correlated with the level of viral RNAs in the bee pupae, the relative densities of the samples in the dot blots and the number of mites were analyzed with linear regression. The results demonstrated that the number of mites was highly correlated with the levels of viral RNAs of both KBV ($P < 0.0001$, $R^2 = 0.31$) and DWV ($P < 0.0001$, $R^2 = 0.42$) (Fig. 2).

We further analyzed the presence of RNAs of KBV, DWV, and SBV in the bee samples by Northern hybridization. In the ethidium-bromide-stained gel, an RNA band of about 9–10 kb was clearly visible in the RNA samples from mite-infested pupae (Fig. 3, left panel). This RNA band is consistent with the genome sizes of KBV, DWV, or SBV. When analyzed with the KBV or SBV probe, no hybridization to this RNA band was evident even after exposure for 48 h at −80 °C. However, this band hybridized strongly to the DWV probe, and the hybridization signal was clearly detectable after exposure to X-ray film for as short as 5 min (Fig. 3, right panel). This indicated that the mite-infested bee pupae had extremely high DWV RNA titers as compared to the mite-free pupae.

The presence of KBV RNA in adult and nymph mites

To further investigate the role of the varroa mites in activating and/or transmitting bee viruses, we performed RT-PCR analyses in the mite samples for the presence of KBV RNA and compared the results with those of the dot blot analyses (Table 1). Among the 19 selected bee samples, 12 samples hybridized to the KBV probe. RT-PCR detected the presence of KBV RNA in most of the nymph mite samples from these KBV dot-blot-positive pupae, whereas only 3 adult mite samples from these KBV positive pupae had PCR-detectable KBV RNA. One mite sample from KBV dot-blot-negative pupa had strong RT-PCR reactions.

Activation of endogenous viruses in bee pupae by artificially introduced mites

To demonstrate the role of varroa mites in activating bee viruses, we sampled honey bee pupae from a mite-free colony. This colony had low levels of persistent KBV and DWV infections that could only be detected by the more sensitive RT-PCR method. This colony appeared healthy without any signs of viral infection. Two groups of bees served as controls: six pupae were immediately frozen as baseline controls; and another six pupae were incubated in tubes for 5 days. The levels of KBV and DWV RNAs were not significantly different between these two groups by dot blot (ANOVA, $P > 0.99$). After artificial introduction of adult mites, significantly higher levels of KBV, DWV, and SBV RNAs were detected by dot blot analysis in pupae with mites than pupae without mites (ANOVA, $P = 0.002$) (Fig. 4). Moreover, ELISA analyses using three antibodies

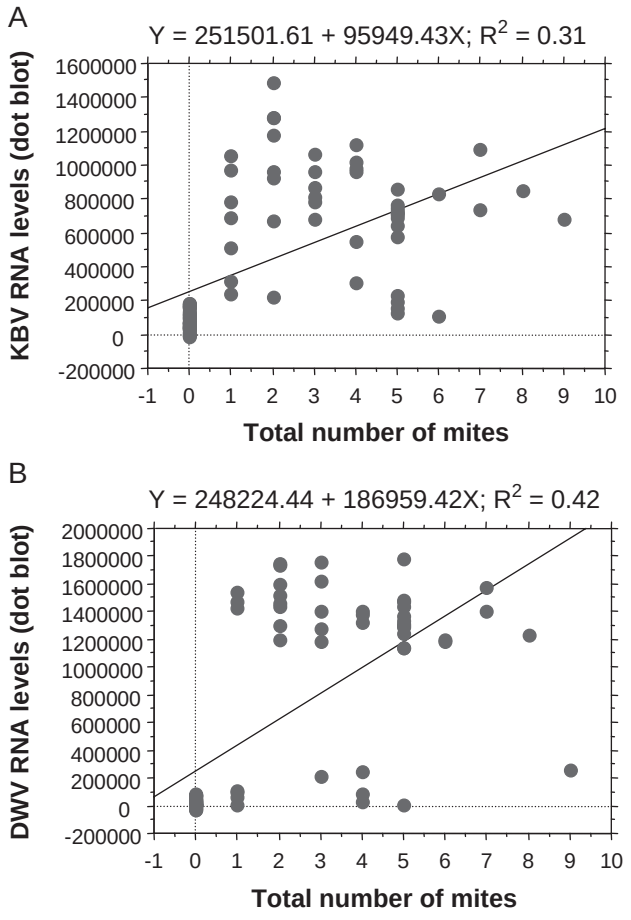

Fig. 2. The RNA levels of KBV (A) and DWV (B) within bee pupae were positively related to the number of varroa mites. Linear regression was used to analyze the dot blot data and mite density and was highly significant (ANOVA, $P < 0.0001$).

Table 1
Detection of KBV RNA in bee pupae, female adult mites, and nymphal mites

KBV in bee pupae (dot blot)	Number of mites		KBV in mites (RT-PCR)	
	Adult	Nymph	Adult	Nymph
–	2	3	–	+
–	1	0	+	
–	1	0	–	
–	1	1	+	–
–	1	0	–	
–	3	2	+	+
–	3	1	–	+
+	3	6	–	+
+	2	1	–	ND
+	1	1	–	+
+	1	1	+	+
+	2	2	–	+
+	2	1	–	+
+	1	2	–	+
+	1	2	–	+
+	2	3	+	+
+	1	1	–	+
+	1	1	–	+
+	1	3	+	+

Nineteen bee pupae with various numbers of varroa female adults and nymphs were selected and analyzed for the presence of KBV in bee pupae with dot blot and in mites by RT-PCR. ND, not determined.

against KBV structural proteins also demonstrated significantly increased titers of the KBV structural proteins in pupae incubated with varroa mites (ANOVA, $P \leq 0.04$ for anti-V4 and anti-Odt) (Fig. 5).

To corroborate the data from the natural mite infestation, we further manipulated the number of varroa mites in the artificial introduction experiment. When the levels of viral RNA and structural proteins and the number of mites were analyzed with linear regression, a strong correlation was detected between the number of mites and the titers of KBV

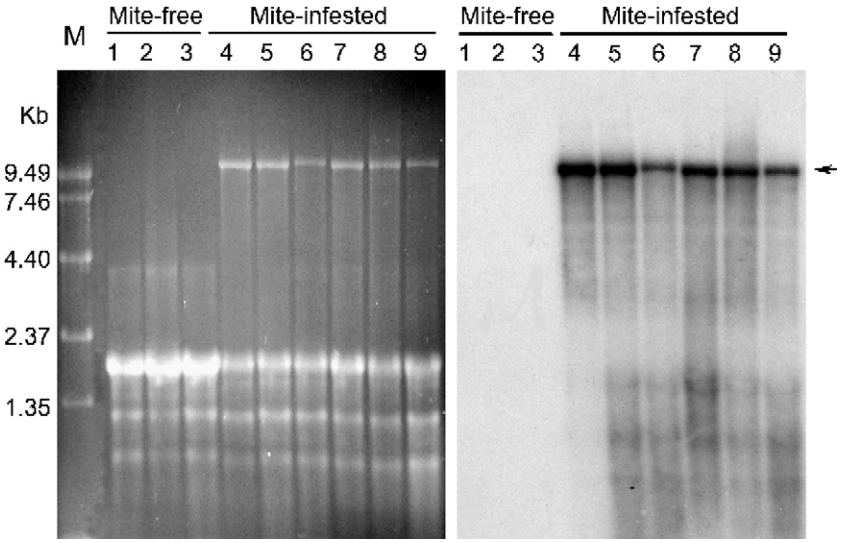

Fig. 3. Detection of DWV genomic RNA in honey bee pupae by Northern analysis. Total RNA (5 μg) from three mite-free and six mite-infested bee pupae (5 μg) was separated in a 1.0% agarose gel. Left panel shows ethidium bromide staining of the gel, and right panel shows the hybridization to the DWV probe. The film was exposed at −80 °C for 4.5 h. RNA markers (M) are in kilobases (kb). The arrow indicates the hybridizing band.

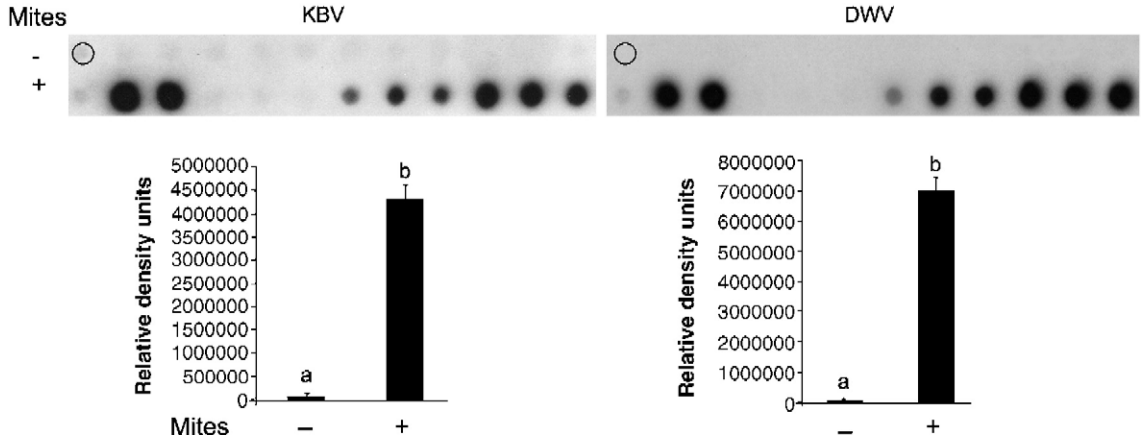

Fig. 4. Dot blot analysis of KBV and DWV RNAs in pupae without mites and pupae with artificially introduced mites. Upper panel shows the results of duplicate membranes hybridized to the KBV and DWV probes, respectively. The total RNA (15 μg) was from bee pupae without (−) mites and pupae with artificially introduced mites (+). Circles indicate negative controls (15 μg of total RNA, *H. zea*) for background. Lower panel indicates the densitometric analyses of the hybridization signals and statistical comparisons of hybridization between the bee samples with mites (+) and without mites (−) by ANOVA. The different letters indicate significance at the $P < 0.0001$ level.

(ANOVA, $P = 0.008$; $R^2 \geq 0.14$). In addition, DWV RNA levels were also positively correlated with the number of mites (ANOVA, $P = 0.0017$, $R^2 = 0.37$).

Discussion

At least 18 viruses have been identified from honey bees (Allen and Ball, 1996); many of them cause persistent infections in seemingly healthy bee colonies (Tentcheva et al., 2004). Although little is known about the dynamics of these viruses in bee colonies, severe viral diseases have frequently been encountered in bee colonies with varroa mite infestations. Using a serological test, Ball and Allen (1988) revealed that significantly higher ABPV titers were found in mite-infested honey bee colonies than uninfested colonies, suggesting that mites potentially activated the latent viruses. Further findings of honey bee viruses in mites support a putative role of mites in vectoring the viruses (Hung et al., 2000; Ongus et al., 2004; Tentcheva et al., 2004; Shen, 2003; Shen et al., 2005). These observations are consistent with the proposal that the varroa mite may transmit the viruses among bees and its feeding might induce or activate viral replication in some ways (Ball, 1983; Bailey and Ball, 1991). To further incriminate the roles of varroa mites in honey bee viral diseases, we quantitatively compared viral titers in bees with and without mites and found that significantly higher levels of viral RNAs of both KBV and DWV were associated with mite infestations. This finding was further corroborated by experiments with artificial introduction of mites to honey bee pupae.

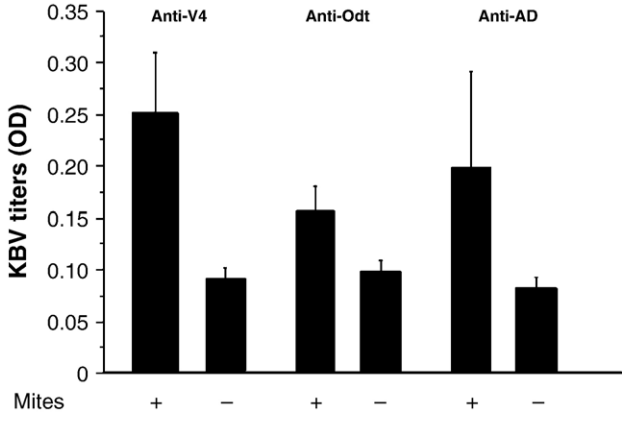

Fig. 5. KBV structural proteins in pupae without mites (−) and pupae with artificially introduced mites (+). KBV titers (optical density — OD) were determined by ELISA using three KBV antibodies (anti-V4, anti-Odt, and anti-AD).

Varroa infestation is an important factor of bee mortality and colony collapses. Sometimes, varroa kills an apparently healthy colony in a few months. However, little is known about how varroa mites kill bees. Because of its frequent co-occurrence with severe viral infections in honey bee colonies, it is considered that mites could activate latent viral infections (Allen and Ball, 1996). In supporting this assumption, we have shown that both the percentage of bee pupae with detectable RNAs for KBV and DWV and the titers of these viral RNAs were significantly higher in mite-infested samples. Moreover, by sampling adult female mites from 12 KBV heavily infected bee pupae, we could not detect KBV viral RNAs in 75% of mites by a sensitive RT-PCR method, suggesting that the higher viral titers associated with "clean" adult mites were likely the result of activation of viral replication. Although the exact mechanisms of activation are not clear, we speculate that it is at least partially due to immunosuppression in honey bees. Normally, viral infections in honey bees are probably latent, persistent, and kept under control by host

immunity. Weakening of the bees from blood loss and possible suppression of the bee immune system by mite saliva during feeding may result in active viral replication. It is well known that tick saliva contains immunosuppressive proteins that facilitate the transmission/establishment of tick-borne pathogens (Wikel, 1999). Although the biochemical components of mite saliva are not characterized, our recent work has shown that feeding by mites suppressed both the humoral and cellular immune responses of honey bees (Yang, 2004; Yang and Cox-Foster, 2005). Insect antiviral response is a well-known phenomenon, systemic inhibition of the insect immunity leads to accelerated replication of insect viruses and more severe pathology (Washburn et al., 1996, 2000). Likewise, the immunosuppressive effect of mite saliva may be responsible for the activation of latent persistent viral infections in honey bees.

We have shown that persistent viral infections occur in seemingly healthy honey bee colonies, and the viruses are probably transmitted in the colonies via multiple routes (Shen, 2003; Shen et al., 2005). Viral genomes (KBV, DWV, and SBV) have been detected in varroa mites from these colonies, which suggests that varroa may vector these viruses. In this report, we have found KBV RNA in the majority of immature mites and certain mature females from bee pupae with high levels of viral infections. However, because infection of mites by honey bee viruses has not been experimentally confirmed, it is not clear whether the viral RNA detected in mites is from direct infections of mite tissues or contained in the ingested bee hemolymph. Nor is it clear how long the viruses obtained from honey bees could persist in the mites. Yet, we have found one sample with a high level of KBV in an adult mite but no detectable viral RNA in the bee pupa, suggesting that KBV could persist in adult mites. Nonetheless, our finding of KBV in mite salivary secretions (Shen, 2003; Shen et al., 2005) further supports a role for the varroa mites in transmitting viral diseases among honey bees. Whether varroa serves as a biological or mechanical vector of honey bee viral diseases needs to be investigated.

We have demonstrated that natural infestation and artificial introduction of mites elevated viral RNA levels in honey bee pupae. It is interesting to note the 70% of the pupae naturally infested with mites were co-infected by DWV and KBV, and there was a significant association between the two viruses. Currently, it is not known if these viruses infect the same tissues or the same cells, but our results suggested the lack of obvious interference between the two viruses at the organismal level. However, given that the labeling and hybridization were carried out under identical conditions, the apparently stronger hybridization of bee RNA preparations to the DWV probe may imply higher levels of DWV replication in the bee samples. This may be related to the observations of higher DWV prevalence in honey bee colonies in recent years (Martin, 2001; Chen et al., 2005).

During our study, low levels of SBV infection have been consistently detected in the bee samples regardless of mite infestation. Dramatically different from KBV and DWV, SBV's titer was not significantly elevated by varroa infestation. This result is intriguing given that the genomes of SBV and DWV both resemble mammalian picornaviruses and they share 21–32% identities in amino acid sequences. Related to this observation are the different patterns of seasonal dynamics in the prevalence of these viruses. In several bee colonies, we detected higher SBV levels in the spring, when the levels of the other two viruses were relatively low. However, as the season progressed, SBV became rare and the incidences and virus titers of KBV and DWV were elevated (Shen, 2003; Cox-Foster et al., unpublished). Whether KBV and DWV interfere with SBV replication and cause its seasonal reduction is not clear. Varroa mites immunosuppress honey bees by depressing the expression of immunity-related genes, and DWV viral RNA titers are negatively correlated to the expression of the immunity-related enzymes (Yang and Cox-Foster, 2005). Both varroa infestation and bacterial exposure (heat-killed *Escherichia coli*) are required for DWV to replicate approximately a million-fold within 10 h (Yang and Cox-Foster, 2005), however, the same is not seen in SBV titers (Yang, 2004). Elevated titer of DWV is only found in hemocytes of mite-infested deformed-wing bees, whereas SBV is detected at approximately the same low level in hemocytes of mite-infested deformed- and normal-wing bees. This suggests that the replication or stability of SBV and DWV RNA is regulated differently. We are currently investigating the causal mechanism underlying this interesting phenomenon.

To study viral infections in honey bees, it would be ideal to utilize virus-free bees and mites and purified species of viruses. However, the ability to obtain absolutely virus-free bees or mites may be difficult. It is possible that the viral infections can persist at levels below detection by certain methods. In our bee colony used for mite introduction, KBV infection was initially not detected by ELISA, but the more sensitive PCR method revealed low-level KBV infection in the same samples (Shen et al., 2005). Furthermore, it is possible that viral infections vary greatly among bee individuals. For example, the Kakugo virus (a virus closely related to DWV with 99% sequence homology) is not detected in foraging bees but found at high levels in guard bees from the same colony (Fujiyuki et al., 2004). Even though we have found queens and colonies that appear to be virus-free for KBV, DWV, and SBV by PCR, it is still possible that one or more of the other 18 viruses reported to infect bees are present in theses colonies.

In conclusion, we have provided evidence for the activation of persistent viral infections in honey bees by the varroa mites during natural and laboratory artificial infestations. The correlation between the mite numbers and the viral titers in honey bee pupae further implicates the mites as an important stress factor. The detection of honey

bee viruses in mites and more importantly in mite saliva also indicates a role of the mites in vectoring the viruses. These intricate interactions between the mites, honey bees, and the viruses, which may determine the health status of honey bee colonies, require detailed investigations.

Materials and methods

Sampling of bee pupae with natural varroa infestation

Honey bee pupae were collected from Colony 14 in the Penn State Apiary, University Park, Pennsylvania. It was a 2-year-old colony maintained with high levels of varroa mites. It was infected by KBV, DWV, and SBV but exhibited little or no symptoms of viral infections. Brood cells were opened to collect individual pupae, and the number of mites from every pupa was recorded. Individual bee and varroa samples were kept separately in microfuge tubes. Bee pupae without varroa served as negative controls. A total of 36 mite-free pupae and 46 pupae with different levels of varroa mites were collected and stored at −80 °C for analysis of viral infections.

Artificial introduction of varroa mites to bee pupae

Twenty-four 4- to 5-day-old bee pupae were taken from Colony 205 in the Airport Apiary and confirmed to be varroa mite-free. Six pupae were randomly selected and immediately frozen at −80 °C to serve as baseline controls. Another group of six pupae was incubated in microfuge tubes with air holes at 35 °C, 50% relative humidity for 5 days. For the third group of bee pupae ($N = 12$), each pupa was incubated with 5–10 female varroa mites under the same conditions for 5 days. The female varroa mites were taken from Colony 14 in the Penn State Apiary, which was 4 miles away from the Airport Apiary.

Sample preparation for ELISA, dot blot, and reverse transcriptase-polymerase chain reaction (RT-PCR)

Each frozen bee pupa was cut laterally, with one half being used for protein extraction and the other half for RNA isolation. For ELISA, the samples were individually homogenized in phosphate-buffered saline (PBS, pH 7.6) and clarified with chloroform (Shen et al., 2005). Supernatants were kept on ice and used directly for ELISA or saved at −20 °C for future use. Total RNA was isolated from the bee samples using the Trizol Reagent (Invitrogen). RNA samples from mites were prepared similarly using either individual mites or groups of mites.

ELISA

Each well of 96-well microplates was coated with 50 μl of protein extract from bees or varroa mites in 150 μl of sodium carbonate buffer (pH 9.8) at 4 °C overnight. After rinsing each well three times with PBS-T (PBS with 0.05% Tween-20), diluted antibodies (1:2000) in PBS-T containing 2% polyvinylpropylene and 0.2% bovine serum albumin were added, and the wells were incubated at 37 °C for 3 h. After rinsing each well three times with PBS-T, the wells were incubated with horseradish-peroxidase-conjugated protein-A (Sigma) (final concentration, 0.08 μg/ml) at 37 °C for 2 h. Immobilized enzyme was detected colorimetrically by adding peroxidase substrate 3,3′,5,5′-tetramethyl benzidine (0.1 mg/ml). The endpoint absorbance was measured at 450 nm using a Spectra Max 250 microplate reader (Molecular Devices). Healthy bees negative by RT-PCR for KBV, DWV, and SBV infections were used as ELISA negative controls. Twice the average level of reaction of each antibody with healthy bees was subtracted from the absorbance of each sample to correct for the background level of reaction and to set the baseline for a positive reaction. Three KBV polyclonal antisera were used in ELISA tests. Two structural recombinant proteins of KBV (Odt and AD) were purified and used to raise polyclonal antisera (Pocono Rabbit Farm and Laboratory Inc., Canadensis, PA) (Shen, 2003; Shen et al., 2005). The antiserum against another structural protein, V_4, was produced against KBV VP_4 (Stoltz et al., 1995) and received as a gift from Dr. Don Stoltz, Dalhousie University.

RT-PCR

Primers were designed for their specificity to KBV (GenBank accession number AY275710; KBV-F: ATGACGATGATGAGTTCAAG and KBV-R: AATTGCAAGACCTGCATC), or DWV (GenBank accession number AY292384; DWV-F: ACGACACAACATCCTGTAG and DWV-R: TAAACTAGGTTGGACTGGAA), or SBV (GenBank accession number AF092924; SBV-F: CACTCAACTTACACAAAAAC and SBV-R: CATTAACTACTCTCACTTTC). Primers (actin-F: ATGAAGATCCTTACAGAAAG and actin-R: TCTTGTTTAGAGATCCACAT) were used to amplify 514 bp of the honey bee actin gene, which was used as an internal control. cDNA was synthesized using the M-MLV reverse transcriptase (Promega). PCR was carried out using a program of initial denaturing for 5 min at 94 °C and 35 cycles of 94 °C for 20 s, 50 °C for 20 s, and 72 °C for 1 min. Five microliters of the RT-PCR products were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and imaged using a Kodak digital image system. A negative control lacking template DNA and a positive cDNA control were performed for each PCR reaction.

Dot and Northern blots

Fifteen micrograms of total RNA of each bee sample were spotted on a duplicate set of nylon membranes using a dot blot apparatus. Total RNA from *Helicoverpa zea* (Lepidop-

tera: Noctuidae) was included in each membrane as a background control. For Northern analysis, 5 μg total RNA from each of the three mite-free and six mite-infested pupae was separated in a 1.0% formaldehyde gel. Gel transfer to a nylon membrane and UV-cross linking were performed using standard protocols (Sambrook et al., 1989). KBV, SBV, and DWV RT-PCR products were gel-purified and used for probe labeling with P^{32}-dATP using the Prime-a-Gene kit (Promega). The blots were hybridized separately with labeled cDNAs from KBV, SBV, and DWV. High-stringency hybridization and wash were performed using standard protocols (Sambrook et al., 1989). The membranes were exposed to X-ray films, which were analyzed with the Personal Densitometer SI (Molecular Dynamics). The relative intensity of each hybridization signal was calculated by subtracting background hybridization with the ImageQuant software.

Data analysis

StatView statistical package (SAS Institute Inc., version 5.0.1) was used to analyze the data via ANOVA, linear regression, and Fisher's PLSD.

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