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Short Communication

# *Varroa destructor* is an effective vector of Israeli acute paralysis virus in the honeybee, *Apis mellifera*

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The Israeli acute paralysis virus (IAPV) is a significant marker of honeybee colony collapse disorder (CCD). In the present work, we provide the first evidence that *Varroa destructor* is IAPV replication-competent and capable of vectoring IAPV in honeybees. The honeybees became infected with IAPV after exposure to *Varroa* mites that carried the virus. The copy number of IAPV in bees was positively correlated with the density of *Varroa* mites and time period of exposure to *Varroa* mites. Further, we showed that the mite–virus association could possibly reduce host immunity and therefore promote elevated levels of virus replication. This study defines an active role of *Varroa* mites in IAPV transmission and sheds light on the epidemiology of IAPV infection in honeybees.

Varroatosis is one of the most serious honeybee diseases caused by a parasitic mite, *Varroa destructor*. *Varroa* mites use their piercing mouthparts to suck out haemolymph from honeybees. The repeated feeding results in a decline of colony vigour, shortened life span of the honeybees and eventual perishing of colonies (Rosenkranz *et al.*, 2010). Further, the feeding of *Varroa* mites gives mites the potential to act as vectors of bee diseases, presenting additional threats to bee health. The role of *Varroa* mite as a vector in the transmission of bee viruses has been well documented (Bowen-Walker *et al.*, 1999; Chen *et al.*, 2004; Shen *et al.*, 2005). However, it has not yet been demonstrated if this mite could act as a vector of Israeli acute paralysis virus (IAPV), a virus that was tightly correlated with honeybee colony collapse disorder (CCD), a malady that has decimated honeybee colonies across the USA (Cox-Foster *et al.*, 2007; vanEngelsdorp *et al.*, 2007) and around the world (Neumann & Carreck, 2010). The present study was undertaken to determine the possible role of *Varroa* mites in the transmission of IAPV and in the promotion of its replication in honeybees. Moreover, the expression of immune-related gene transcripts, *apidaecin* and *eater* that are involved in honeybee humoral and cellular immunity (Ertürk-Hasdemir & Silverman, 2005; Evans *et al.*, 2006; Kocks *et al.*, 2005; Simone *et al.*, 2009) was measured in *Varroa*-challenged bees. *Apidaecin* is a proline-rich peptide and one of the most prominent components of the honeybee humoral defence against microbial invasion (Evans *et al.* 2006). *Eater* is a member of epidermal growth factor-family protein involved cellular immunity, as it plays an important role in phagocytosis (Ertürk-Hasdemir & Silverman, 2005; Kocks *et al.*, 2005).

Two strong bee colonies (i.e. with at least seven frames filled with capped brood and food, and covered with adult bees) and two weak colonies (i.e. with a low-adult bee population and fewer than four frames with a small patch of capped brood) that were determined to be free of IAPV infection by RT-PCR assay were selected for transmission studies. Further, the expression levels of vitellogenin, an indicator of the general health of the colonies (Amdam *et al.*, 2005; Simone *et al.*, 2009), were determined for the two selected groups. The relative transcript levels of vitellogenin in bees from strong colonies were at least threefold higher than in bees from weak colonies with a peak of $4.46 \pm 0.31$ ($t=3.084$, $P=0.0059$), which confirmed the health conditions visually determined above. *Varroa* mites that were to be used in transmission assays were collected from a colony that was heavily infested with mites; both honeybees and mites were shown to have IAPV infections by RT-PCR assay. The ubiquitous presence of deformed wing virus (Lanzi *et al.*, 2006) in bees and of *Varroa destructor* virus-1 (VDV-1) (Ongus *et al.*, 2004) in *Varroa* mites was considered as a background infection.

The transmission studies were set up following the design illustrated in Fig. 1. Briefly, newly emerged bees from both strong and weak colonies were divided into four subgroups ($n=40$) individually, based on the level of *Varroa* mites (0, 10, 20 and 30 %) they would receive in a bee rearing cup (Evans *et al.*, 2009). In addition, for both strong and weak colonies, a subgroup of ten newly emerged bees was collected prior to transmission assay and used as negative

Supplementary figures are available with the online version of this paper.

control (defined as day 0). The first half of bees, along with half of experimental *Varroa* mites, were collected from each cup at day 3 after mite infestation, and the second half of bees and *Varroa* mites, were collected at day 7. The quantification of IAPV was performed by TaqMan real-time quantitative PCR (qRT-PCR). A PCR product of 586 bp was generated from the following pair of IAPV primers (sense: 5′-GCGGAGAATATAAGGCTCAG-3′; anti-sense: 5′-CTTGCAAGATAAGAAAGGGGG-3′). A 0.2 μM TaqMan probe (5′-FAM-CGCCTGCACTGTCGTCATTA-GTTA-TAMRA-3′), and 500 ng total RNA were incorporated in the reaction mixture of Access RT-PCR system (Promega). The absolute quantification method was used for analysing the qRT-PCR data of IAPV. The copy numbers of the IAPV were determined by relating the $C_t$ value of each sample to an established standard curve. The standard curve was established by plotting the logarithm of initial quantities of 10-fold serial diluted IAPV-recombinant plasmid, ranging from $10^3$ to $10^{14}$ copies, against corresponding threshold value ($C_t$). A linear relationship was observed between $C_t$ values and input recombinant plasmid DNA (Supplementary Fig. S1, available in JGV Online). The PCR amplification efficiency was calculated as 157 % based on the slope of the standard curve (slope=−2.43), by using the formula: $E=10^{(-1/\text{slope})}-1$ (Application Note: http://www.stratagene.com/lit_items/appnotes10:pdf), confirming the validity of the assay used in this study.

The detection of negative-strand RNA of IAPV was conducted to assess whether *Varroa* mites could allow replication of IAPV. Briefly, the cDNA complementary to the negative-strand RNA of IAPV was synthesized from RNA extracted from *Varroa* mites, with Tag-IAPV-sense primer (5′-agcctgcgcacgtggGCGGAGAATATAAGGCTC-AG-3′. The sequence of Tag is shown in lower case (Yue & Genersch, 2005). To prevent amplification of non-strand-specific products, the synthesized cDNAs were column purified (Qiagen) to remove short fragments of oligonucleotides and residues of enzymic reagents (Boncristiani *et al.*, 2009). The purified cDNAs were then PCR amplified with Tag and IAPV-antisense primer. The specificity of PCR products was confirmed by sequencing analysis.

The relative quantification of *apidaecin* and *eater* transcripts was performed by SYBR Green qRT-PCR. The interpretation of qRT-PCR data was conducted using the comparative $C_t$ method ($\Delta\Delta C_t$ method) as described previously (Chen *et al.*, 2005). After the validation of approximately equal amplification efficiencies of immune transcripts and the reference gene (Supplementary Fig. S2a, b, available in JGV Online), the expression of each immune transcript was determined by normalizing the $C_t$ value of the target to the reference gene $\beta$-actin (Chen *et al.*, 2005) and relative to a calibrator, a sample representing the minimal level of the transcript. Negative controls from both healthy and weak groups (bees at day 0), showing the lowest level of gene expression and not showing significant difference between each other, were used as calibrator individually. The relative quantities of immune transcript expression in bees are expressed as an *n*-fold change relative to the calibrator following the equation $2^{-\Delta\Delta Ct}$.

Normality of data was checked by Shapiro-Wilk test, using SYSTAT software, and Chi-squared and Jarque-Bera tests, using PAST software (Palaeontological Statistic software package), while homoscedasticity was checked with Levene's test, using SYSTAT software. IAPV, *apidaecin* and *eater* data were translated as $\log_{10}$ (original data + 1) before analysis to obtain a normal distribution. A three-way factorial ANOVA was used to compare genome

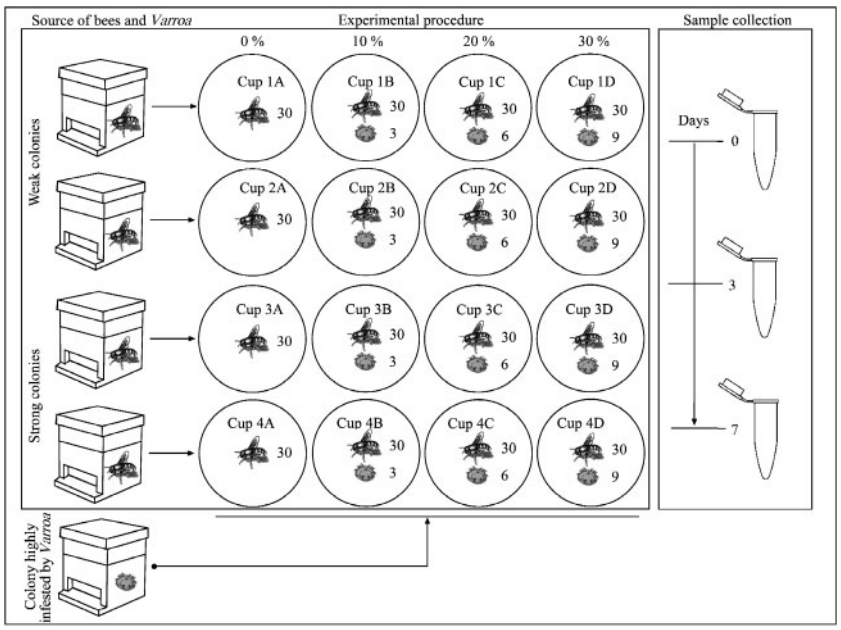


**Fig. 1.** Experimental design to assess the role of *Varroa* mites in the IAPV transmission. Bees were transferred into rearing cups and exposed to different levels of *Varroa* mites. Samples of bees and associated mites were collected at days 3 and 7 post-exposure. Bees collected prior to transmission assay (day 0) were used as negative control.

equivalents of the virus and the relative levels of immune transcripts considering three factorial levels: the colony (strong versus weak), the *Varroa* mite inoculation (0, 10, 20 and 30 %) and the day post-treatment (3 versus 7). In order to measure the strength of the linear relationship, the coefficient of correlation, $r^2$ (Zar, 2009), was calculated between the viral copy number and the per cent of *Varroa* mite, as well as between the relative expression of immune genes and the per cent of *Varroa*-mite exposure. To test the effects of *Varroa* mite inoculation level on copies of IAPV among colonies (i.e. strong and weak) and among replicates within colony, two separate analyses of covariance (ANCOVA), at days 3 and 7, were carried out, with colony as main effect and replicates nested within colony.

While all bees from negative control as well as bees not exposed to *Varroa* mites tested negative for IAPV, bees that were exposed to *Varroa* mites became IAPV positive. The copy numbers of IAPV were significantly and positively correlated with the level of *Varroa* mites introduced in bees from both strong colonies (day 3: $r^2=0.86$, $P<0.001$; day 7: $r^2=0.84$, $P<0.001$) and weak colonies (day 3: $r^2=0.98$, $P<0.001$; day 7: $r^2=0.95$, $P<0.001$). As shown in Fig. 2(a, b), the more mites introduced, the higher IAPV copy number was detected in infected bees, which also increased over time. The copy number of IAPV in bees at day 7 was significantly higher than that in bees at day 3 ($F=14.09$; d.f. 1, 32; $P<0.01$). Bees originating from strong and weak colonies showed different levels of response to the challenge of *Varroa* mites. The IAPV concentrations in bees originating from strong colonies were significantly lower (Fig. 2a) than bees from weak colonies (Fig. 2b), at the different levels of *Varroa*-mite challenge ($F=59.17$; d.f. 1, 32; $P<0.001$). While the copy number of IAPV in bees increased significantly as the load of *Varroa* mites increased ($F=320.07$; d.f. 3, 32; $P<0.001$), the magnitude of the IAPV titre change in bees from strong colonies was lower than in weak colonies. In fact, the effect of replicates was not significant, both at days 3 and 7, indicating that the relationship between copies of IAPV and levels of *Varroa* is the same for all the replicates, within each colony type. In contrast, the relationship between copies of IAPV and levels of *Varroa* were significantly different between strong and weak colonies, as indicated by significant differences of slopes ($F=18.1$; d.f.$=1$, 12; $P<0.001$ at day 7; $F=23.2$; d.f.$=1$, 12; $P<0.001$ at day 3) and intercepts ($F=14.7$; d.f.$=1$, 12; $P<0.001$ at day 7; $F=10.5$; d.f.$=1$, 12; $P<0.001$ at day 3).

While evaluation of *Varroa* mites prior to transmission assays showed that 70 % *Varroa* mites (14/20) collected from the field were positive for IAPV, 100 % of the *Varroa* mites (72/72) originating from the same colony and collected after the inoculating experiment tested positive for IAPV. Further, the strand-specific RT-PCR assay showed the presence of negative-strand IAPV RNA in all *Varroa*-mite samples examined (Fig. 2c). Like other positive-stranded RNA viruses, the replication of IAPV (Maori *et al.*, 2007) proceeds via the production of negative-stranded intermediates. Therefore, the presence of negative-strand RNA of IAPV in *Varroa* mite appears to be evidence of virus replication and suggests that the

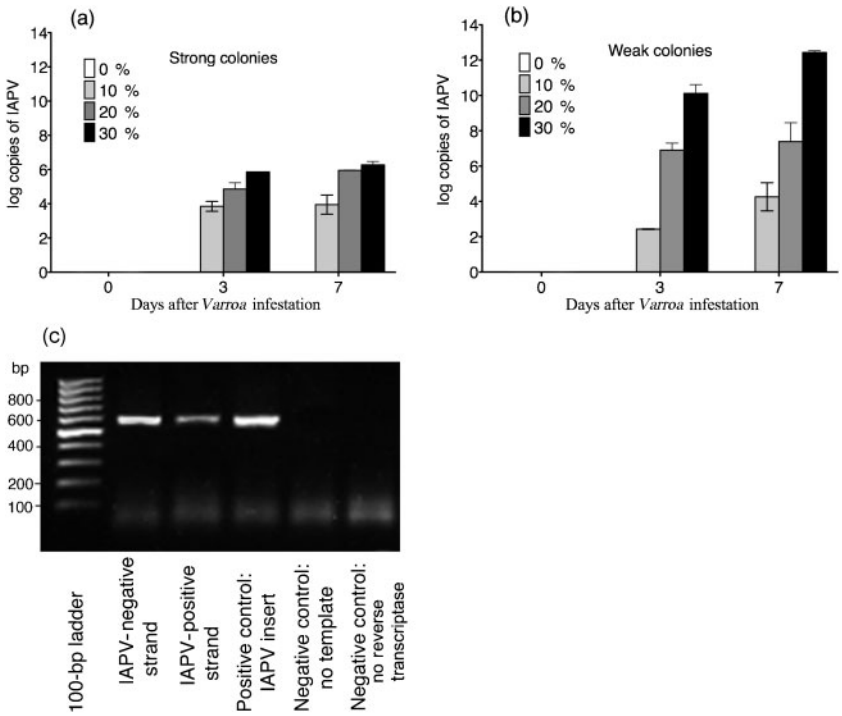


**Fig. 2.** Absolute quantification of IAPV copy number in bees at days 0, 3 and 7 post-exposure to *Varroa* mites (0, 10, 20 and 30 %). (a) Bees originating from strong colonies. (b) Bees originating from weak colonies. Bees at day 0 and without *Varroa*-mite exposure (0 %) tested negative for IAPV. (c) The strand-specific RT-PCR result of a representative *Varroa* mite, in which IAPV actively replicates.

*Varroa* mite could be a biological vector that supports the replication of the virus. Further studies to confirm this hypothesis will be necessary.

Our results also showed that *Varroa* mites not only served as a vector to transmit the virus but also impacted on the course of IAPV infection by possibly weakening the bees and reducing host immunity, and therefore leading to elevated levels of virus replication. Yang & Cox-Foster (2005) reported a suppression of the immune response by *Varroa*-mite feeding, which subsequently triggered viral replication in bees. The occurrence of immune suppression of *apidaecin* was observed in bees from weak colonies at the highest level of *Varroa* infestation. Moreover, the expression of immune transcripts was dependent upon host health backgrounds, and somehow affected by the cup-induced stress, which triggered gene transcript expression even in the absence of *Varroa*. While the correlation between the expression level of *apidaecin* and the load of *Varroa* mites in bees from strong colonies was not statistically significant ($F=2.53$; d.f. 3, 16; $P=0.09$) (Fig. 3a), the increases in level of *Varroa* exposure were accompanied by a decrease in relative levels of *apidaecin* in bees originating from weak colonies ($F=5.22$; d.f. 3, 16; $P=0.011$) (Fig. 3b). The expression of *eater* was evoked in bees by *Varroa*–IAPV association. While the correlation between the levels of *eater* and the levels of *Varroa*-mite exposure was not statistically significant ($F=2.24$; d.f. 3, 32; $P=0.10$) (Fig. 3c, d), bees originating from weak colonies had a significantly lower relative transcript abundance than those from strong colonies ($F=121.18$; d.f. 1, 32; $P<0.001$) (Fig. 3c, d). This difference may partially explain the results reported in the literature (Gregory *et al.*, 2005; Navajas *et al.*, 2008; Yang & Cox-Foster, 2005), which could have been due to overlooked health conditions of the experimental populations. Host condition is an important factor that influences the bee response to any environmental challenge and the complex cross-talk between stress and immunity. An in-depth understanding at the molecular level of the stress impact on immunomodulation of latent pathogens is of key importance for shedding light on how different environmental factors can influence honeybee population stability.

Collectively, our laboratory data show that: (i) *Varroa* mites are capable of transmitting IAPV in honeybees; (ii) there is a significant positive correlation between the rate of IAPV transmission and the density of *Varroa* mite; (iii) mite-to-mite indirect transmission is possible by co-feeding on the same bees of the virus-positive mites and virus-negative mites; (iv) *Varroa* mites are IAPV replication-competent; and (v) host-health conditions could alter outcomes of virus transmission and host–vector–virus interactions in a significant way. Based on this, we can conclude that *Varroa* mites provide a plausible route of IAPV transmission in the field, and may significantly contribute to the honeybee disease problems often associated with colony collapse.

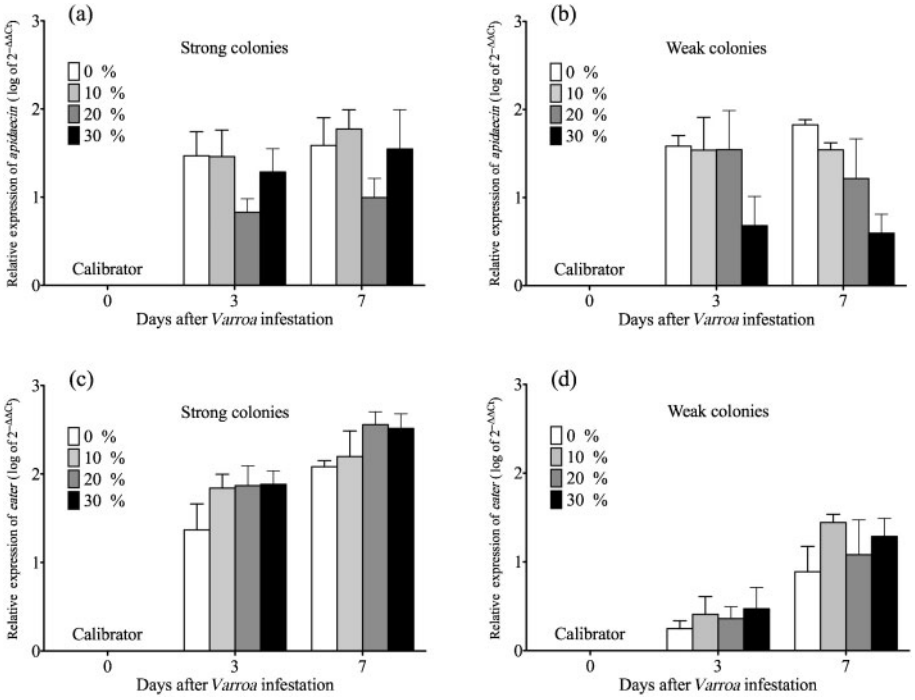


**Fig. 3.** Relative expression of *apidaecin* transcript in response to *Varroa*–IAPV association in bees originating from strong colonies (a) and from weak colonies (b). Relative expression of *eater* transcript in response to *Varroa*–IAPV association in bees originating from strong colonies (c) and from weak colonies (d).

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## References

**Amdam, G.V., Aase, A. L. T. O., Seehuus, S.-C., Kim Fondrk, M., Norberg, K. & Hartfelder, K. (2005).** Social reversal of immunosenescence in honey bee workers. *Exp Gerontol* **40**, 939–947.

**Boncristiani, H. F., Prisco, G. D., Pettis, J. S., Hamilton, M. & Chen, Y. P. (2009).** Molecular approaches to the analysis of deformed wings virus replication and pathogenesis in honey bees, *Apis mellifera*. *Virol J* **6**, 221, doi: 10.1186/1743-422X-6-221.

**Bowen-Walker, P. L., Martin, S. J. & Gunn, A. (1999).** The transmission of deformed wing virus between honey bees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni* Oud. *J Invertebr Pathol* **73**, 101–106.

**Chen, Y. P., Pettis, J. S., Evans, J. D., Kramer, M. & Feldlaufer, M. F. (2004).** Molecular evidence for transmission of Kashmir bee virus in honey bee colonies by ectoparasitic mite, *Varroa destructor*. *Apidologie (Celle)* **35**, 441–448.

**Chen, Y. P., Higgins, J. A. & Feldlaufer, M. F. (2005).** Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in honeybee. *Appl Environ Microbiol* **71**, 436–441.

**Cox-Foster, D. L., Conlan, S., Holmes, E., Palacios, G., Evans, J. D., Moran, N. A., Quan, P. L., Briese, T., Hornig, M. & other authors (2007).** A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* **318**, 283–287.

**Ertürk-Hasdemir, D. & Silverman, N. (2005).** Eater: a big bite into phagocytosis. *Cell* **123**, 190–192.

**Evans, J. D., Aronstein, K. A., Chen, Y., Hetru, C., Imler, J.-L., Jiang, H., Kanost, M., Thompson, G., Zou, Z. & Hultmark, D. (2006).** Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Mol Biol* **15**, 645–656.

**Evans, J. D., Chen, Y. P., Pettis, J. & Williams, V. (2009).** Bee cups: single-use cages for honey bee experiments. *J Apic Res* **48**, 300–302.

**Gregory, P. G., Evans, J. D., Rinderer, T. & de Guzman, L. (2005).** Conditional immune-gene suppression of honey bees parasitized by *Varroa* mites. *J Insect Sci* **5**, 7.

**Kocks, C., Cho, J. H., Nehme, N., Ulvila, J., Pearson, A. M., Meister, M., Strom, C., Conto, S. L., Hetru, C. & other authors (2005).** Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in *Drosophila*. *Cell* **123**, 335–346.

**Lanzi, G., de Miranda, J. R., Boniotti, M. B., Cameron, C. E., Lavazza, A., Capucci, L., Camazine, S. M. & Rossi, C. (2006).** Molecular and biological characterization of deformed wing virus of honey bees (*Apis mellifera* L.). *J Virol* **80**, 4998–5009.

**Maori, E., Lavi, S., Mozes-Koch, R., Gantman, Y., Peretz, Y., Edelbaum, O., Tanne, E. & Sela, I. (2007).** Isolation and characterization of Israeli acute paralysis virus, a dicistrovirus affecting honeybees in Israel: evidence for diversity due to intra- and inter-species recombination. *J Gen Virol* **88**, 3428–3438.

**Navajas, M., Migeon, A., Alaux, C., Cros-Arteil, S., Martin-Magniette, M. L., Robinson, G. E., Evans, J. D., Crauser, D. & Le Conte, Y. (2008).** Differential gene expression of the honey bee *Apis mellifera* associated with *Varroa destructor* infection. *BMC Genomics* **9**, 301.

**Neumann, P. & Carreck, N. L. (2010).** Honey bee colony losses. *J Apic Res* **49**, 1–6.

**Ongus, J. R., Peters, D., Bonmatin, J.-M., Bengsch, E., Vlak, J. M. & van Oers, M. M. (2004).** Complete sequence of a picorna-like virus of the genus *Iflavirus* replicating in the mite *Varroa destructor*. *J Gen Virol* **85**, 3747–3755.

**Rosenkranz, P., Aumeier, P. & Ziegelmann, B. (2010).** Biology and control of *Varroa destructor*. *J Invertebr Pathol* **103**, S96–S119.

**Shen, M., Yang, X. L., Cox-Foster, D. & Cui, L. W. (2005).** The role of *Varroa* mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. *Virology* **342**, 141–149.

**Simone, M., Evans, J. D. & Marla, S. (2009).** Resin collection and social immunity in honey bees. *Evolution* **63**, 3016–3022.

**vanEngelsdorp, D., Underwood, R., Caron, D. & Hayes, J., Jr (2007).** An estimate of managed colony losses in the winter of 2006 – 2007: a report commissioned by the Apiary Inspectors of America. *Am Bee J* **147**, 599–603.

**Yang, X. & Cox-Foster, D. L. (2005).** Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. *Proc Natl Acad Sci U S A* **102**, 7470–7475.

**Yue, C. & Genersch, E. (2005).** RT-PCR analysis of deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). *J Gen Virol* **86**, 3419–3424.

**Zar, J. H. (2009).** *Biostatistical Analysis*, 5th edn. London: Pearson Education International.