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IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion

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Abstract

Colony Collapse Disorder (CCD) has been associated with Israeli acute paralysis virus (IAPV). CCD poses a serious threat to apiculture and agriculture as a whole, due to the consequent inability to provide the necessary amount of bees for pollination of critical crops. Here we report on RNAi-silencing of IAPV infection by feeding bees with double-stranded RNA, as an efficient and feasible way of controlling this viral disease. The association of CCD with IAPV is discussed, as well as the potential of controlling CCD.

Keywords: IAPV, bees, CCD, silencing, ingestion.

Introduction

The importance of honeybees to the global world economy far surpasses their contribution in terms of honey production, because beehives are used for the pollination of many major fruit crops. The United States Department of Agriculture (USDA) now estimates that every third bite we consume in our diet is dependent upon honeybee pollination. In the US alone, the total contribution of pollination in terms of added value to fruit crops exceeds $15 billion per annum (Kaplan, 2007).

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Colony Collapse Disorder (CCD) poses a serious threat to apiculture and agriculture worldwide and CCD-related losses (direct and indirect) have been estimated at $75 billion (Swinton *et al*., 2007). A 2006 report on 'Fall-Dwindle Disease' (FCD) in the US described the alarming unexplained death of bees, seriously affecting apiaries in the east coast and, to a lesser extent in the west coast (vanEngelsdorp *et al*., 2006). Two years earlier, beekeepers in the northern US had already noted unique colony declines consistent with CCD, as reported in Mid-Atlantic Apiculture (www.ento.psu.edu/MAAREC/ColonyCollapseDisorderInfo.html; 2008).

CCD is characterized by rapid loss of the colony's adult bee population. At the final stages of collapse, the queen is attended by only a few newly emerged adult bees. The involvement of pathogens in CCD is supported by preliminary evidence of its transmissibility through reuse of equipment from CCD colonies, and the prevention of such transmission by irradiation (Pettis *et al*., 2007).

Israeli acute paralysis virus (IAPV) has been characterized as a bee-affecting dicistrovirus (Maori *et al*., 2007b). Recently, DNA versions of genomic segments of non-retro RNA viruses have been found in their respective host genomes, and the reciprocal exchange of genome sequences between host and virus has been demonstrated (Tanne & Sela, 2005; Maori *et al*., 2007a). In particular, IAPV and bees exchange genomic sequences, and bees carrying segments of the viral sequences are resistant to IAPV (Maori *et al*., 2007a). Most recently, a metagenomic survey has indicated a close association between CCD and IAPV (Cox-Foster *et al*., 2007).

RNA silencing down regulates gene expression by degrading RNAs in a sequence-specific manner, arresting the translation of a designated mRNA, or engendering transcriptional gene silencing involving DNA methylation and chromatin remodeling (Mathieu & Bender, 2004; Matzke *et al*., 2004; Brodersen & Voinnet, 2006; Zaratiegui *et al*., 2007). The silencing pathway involves the appearance of double-stranded (ds) RNA and its dicing by RNase III-like enzymes to small (21–26 bp) interfering (si) RNAs. The resultant siRNAs guide protein complexes to RNAs carrying homologous sequences and target them for RNA degradation, DNA methylation or chromatin remodeling. The ubiquity of silencing mechanisms has been demonstrated across kingdoms. In

plants and animals, direct application of dsRNA to tissue prior to or simultaneous with virus inoculation has been shown to confer resistance to the homologous virus (Tenllado & Diaz-Ruiz, 2001; Radhakrishnan *et al*., 2007).

Successful silencing of nematode genes by feeding dsRNA of the pertinent sequences has been reported (Timmons & Fire, 1998; Timmons *et al*., 2001). Cases of moderate silencing of insects' gene expression have also been reported (Araujo *et al*., 2006; Aronstein *et al*., 2006; Turner *et al*., 2006). The present paper reports that by feeding bees with dsRNA of IAPV sequences, IAPV infection is silenced, and bee mortality is prevented.

Results

Experiments were carried out to examine whether ingestion of dsRNAs of IAPV sequences would protect bees from subsequent IAPV infection. To that end, we synthesized, *in vitro* (from plasmid inserts bounded by opposing T7 promoters, see Experimental Procedures) dsRNAs corresponding to two different segments of the IAPV genome: a segment of the structural protein open reading frame (ORF); and a segment of the intergenic region (see Table 1 for details). The dsRNA corresponding to the structural polyprotein ORF was selected because we have previously found this sequence to be integrated into the bee genome (Maori *et al*., 2007a). The dsRNA of the intergenic region sequence was found by Cox-Foster *et al*. (2007) to be a differentiating factor in CCD-affected vs. non-affected bees. Both dsRNA sequences were compared using Basic Local Alignment Search Tool (BLAST) against the bee genome, and (as required by the US Environmental Protection Agency (EPA) did not contain any 20-bp-long dsRNA sequence identical to any bee sequence, ruling out the possibility of off-target effects.

Bees were colonized in mini-hives, 200 bees per hive, including a reproductive queen, and kept in an environmentally controlled room. Firstly, we determined the IAPV concentration needed for optimal inoculation of the experimental hives (Fig. 1). In order to determine dsRNA activity against a high dose of virus (but not too high, as it might not be relevant to natural infection), bees were fed-inoculated with 10 ng/μl of IAPV. On day seven, this dose had the same effect as all doses between 0.1 and 100 ng/μl. Then, the following treatments were applied, (each treatment was applied to three hives and the entire experiment was repeated twice). IAPV inoculum was applied: (i) without dsRNA treatment; (ii) with feeding of an unrelated dsRNA (of green fluorescence protein; (GFP); (iii) with feeding of IAPV-dsRNA. One set of control hives was left untreated (see Experimental Procedures for details). Figure 2 presents the survival rates of bees following the various treatments.

The gradual decline in bee numbers in the control hives may have been due to the experimental conditions, i.e. a small enclosed population. However, Fig. 2 clearly demonstrates protection of bees fed on IAPV-dsRNA from subsequent IAPV infection, whereas feeding of unrelated dsRNA (that of GFP) did not protect the bees from infection. The dying bees in the dsRNA-treated hives did not develop symptoms of viral infection, and their mortality rate was similar to that in the control hives. Therefore, it is unlikely that their death was a result of viral infection. Ingestion of

Table 1. List of primers used for PCR

Primers & Purpose 5′ → 3′	Amplified sequence	Product size (bp)
IAPV: RT-PCR detection		
F: AGACACCAATCACGGACCTCAC R: GAGATTGTTTGAGAGGGGTGG	8860–8997 (GenBank accession no. NC_009025)	137
Honeybee β-Actin: RT-PCR detection		
F: ATGAAGATCCTTACAGAAAG R: TCTTGTTTAGAGATCCACAT	686–1200 (GenBank accession no. XM_393368)	514
IAPV: dsRNA synthesis		
F: **TAATACGACTCACTATAGGGCGA**CCA CCCCTCTCAAACAATCTCAAACA R: **TAATACGACTCACTATAGGGCGA**TA TATCCAGTTCAAGTGTCGGTTTTC	8977–9385 (GenBank accession no. NC_009025)	408 excluding the T7 promoter sequence (bold)
IAPV: dsRNA synthesis		
F: **TAATACGACTCACTATAGGGCGA**GAC ACAATTCTTGAAATGCCAAACT R: **TAATACGACTCACTATAGGGCGA**CAT GTGTTACCATACGACTGCTGTAA	6168–6594 (GenBank accession no. NC_009025)	426 excluding the T7 promoter sequence (bold)
GFP: dsRNA synthesis		
F: **TAATACGACTCACTATAGGGCGA**GC CAACACTTGTCACTACTTTCTCTT R: **TAATACGACTCACTATAGGGCGA**AG GTAATGGTTGTCTGGTAAAAGGAC	254–685 (GenBank accession no. U87625)	431 excluding the T7 promoter sequence (bold)
Honeybee (β-Actin): real-time PCR		
F: TGCCAACACTGTCCTTTCTG R: TTGCATTCTATCTGCGATTCC	1000–1060 (GenBank accession no.: XM_393368)	61

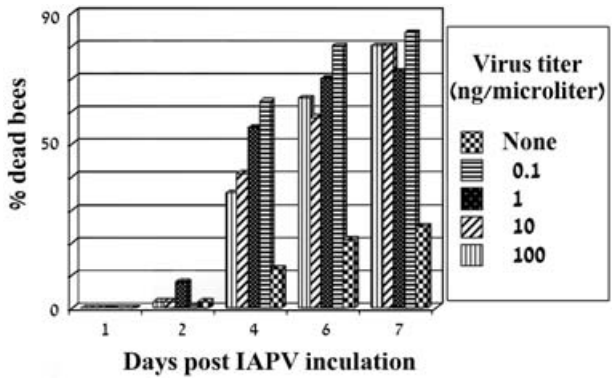

Figure 1. Titration of IAPV and the engendered bee mortality.

dsRNAs (of IAPV and GFP) without subsequent IAPV inoculation did not affect bee numbers relative to controls. In a follow-up field test, neither bee count nor honey production were affected by dsRNA treatments (data not shown), indicating non-toxicity of the dsRNA. PCR analysis (Fig. 3a; see Table 1 for primer designations) demonstrated that IAPV-dsRNA-treated bees carry only residual virus, whereas untreated, and GFP-dsRNA-treated bees carry the virus. As indicated by the internal actin controls, all samples contained comparable amounts of template RNA. The more sensitive and quantitative real-time PCR assay indicated a basal level of IAPV-RNA in some of the non-inoculated bees as well (data not shown), possibly due to transcription from integrated IAPV sequences (Maori *et al.*, 2007b). A decline in IAPV level was detected in bee populations treated with IAPV-dsRNA. On the fourth day after inoculation, the titer of IAPV, as determined by real-time qPCR, in IAPV-dsRNA-treated bees was approximately 2-log less than in bees that had not been treated with IAPV-dsRNA, or that had been treated with unrelated dsRNA (Fig. 3b). Thus, protection from IAPV-related mortality by ingestion of IAPV-dsRNA was demonstrated. It is noteworthy that, with very few exceptions, the queens and a few nursing bees survived IAPV infection, a situation reminiscent of CCD-affected hives.

Subsequent experiments were performed to verify that the ingested dsRNA had accumulated in the bees and that IAPV control was the result of RNA silencing. The presence of dsRNA in bees (RNase A- and DNase I-resistant and RNase III-sensitive, Fig. 4a) indicated successful ingestion of the dsRNA. Furthermore, the presence of IAPV-specific siRNA (21-, 22-, and 25-bp long) in bees fed IAPV-dsRNA (Fig. 4b) indicated that an RNAi-related pathway had been initiated by the ingested dsRNA, most probably leading to the afore-demonstrated silencing of IAPV.

Discussion

The presented study demonstrates that IAPV-RNA can be silenced in bees by ingestion of a segment of IAPV-dsRNA and indicates that an RNAi-related pathway of silencing leads to viral RNA degradation. The described dsRNA-engendered silencing was sufficient to greatly reduce bee mortality resulting from IAPV infection. Treatment with dsRNA may be developed to be effective in the field, protecting hives from IAPV, and possibly from CCD. Indeed, in a subsequent field experiment (James Ellis, Wayne Hunter, Nitzan Paldi, Dennis Van Engelsdorp, Eyal Maori and Ilan Sela, unpublished results) application of IAPV-specific dsRNA protected hives from collapse. Furthermore, the dsRNA-treated hives, as measured by brood counting and honey production, were stronger and more foragers returned to them. Upon completion of a series of similar field tests, a simple method for the significant control of IAPV and CCD could potentially be devised.

RNA sequences corresponding to IAPV have been found to serve as differential markers for CCD-affected vs. non-affected bees (Cox-Foster *et al.*, 2007). The IAPV-CCD association may result from IAPV infection (possibly strain-specific) and/or genome-derived transcripts of IAPV sequences. Therefore, IAPV infection, or a history of IAPV

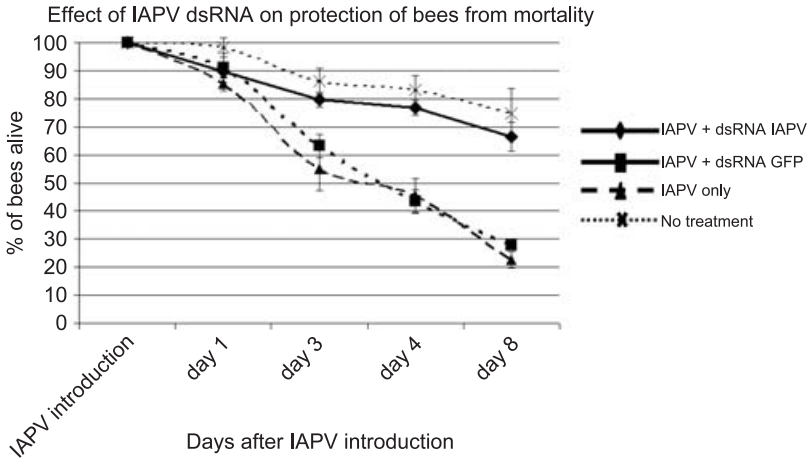

Figure 2. Bee survival following IAPV infection under different treatments. Rate of mortality differed between treatments (two-way ANOVA (www.chem.agilent.com/cag/bsp/products/gsg×/Downloads/pdf/two-way_anova.pdf). Day × Treatment: $F_{3,82} = 3.42$, $P = 0.02$). Among bees inoculated with IAPV (on day 0), mortality was reduced in those treated with IAPV-dsRNA relative to those that were not treated or that were treated with GFP-dsRNA (LS means contrast, $F_{1,82} = 9.74$, $P = 0.002$). Mortality of bees treated with IAPV-dsRNA and inoculated with IAPV tended to increase relative to the non-infected bees, though the difference did not reach statistical significance (LS means contrast, $F_{1,82} = 3.25$, NS). Data are mean (± SE) estimated percent of live bees. Statistical analyses were performed on arcsin square-root transformed proportions using JMP version 7.

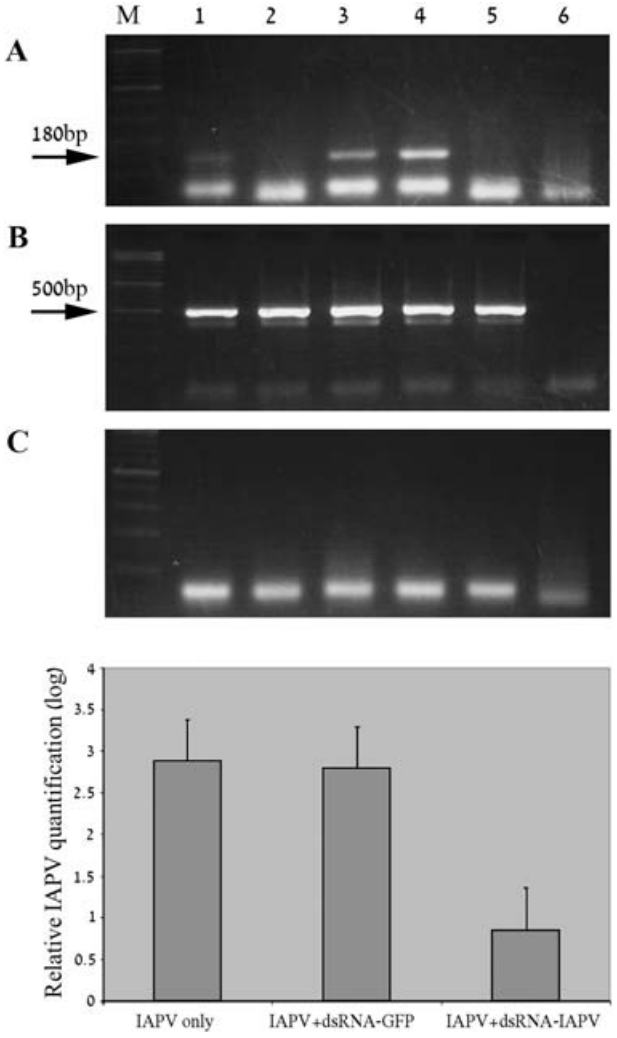

Figure 3. a. RT-PCR for the presence of IAPV. Panel A represents PCR products with primers specific to IAPV. Panel B represents PCR products with actin-specific primers (internal positive controls). Panel C demonstrates that no product was amplified in the absence of reverse transcriptase, indicating the absence of DNA in the template. Total RNA was extracted (8 days after inoculation) from the following samples. Lanes 1 & 2: from bees treated with IAPV-dsRNA followed by IAPV inoculation. Lane 3: from bees treated with GFP-dsRNA followed by IAPV inoculation. Lane 4: from IAPV-inoculated untreated bees. Lane 5: from untreated, non-inoculated bees. Lane 6: negative control without template. b. Relative virus titers (as determined by real-time PCR) 4 days after IAPV inoculation in untreated bees and in bees treated with GFP-dsRNA and IAPV-dsRNA. Data were calibrated against virus titer in non-inoculated bees.

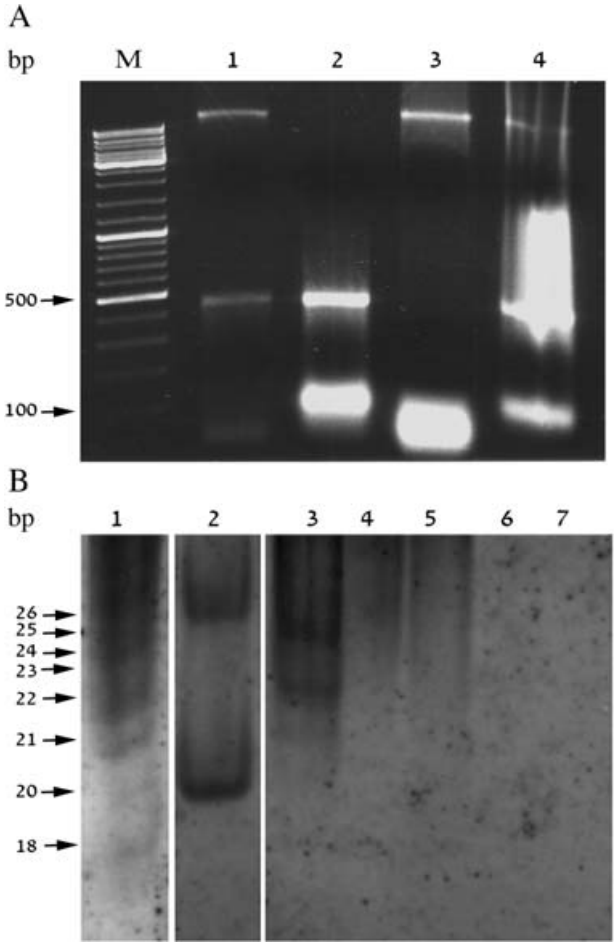

Figure 4. a. dsRNA accumulation in bees. Electrophoretic patterns of total bee RNA extracted from dsRNA-fed bees. Lane 1: total RNA after digestion with RNase A. Lane 2: total RNA after digestion with DNase I. Lane 3: total RNA after digestion with RNase A + RNase III. Lane 4: untreated extract of total RNA. b. Presence of IAPV-siRNA in bees fed on IAPV-dsRNA. The blot was reacted with a probe representing a segment of IAPV's structural protein. Lane 1: IAPV-dsRNA digested with RNase III, resulting in 18- to 26-bp fragments. Lane 2: synthetic primers for IAPV as size markers for 20- and 26-bp fragments. Lane 3: electrophoretic pattern of total RNA extracted from bees fed on IAPV-dsRNA. Lane 4: blot of total RNA extracted from bees fed on GFP-dsRNA. Lanes 5 & 6: synthetic IAPV-dsRNA and GFP-dsRNA (respectively). The ca. 400-bp dsRNA was excluded from the siRNA gel. Lane 7: total RNA from untreated bees. Arrows indicate sizes in bp.

infection, is a determinant in the development of CCD. In and of itself, however, this does not indicate that IAPV is the sole cause of CCD. In either case, IAPV resistance may prevent CCD development. Maori *et al.* (2007b) have shown that integration of segments of the IAPV genome into the bee genome brings about resistance to IAPV. The exact locus of integration may, however, determine the appearance of this phenotype: over 90% of bees inoculated in the laboratory with IAPV die, and 70 to 80% of the bees fed IAPV in our study died within a week (Fig. 1); on the other hand, a survey in Israel has indicated that about 30% of live bees carry IAPV sequences in their genome (Maori *et al.*, 2007b).

The appearance of CCD may indicate a past history of IAPV infection rather than a concurrent one. We speculate that IAPV-resistant bees, or progeny of such bees (if a queen or a drone has been affected and survived), have inherited the IAPV sequences, and are primed for CCD development by another agent, such as the varroa mite, other parasites, or environmental factors. Indeed we have found IAPV in varroa as well as viral segments in the varroa genome (E. Maori, S. Lavi, E. Tanne & I. Sela, unpublished). These agents are abundant in all colonies, regardless

of whether they are affected by CCD, and therefore have not been found as differential factors discerning CCD from non-CCD hives. In this respect, the conflicting views recently expressed by an exchange of letters in Science Magazine (Anderson & East, 2008; Cox-Foster *et al.*, 2008) questioning the IAPV-CCD association, as well as the argument that IAPV should be uncoupled from CCD due to its presence in the US prior to the appearance of CCD (Chen & Evans, 2007), may well be irrelevant.

Based on the postulation that IAPV is a causal agent or a prerequisite for CCD, protection from IAPV infection should lead to the prevention of future development of CCD. The silencing approach also degrades transcripts of IAPV sequences (as suggested by the real-time qPCR results). Therefore, the results presented in this paper could potentially be used to overcome CCD.

dsRNA absorption into the bee has been demonstrated by Aronstein *et al*. (2006) and a transmembrane protein (the bee SID-I homologue) was implicated in the mediation of dsRNA acquisition. Araujo *et al*. (2006), using dsRNA ingestion, were able to silence nitrophorin 2 in *Rhodnius prolixus* by approximately 42%. Similarly, Turner *et al*. (2006) were able to reduce carboxylesterase expression and transcript level of pheromone-binding protein in *Epiphyas postvittana* about fivefold by ingestion of the respective dsRNA. Here we report an approximately 2-log difference in the level of IAPV-RNA between control and dsRNA-ingesting bees. We cannot presently explain the high silencing efficiency which led to protection from IAPV infection in this report.

It should also be noted that by expanding the procedure described herein, we are currently developing a protocol for simultaneous protection against multiple viral infections.

Experimental procedures

IAPV titration

Bees were placed in plastic containers, 50 bees per container, and kept in the dark at 30 °C. A small dish containing 2 ml of 50% (w/v) sucrose and another dish containing 1 ml of water were placed in each container and were replaced daily. At the beginning of the experiment, various concentrations of IAPV were prepared. A virus solution (900 μl) was added to the sucrose for the first feeding. No further virus was added. The final concentrations of IAPV applied to the bees were (per μl): 100 pg, 1 ng, 10 ng, and 100 ng.

dsRNA preparation

IAPV sequences corresponding to the intergenic region (bases 6168–6594; gi|124494152; 426 b) and to a viral sequence known to integrate into the bee genome (bases 8977–9410; gi|124494152; 433 b) were used for cloning. The two sequences were PCR-amplified with IAPV-specific primers carrying a 5′ tail of the T7 promoter. Each PCR product was T/A-cloned into the plasmid pDrive. The inserts were PCR-amplified, and since they carried T7 promoters at both ends were used as templates for T7-depended in-vitro transcription, resulting in ds RNA product of the inserted sequence. Following propagation of plasmid DNA, the viral fragments, including the T7 promoters, were excised, gel-purified, and used as templates for T7-directed transcription *in vitro*. The reaction product was subjected to DNase digestion followed by phenol extraction and ethanol precipitation. The final preparation was dissolved in nuclease-free water.

Prevention of IAPV infection using IAPV-specific dsRNA

Queen bees, together with approximately 200 worker bees, were placed in mini-hives fitted with one previously constructed mini-comb. The mini-hives were closed and placed in a temperature-controlled room (30 °C). Every night, pollen-supplement patties (5 g each) were placed on top of each comb and 10 ml of 50% sucrose solution was introduced into the hive in a sterile Petri dish. This was continued for seven days and subsequently, only hives in which queens started to lay eggs were included in the experiments. Araujo *et al*. (2006) applied 13–15 μg dsRNA per insect. Bearing in mind the need to develop the concept described herein into a practical method, we reduced the amount of dsRNA to an average of 1 μg per insect. Therefore, except for control hives, 200 μg of a mixture of the two dsRNA preparations (calculated as approximately 1 μg per bee) was added to each hive daily in 10 ml of 50% sucrose solution. On the third evening, IAPV was added to the sucrose solutions to a final concentration of 10 ng/μl, and dsRNA was supplemented daily for another three days. Samples of live and dead bees (larvae and adults) were collected daily from each hive, immediately frozen in liquid nitrogen and kept at –70 °C for further analysis. One group of hives was supplemented with IAPV-specific dsRNA. Another group was supplemented with unrelated dsRNA (GFP-specific; prepared as described for IAPV-specific dsRNA). To test dsRNA toxicity, another group of hives was provided with IAPV-specific dsRNA, but was not IAPV-inoculated. Two sets of hives served as additional controls: hives that were not treated with dsRNA and were not inoculated with IAPV; and hives that were not treated with dsRNA, but were inoculated with IAPV. The hive-combs were photographed daily, and the number of remaining live bees was monitored.

Molecular procedures

Cloning, transcription, DNA and RNA preparations, PCR and RT-PCR were carried out according to published protocols (Sambrook & Russell, 2001) or protocols from the kits' manufacturers.

Northern analyses for siRNA were probed with a DIG-labeled probe (Roche Diagnostics Indianapolis, IN, USA) of a sequence corresponding to sequence used as template for the dsRNA synthesis. A commercial kit for small RNA size markers, as well as synthetic primers of IAPV-structural protein ORF served as size markers of 20 bases and 26 bases long (5′ GATTTGTCTGTCTCCCAGTG 3′ and 5′ AGATTTGTCTGTCTCCCAGTGCACAT 3′, respectively).

PCR and Real-time PCR

The RT step in RT-PCR was performed with with 'high Capacity cDNA Reverse Transcription Kit' of Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Complimentary DNA was synthesized by using random hexameres, according to the manufacturer protocol. Both IAPV and bee actin sequences were amplified from the same cDNA. Every reaction was repeated 3–5 times. In every assay we used an extract from a single bee.

Real-time quantitative (q) PCR was performed by LightCycler 480 (Roche) and was analyzed with the instrument's software. The employed primers are presented in Table 1. The employed probes

were from Roche, cat. no. 157 for bee actin and cat. no. 161 for IAPV. The real-time program was as follows: 95 °C for 10 min, followed by 45 cycles, each cycle consisting of 95 °C for 10 s and 60 °C for 30 s. At the end samples were subjected to 40 °C for 30 s.

Acknowledgments

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References

Anderson, D. and East, I.J. (2008) The latest buzz about coloney collapse disorder. *Science* **319**: 724.

Araujo, R.N., Santos, A., Pinto, F.S., Gontijo, N.F., Lehane, M.J. and Pereira, M.H. (2006) RNA interference of the salivary gland nitrophorin 2 in the triatomine bug Rhodnius prolixus (Hemiptera : Reduviidae) by dsRNA ingestion or injection. *Insect Biochem Mol Biol* **36**: 683–693.

Aronstein, K., Pankiw, T. and Saldivar, E. (2006) SID-I is implicated in systemic gene silencing in the honey bee *J Apiculture Res Bee World* **45**: 20–24.

Brodersen, P. and Voinnet, O. (2006) The diversity of RNA silencing in plants. *Trends Genet* **22**: 268–280.

Chen, Y. and Evans, J.D. (2007) Historical presence of Israeli acute paralysis virus in the United States. *Am Bee J* **147**: 1027–1028.

Cox-Foster, D., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., *et al*. (2008) Response to the latest buzz about colony collapse disorder. *Science* **319**: 725.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., *et al*. (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* **318**: 283–287.

Kaplan, K. (2007) *Genetic Survey Finds Association Between CCD and Virus*. http://www.ars.usda.gov/IS/pr/2007/070906.htm.

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

Maori, E., Tanne, E. and Sela, I. (2007b) Reciprocal sequence exchange between non-retro viruses and hosts leading to the appearance of new host phenotypes. *Virology* **362**: 342–349.

Mathieu, O. and Bender, J. (2004) RNA directed methylation. *J Cell Sci* **117**: 4881–4888.

Matzke, M., Aufsatz, W., Kanno, T., Daxinger, L., Papp, I., Mette, A.F., *et al*. (2004) Genetic analysis of RNA-mediated transcriptional gene silencing. *Biochim Biophys Acta* **1677**: 129–141.

Pettis, J., vanEngelsdorp, D. and Cox-Foster, D. (2007) Colony collapse disorder working group pathogen sub-group progress report *Am Bee J* **147**: 595–597.

Radhakrishnan, S.K., Layden, T.J. and Gartel, A.L. (2007) RNA interference as a new strategy against viral hepatitis. *Virology* **323**: 173–181.

Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Swinton, S.M., Lupi, F., Robertson, G.P. and Hamilton, S.K. (2007) Ecosystem services and agriculture: cultivating agricultural ecosystems for diverse benefits. *Ecol Econ* **64**: 245–252.

Tanne, E. and Sela, I. (2005) Occurrence of a DNA sequence of anon-retro RNA virus in the host plant genome and its expression: evidence for recombination between viral and host RNAs. *Virology* **332**: 614–622.

Tenllado, F. and Diaz-Ruiz, J.R. (2001) Double-stranded RNA-mediated interference with plant virus infection. *J Virol* **75**: 12288–12297.

Timmons, L. and Fire, A. (1998) Specific interference by ingested dsRNA. *Nature* **29**: 854.

Timmons, L., Court, D.L. and Fire, A. (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. *Gene* **263**: 103–112.

Turner, C.T., Davy, M.W., MacDiarmid, R.M., Plummer, K.M., Birch, N.P. and Newcomb, R.D. (2006) RNA interference in the light brown apple moth, Epiphyas postvittana (Walker) induced by double-stranded RNA feeding. *Insect Mol Biol* **15**: 383–391.

vanEngelsdorp, D., Cox Foster, D., Frazier, M., Ostiguy, N. and Hayes, J. (2006) 'Fall-Dwindle Disease': a preliminary report. http://www.ento.psu.edu/MAAREC/pressreleases/FallDwindleUpdate0107.pdf

Zaratiegui, M., Irvine, D. and Martienssen, R. (2007) Noncoding RNAs and gene silencing. *Cell* **128**: 763–776.