Recombinants between Deformed wing virus and Varroa destructor virus-1 may prevail in *Varroa destructor*-infested honeybee colonies.

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Abstract

We have used high-throughput Illumina sequencing to identify novel recombinants between deformed wing virus (DWV) and Varroa destructor virus-1 (VDV-1), which accumulate to higher levels than DWV in both honeybees and *Varroa destructor* mites. The recombinants, VDV-1_{VVD} and VDV-1_{DVD}, exhibit crossovers between the 5'-untranslated region (5'-UTR), and/or the regions encoding the structural (capsid) and non-structural viral proteins. This implies the genomes are modular and that each region may evolve independently, as demonstrated in human enteroviruses. Individual honeybee pupae were infected with a mixture of observed recombinants and DWV. The strong correlation between VDV-1_{DVD} levels in honeybee pupae and the associated mites was observed, suggesting that this recombinant, with a DWV-derived 5'-UTR and non-structural protein region flanking VDV-1-derived capsid encoding region, is better adapted to transmission between *V. destructor* and honeybees than the parental DWV or a recombinant bearing the VDV-1-derived 5'-UTR (VDV-1_{VVD}).

RNA recombination events and their impact on virus evolution have been well studied for the members of *Picornaviridae* family (order *Picornavirales*) infecting mammals (Simmonds, 2006), while the importance of recombination between the members of the order infecting invertebrate hosts, including the honeybee (*Apis mellifera),* remains unexplored. The honeybee viruses known to date are RNA viruses which belong either to the families *Dicistroviridae* or *Iflaviridae* , both of the order *Picornavirales*, and the, as yet, unclassified chronic bee paralysis virus (Chen & Siede, 2007). Among Iflaviruses, deformed wing virus (DWV), has been the focus of recent attention due to high prevalence and association with pathological conditions in honeybees (Lanzi *et al*., 2006; de Miranda & Genersch, 2010). DWV is closely related to kakugo virus (KV) with 96% nucleotide and 98% amino acid identity (Fujiyuki *et al*., 2004), and Varroa destructor virus-1 (VDV-1) with 84 % nucleotide (95% amino acid) identity (Ongus *et al.,* 2004). Genome organization of these viruses is typical for the genus *Iflavirus*. The 10kb positive-strand RNA genome has an extended 1.1kb 5'-proximal untranslated (5'-UTR) region containing an internal ribosome entry site (IRES) (Ongus *et al.,* 2006) is polyadenylated at the 3'-terminus and encodes a single polypeptide which is co- and/or post-translationally cleaved to release the viral structural and non-structural proteins. The N-terminal part of the polyprotein includes a postulated papain-like leader polypeptide (L protein) and the structural proteins, while the C-terminal part includes the conserved non-structural proteins with the recognizable protein motifs common to an RNA helicase, a picornavirus 3C-like protease and an RNA-dependent RNA polymerase, together with the predicted viral genome-linked protein (VPg) (Lanzi *et al*., 2006). The host range of VDV-1 includes both the honeybee and its ectoparasite, the mite *Varroa destructor*, which feeds on honeybee haemolymph.

Varroa destructor originated in Asia and arrived in the UK in the 1980's (de Miranda & Genersch, 2010). Infestation of honeybee colonies with *Varroa* results in a dramatic increase of DWV levels, coincident with appearance of honeybees with wing deformities

(Highfield *et al*., 2009). There are indications that DWV infection, rather than *Varroa* itself, causes pathology in honeybees (Gisder *et al*., 2009). It has been suggested that increase of DWV levels in *Varroa*-infested bees is mediated by suppression of the antiviral response by *Varroa*, by inoculation of DWV to honeybee haemolymph, or by combination of both mechanisms (Yang & Cox-Foster 2005). There is no agreement on whether DWV replicates in *Varroa.* The negative strands of DWV RNA were detected in *Varroa* by Ongus *et al.*, 2004, and in an independent study, which also reported high levels of DWV in honeybee pupae associated with the mites in which replication of DWV took place (Yue & Genersch, 2005). However, a recent study showed that DWV acquired by *Varroa* feeding on DWV-infected honeybees accumulated in the midgut lumen, but did not replicate (Santillán-Galicia *et al*., 2008). This apparent contradiction may result from RT-PCR screening for DWV using primers which may not differentiate between VDV-1, DWV and/or strains of these viruses. Therefore comprehensive characterization of virus diversity would be important to identify DWV and/or VDV-1 strains carrying changes which can fundamentally influence virus tropism, host range and pathogenesis.

We have used high-throughput Illumina sequencing to analyze virus diversity in honeybees from a *Varroa*-infested colony from Warwick-HRI apiary. Since DWV infection causes the most severe pathology in honeybees when insects are infested by mites at the pupal stage (Yue & Genersch, 2005), we sourced 40 capped honeybee pupae, approximately two thirds of which were *Varroa*-infested. The virus was purified using CsCl gradient centrifugation as described previously (Ryabov *et al.*, 2009). Total RNA was extracted from the virus preparation (Fig. 1b) using RNAeasy (Qiagen) and used for high-throughput sequencing and RT-PCR. A library of approximately 150 nt cDNA fragments was prepared according to the Illumina protocol and sequenced using an Illumina GAII in a 72 bp paired-end run. In total, approximately 3 x 10^7 cDNA mate-paired reads were produced. *De novo* assembled contigs were compared using BLAST with nucleotide sequences in GenBank. We

found that the contigs showed highest identity levels with either DWV or VDV-1, and somewhat lower levels of similarity to KV. No contigs with similarity to other viral sequences were found. Having identified DWV and VDV-1 sequences in the cDNA library, we competitively aligned the mate-pair reads to reference DWV and VDV-1 sequences using Bowtie (Langmead *et al*., 2009), allowing for up to three sequence changes per aligned read including one insertion/deletion. Analysis of coverage of individual aligned reads (Fig. 1a) and *de novo* assembled contigs (data not shown) to DWV and VDV-1 reference sequences showed a striking and unexpected pattern. In the 5'-proximal 900nt region (almost the complete 5'-UTR) most reads preferentially aligned to the DWV genome, with a minority of reads aligning to the VDV-1 genome (Fig. 1a). In the central region from approximately 900 to 5900nt (spanning the region encoding the L-protein and capsid proteins, flanked by short regions of the 5'-UTR and the helicase gene) sequence reads aligned in large numbers to both DWV and VDV-1 genomes. In the remainder of the genome (3' to nt. 5900, encoding the non-structural proteins and the 3'-UTR) all reads preferentially aligned to DWV, with none aligning to VDV-1. These results suggest that the sample contained recombinants between VDV-1 and DWV RNA genomes with recombination break-points within the 3'end of the 5'-UTR and the region encoding the helicase.

To precisely locate recombination break-points and identify recombinant variants, we amplified a series of cDNA fragments, both full-genome and partial (Fig. 1c), using cDNA from the viral RNA preparation and DWV- and VDV-1-specific primers designed according to our Illumina contigs and the DWV and VDV-1 sequences previously deposited in GenBank (Supplementary Table S1). Reverse transcription was carried out using Superscript II or Superscript III enzymes (Invitrogen) and cDNA fragments were amplified using proof-reading thermostable Phusion DNA polymerase (Finnzyme). RT-PCR fragments (Fig. 1c) were cloned into pTOPO-XL (Invitrogen) and sequenced using the Big Dye terminator cycle sequencing kit (Applied Biosystems).

Subsequent sequence analysis demonstrated the presence of three distinct types of genome within the tested virus population. A proportion exhibited >98% nucleotide identity with DWV throughout the sequenced region (clones HM162355, HM162356, HM1623568, HM162361; Fig. 1c), whereas the remainder were recombinants between DWV and VDV-1. From multiple independent RT-PCR reactions (Fig. 1c) we identified two distinct recombinant genomes; VDV-1_{DVD} (Genbank #HM067437) with recombination crossovers at nucleotide 946 and within the region 5787-5821 (the sequence identity between VDV-1 and DWV in the latter region prevents more accurate mapping), resulting in a genome encoding the VDV-1 structural proteins and the amino-terminal half of the helicase flanked by the DWV-derived 5'-UTR and DWV-derived sequences encoding the remainder of the helicase and other non-structural proteins, together with the 3'-UTR, hence the indication DVD for this recombinant (Fig. 1e). Similarly, the recombinant VDV-1_{VVD} exhibited a breakpoint in the helicase region, located between nucleotides 5122 and 5153, although in this recombinant all sequences 5' to this position were derived from VDV-1 with the remainder of the genome being DWV-derived (Genbank #HM067438). Notably, although the crossover junctions in the central part of these recombinant genomes were ~600 nt. apart, both occur within the most extensive region of amino-acid identity between parental VDV-1 and DWV genomes (Fig. 1d-f). Positions of the recombination points found in the genome of VDV-1_{DVD}, which was the most abundant component of the virus preparation, were in good agreement with the Illumina sequencing data. Our results suggest the presence of three 'functional' blocks in the genomes of DWV-like viruses (the 5'-UTR, the leader/capsid-coding region and the region encoding the non-structural proteins), which can be exchanged between related viruses and are likely to evolve independently. These leader/capsid and non-structural blocks, which show low ratios of non-synonymous to synonymous substitutions, 0.0288 and 0.0279 respectively, are subject to stabilizing selection, and contain no positively selected sites. Recombination is a well-established mechanism of evolution in the mammalian enteroviruses

(*Picornaviridae)* in which the modular nature of the genome, consisting of the functional domains defined by the 5'-UTR, and the structural and non-structural coding region of the genomes, is well documented (Oberste *et al.,* 2004). Typically, recombination "hotspots", associated with the emergence of novel virus variants, occur between these functional modules (Lukashev 2005; Lukashev *et al*., 2005).

In order to compare biological properties of DWV and the novel DWV-VDV-1 recombinants, we quantified the levels of these viruses in individual honeybee pupae and groups of *Varroa* mites associated with the individual pupae in Warwick-HRI apiary. The pupae, 3 to 8 days after capping, and mites (Table 1) were collected in June 2009 from a single frame of brood comb. The primers used were designed to differentiate between DWV and VDV-1 sequences in three genomic regions, the 5'-UTR, the structural- and the non-structural-coding regions (Supplementary Table S1), with quantification standards of the 5'-UTR and regions encoding the structural proteins being prepared using cloned cDNAs of DWV or VDV-1 (Genbank #HM162355, #HM067438). Oligonucleotides corresponding to nucleotides 8650-8729 of DWV RNA and to nucleotides 8623-8702 of VDV-1 RNA were used as standards for quantification of the region encoding the non-structural proteins of DWV and VDV-1 respectively. Likewise, cDNAs for the β-actin mRNA of both honeybees and *Varroa destructor* were used as quantification standards. Total RNA samples were extracted from pupae and mites, levels of DWV- or VDV-1-specific sequences corresponding to each of the three genomic regions were quantified by qRT-PCR, essentially as in Ryabov *et al*., 2009, from which the amounts of DWV, VDV-1_{VVD} and VDV-1_{DVD} in individual pupae and mite groups was calculated (Table 1). No honeybees or mites containing VDV-1-derived sequences corresponding to the non-structural region of the genome were detected, in agreement with our sequencing results. The results clearly show that individual honeybees were likely to be infected with a mixture of the recombinants and DWV, which may suggest multiple ways of transmission of DWV-like viruses. The number of honeybee pupae with

high levels of recombinants, either VDV-1_{VVD} or VDV-1_{DVD}, was significantly higher than that of pupae with high DWV levels. Interestingly, VDV-1_{DVD} was present in all pupae, DWV in 23/25 pupae (whether mite-infested or not), while VDV-1_{VVD} was present in 15/25 pupae, although it had accumulated to higher levels than DWV. Analysis of the distribution of levels of each virus (DWV, VDV-1_{VVD} and VDV-1_{DVD}) using the Anderson-Darlington test suggested that they all were normally distributed between *Varroa* mites, whereas between honeybee pupae normal distribution was observed only in the case of VDV-$1_{VVD.}$ These results suggest that in honeybees, a bimodal distribution of the normalized levels of DWV and VDV-1_{DVD} was observed, with low and high level peaks between 0.001-0.1 and 100-10000, respectively (Fig. 2a, b). These values are similar to previously reported levels of DWV normalized to levels of β-actin mRNA in symptomless honeybees and honeybees with deformed wings (Young & Cox-Foster, 2005). We speculate that the bimodal distribution of virus levels among honeybees may imply the existence of at least two types of infection patterns, resulting in either low or high levels of virus accumulation, determined either by the ability of honeybee to mount an antivirus response (which may vary due to genetic variation between pupae), by *Varroa* infestation, or by a combination of these factors.

We analyzed possible interdependence between levels of each of the three viruses in honeybee pupae and associated mites and found considerable variation between correlation coefficients (Fig. 2a). While these were relatively low for DWV (0.4780, Spearman test) and VDV-1_{VVD} (0.3224, Pearson test), the correlation coefficient for VDV-1_{DVD} was as high as 0.8088 (Spearman test). These results suggest that VDV-1_{DVD} may be more efficiently transmitted between *Varroa* mites and honeybees. There are several potential reasons why these recombinant viruses, in particular VDV-1_{DVD}, are the most prevalent component of the virus complex in bees and mites. It is possible that the presence of VDV-1-derived capsid in VDV-1_{DVD} and VDV-1_{VVD} may allow their infection of *Varroa*, thereby enhancing their spread between honeybees and *Varroa* in a colony. Therefore, VDV-1_{DVD} and VDV-1_{VVD}

could be more efficiently transmitted horizontally by *Varroa* through direct injection into the haemolymph of developing pupae, than is DWV. This infection route, together with possible immunosuppressive effect of *Varroa*, may result in high replication of VDV-1_{DVD} and VDV-1_{VVD} in bees. The apparent advantage of VDV-1_{DVD} over VDV-1_{VVD} could be explained by the presence of the DWV-derived IRES which may offer optimized or host-specific translation in the cytoplasm of honeybee cells (Ongus *at al.*, 2006). The striking absence of sequences encoding the non-structural proteins of VDV-1 suggests that these proteins may not efficiently drive virus replication in honeybee cells, in turn implying that VDV-1 structural proteins must be accompanied by DWV non-structural proteins to ensure replication of the virus in the honeybee. Alternatively, the acquisition of VDV-1 RNA sequences could aid escape from DWV-generated and DWV-specific RNA interference (RNAi) responses (van Rij & Berezikov, 2009) in honeybees as a consequences of the 17% nt. sequence divergence in the structural protein-coding region of the genome.

Results of this study show that high throughput sequencing can be efficiently used to discover novel virus recombinants in mixed virus populatiuons. We demonstrate that evolution of DWV-related viruses includes recombination of three genome 'modules' (5'-UTR, structural genes, and non-structural genes) and identified two novel recombinants, VDV-1_{DVD}, which contains DWV-derived 5'-UTR, VDV-1-derived structural genes, and DWV-derived non-structural genes, and VDV-1_{VVD}, which contains VDV-1-derived 5'-UTR and structural genes, and DWV-derived non-structural genes. We found that individual bees and mites may harbour a mixture of these recombinants and DWV, with levels of the recombinants exceeding that of DWV, and present data showing that the recombinant VDV-1_{DVD} is probably more efficiently transmitted between *Varroa* and honeybees. These results form the basis for an improved understanding of the roles of DWV, VDV-1 and recombinants thereof in the pathogenesis of deformed wing diseases of honeybees.

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Legends to figures.

Figure 1. Virus diversity in Warwick-HRI apiary. **(a)** Illumina sequencing of viral RNA. Graphs show depth of coverage at genomic loci in DWV (red) or VDV-1 (blue). **(b)** Virus preparation, electron microscopy, bar 50 nm. (c) cDNA fragments used for sequencing, **(d-f)** components of the viral complex, sequences similar to DWV and VDV-1 are in red and blue, respectively. Nucleotide sequence comparison plots generated with SipPlot1.3 (Lole e*t al*., 1999). Positions of amino acid differences between the polyproteins encoded by viruses from Warwick-HRI and reference DWV and VDV-1 genomes indicated as red dots below, or blue dots above, the genome maps, respectively. L – leader protein, VP1 to 3 – structural proteins, VPg – viral protein genomic, 3C-Prot - protease, RdRp – RNA-dependent RNA polymerase, A_n – poly A.

Figure 2. Virus accumulation in individual pupae and associated groups of mite. Virus levels normalized to the levels of the honeybee or *Varroa* β-actin mRNA in **(a)** honeybee and *Varroa* samples for pupae associated with mites and **(b)** *Varroa*-free pupae.

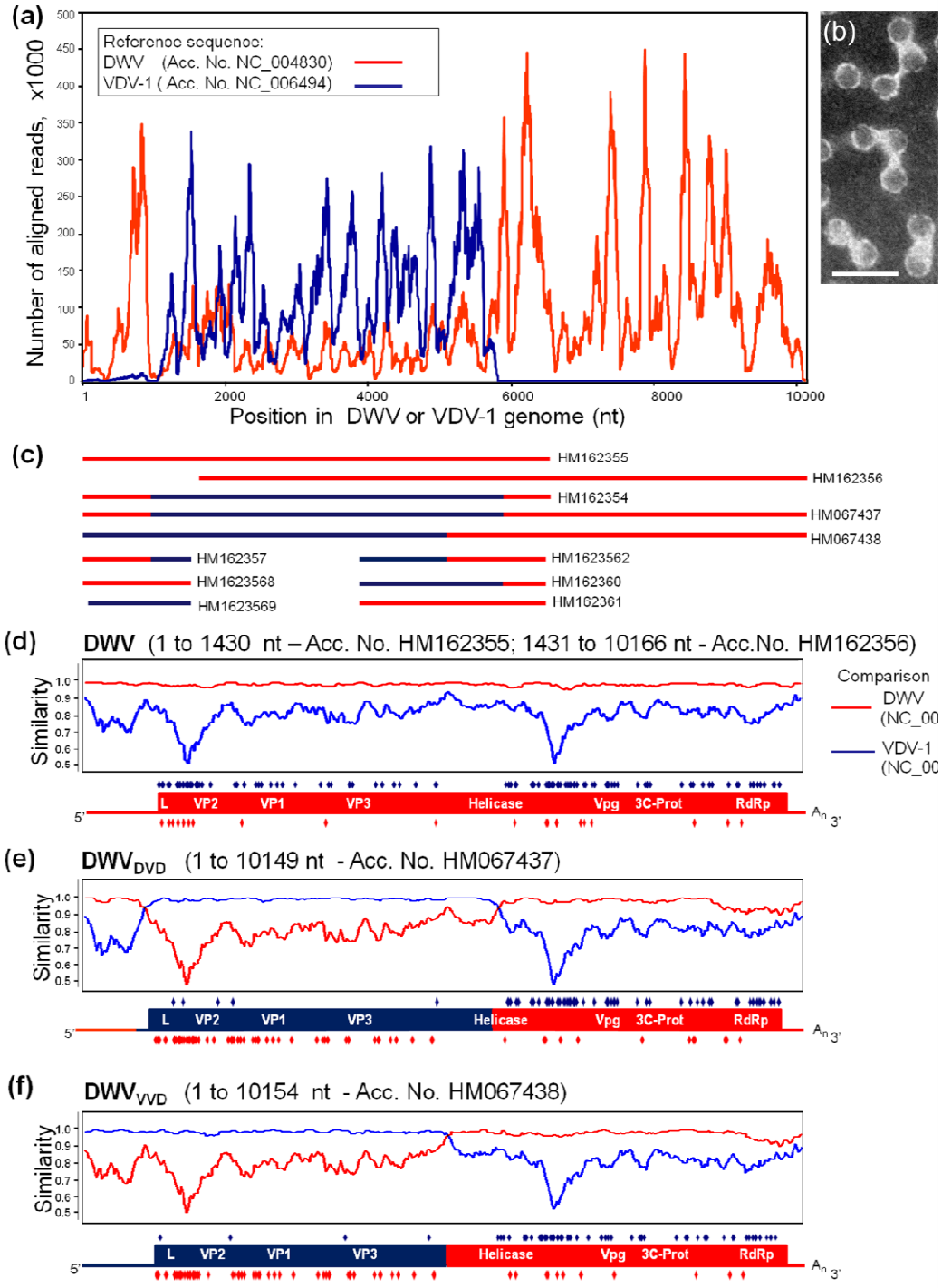
(a)
Reference sequence:
DWV (Acc. No. NC_004830)
VDV-1 (Acc. No. NC_006494)
Number of aligned reads, x1000
Position in DWV or VDV-1 genome (nt)
(b)
(c)
HM162355
HM162356
HM162354
HM067437
HM067438
HM162357
HM1623562
HM1623568
HM162360
HM1623569
HM162361
(d)
DWV (1 to 1430 nt – Acc. No. HM162355; 1431 to 10166 nt - Acc.No. HM162356)
Similarity
Comparison
DWV (NC_00
VDV-1 (NC_00
L VP2 VP1 VP3 Helicase Vpg 3C-Prot RdRp
(e)
DWVDVD (1 to 10149 nt - Acc. No. HM067437)
(f)
DWVVVD (1 to 10154 nt - Acc. No. HM067438)

Figure 1

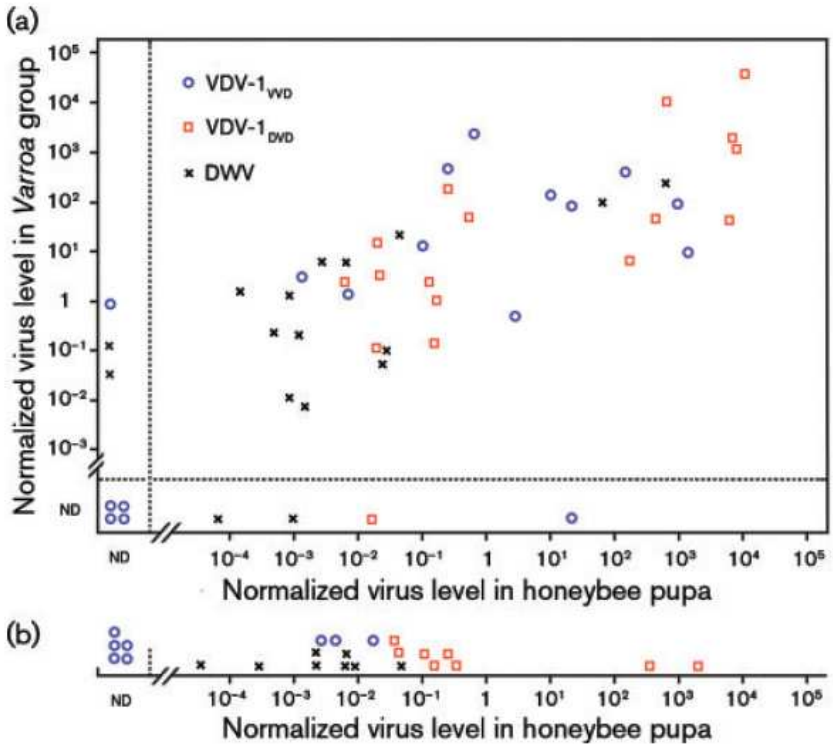
(a)
Normalized virus level in Varroa group
VDV-1VVD
VDV-1DVD
DWV
ND
Normalized virus level in honeybee pupa
(b)
ND
Normalized virus level in honeybee pupa

Figure 2

Table 1. Virus accumulation in individual pupae and associated groups of mite[*].

Bee sample *(days after capping[†])*	**VDV-1_{VVD}**	**VDV-1_{DVD}**	**DWV**	**Mite sample *(mite number in a sample)***	**VDV-1_{VVD}**	**VDV-1_{DVD}**	**DWV**
Bee-11 *(3-5)*	0.98	4.02	2.08	***Varroa*-11** *(4)*	2.14	4.55	2.39
Bee-15 *(3-5*	3.13	3.87	-3.02	***Varroa*-15** *(1)*	0.96	3.11	nd [‡]
Bee-25 *(3-5)*	-1.00	3.82	-2.59	***Varroa*-25** *(2)*	1.11	3.28	0.79
Bee-5 *(3-5*	0.45	3.78	-3.04	***Varroa*-5** *(2)*	-0.31	1.65	0.13
Bee-6 *(3-5)*	nd	3.28	-2.65	*no mites*			
Bee-14 *(3-5)*	2.98	-0.80	-1.61	***Varroa*-14** *(5)*	1.94	-0.82	-1.29
Bee-19 *(3-5*	1.33	2.84	-3.04	***Varroa*-19** *(4)*	1.92	4.00	-1.91
Bee-17 *(3-5)*	nd	2.63	-2.91	***Varroa*-17** *(2)*	nd	1.66	-0.68
Bee-1 *(3-5)*	nd	2.65	-3.40	*no mites*			
Bee-7 *(3-5)*	1.35	2.24	-1.54	***Varroa*-7** *(2)*	nd	0.84	-0.99
Bee-21 *(3-5)*	2.17	-1.71	nd	***Varroa*-21** *(1)*	2.61	-0.95	-0.88
Bee-9 *(3-5)*	nd	-0.90	1.82	***Varroa*-2** *(1)*	nd	0.41	1.98
Bee-2 *(3-5)*	-0.20	-0.24	nd	***Varroa*-2** *(1)*	3.35	1.67	-1.45
Bee-20 *(6-8)*	-2.36	-0.45	-2.65	*no mites*			
Bee-23 *(6-8)*	-0.61	-0.61	-1.36	***Varroa*-23** *(4)*	2.66	2.27	1.35
Bee-16 *(3-5)*	-2.62	-0.60	-1.99	*no mites*			
Bee-3 *(6-8)*	nd	-0.78	-2.80	***Varroa*-3** *(2)*	nd	0.03	-2.09
Bee-24 *(3-5)*	-1.73	-0.87	-4.38	*no mites*			
Bee-12 *(3-5)*	nd	-0.96	-2.16	*no mites*			
Bee-13 *(3-5)*	nd	-1.37	-2.19	*no mites*			
Bee-10 *(3-5)*	nd	-1.48	-1.31	*no mites*			
Bee-8 *(3-5)*	nd	-1.70	-2.15	***Varroa*-8** *(2)*	-0.10	1.15	0.80
Bee-18 *(3-5)*	-2.88	-1.66	-3.83	***Varroa*-18** *(1)*	0.48	0.51	0.18
Bee-4 *(3-5)*	nd	-1.78	-4.20	***Varroa*-4** *(1)*	nd	nd	nd
Bee-22 *(3-5)*	-2.15	-2.20	-3.33	***Varroa*-22** *(1)*	0.16	0.34	-0.64

[*]Virus levels were normalized to the levels of the honeybee or *Varroa* β-actin mRNA and shown as $\log_{10}$ values. [†]Pupae developmental stage was estimated according to (Jay, 1962). [‡] nd – below detection level.

Supplementary Material

Table S1. Oligonucleotides used in this study

Name	Sequence (5'-3')	Description*
712	CGATTTATGCCTTCCATAGC	PCR, DWV/VDV-1 (1-20), F
713	$(T)_{27}$ACTATTATGGTTAAAACTATACTAAAATTAGG	PCR, DWV/VDV-1 (10140-10109), R
1376	CCATGAACAAACATTATGATTA	PCR, VDV-1 (42-63), F
1335	GTACTCGCCTATATCAGTTTCG	PCR, DWV (1432-1453), F
1334	TACGAACTCACCCGCGTCTT	PCR, VDV-1 (1489-1470), R
1336	TATACATTCGCTTGCTTCTTGA	PCR, DWV (1515-1495), R
1315	GCTGAACGAGCTCGCCAG	PCR, DWV (3641-3658), F
1312	GATAGCGTCAGGGTATCGG	PCR, VDV-1 (4102-4120), F
1317	CTTGGAGCTTGAGGCTCTACA	PCR, DWV (6546-6526), R
1377	GCAAGTTGGAGATAATTGTA	qPCR, VDV-1 IRES (141-160), F
1378	CGATACTTACATTCTTCAAGAT	qPCR, VDV-1 IRES (257-236), R
1379	ACAAGTTGGAGTTCACTATC	qPCR, DWV IRES (154-173), F
1380	CTAAAGGTACATTCATACATAAG	qPCR, DWV IRES (271-249), R
1381	CTGTAGTTAAGCGGTTATTAGAA	qPCR, VDV-1 Structural (4890-4912), F
1382	GGTGCTTCTGGAACAGCGGAA	qPCR, VDV-1 Structural, (4986-4966), R
1383	CTGTAGTCAAGCGGTTACTTGAG	qPCR, DWV Structural (4917-4939), F
1384	GGAGCTTCTGGAACGGCAGGT	qPCR, DWV Structural (5013-4993), R
1425	TTCATTAAAACCGCCAGGCTCT	qPCR, VDV-1 Non-structural (8623-8644), F
1426	CAAGTTCAGGTCTCATCCCTCT	qPCR, VDV-1 Non-structural (8723-8702), R
1427	TTCATTAAAGCCACCTGGAACA	qPCR, DWV Non-structural (8650-8671), F
1428	CAAGTTCGGGACGCATTCCACG	qPCR, DWV Non-structural (8750-8729), R
1418	TGAAGGTAGTCTCATGGATAC	qPCR, *Varroa* β-actin (142-122), R
1419	GTCTCTGTTCCAGCCCTCGTTC	qPCR, *Varroa* β-actin (81-102), F
1420	AGGAATGGAAGCTTGCGGTA	qPCR, Honeybee β-actin (919-938), F
1421	AATTTTCATGGTGGATGGTGC	qPCR, Honeybee β-actin (1099-1079), R

* Oligonucleotide application, target (positions in nucleotide sequences, GenBank Accession numbers: DWV- AJ489744, VDV-1 – NC_006494, *Apis mellifera* β-actin mRNA - NM_001185146 , *Varroa destructor* β-actin mRNA - AB242568), polarity (F – Forward, R – reverse)