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Evolutionarily related Sacbrood virus and Deformed wing virus evoke different transcriptional responses in the honeybee which may facilitate horizontal or vertical transmission of these viruses

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Sacbrood virus (SBV) and deformed wing virus (DWV) are evolutionarily related positive-strand RNA viruses, members of the Iflavirus group, which infect the honeybee Apis mellifera, but have strikingly different levels of virulence when transmitted orally. Honeybee larvae orally infected with SBV usually accumulate high levels of the virus, which halts larval development and causes insect death. In contrast, oral DWV infection at the larval stage usually causes asymptomatic infection with low levels of the virus, although high doses of ingested DWV could lead to DWV replicating to high levels. We investigated effects of DWV and SBV infection on the transcriptome of honeybee larvae and pupae using global RNA-Seq and real-time PCR analysis. This showed that high levels of SBV replication resulted in down-regulation of the genes involved in cuticle and muscle development, together with changes in expression of putative immune-related genes. In particular, honeybee larvae with high levels of SBV replication, with and without high levels of DWV replication, showed concerted up-regulated expression of antimicrobial peptides (AMPs), and down-regulated expression of the prophenoloxidase activating enzyme (PPAE) together with up-regulation of the expression of a putative serpin, which could lead to the suppression of the melanisation pathway. The effects of high SBV levels on expression of these immune genes were unlikely to be a consequence of SBV-induced developmental changes, because similar effects were observed in the honeybee pupae infected by injection. We suggest that the effects of SBV infection on the honeybee immunity could be an adaptation to horizontal transmission of the virus. Up-regulation of the expression of AMP genes in the SBV-infected brood may contribute to protection of the SBV virus particles in dead larvae from bacterial degradation. Suppression of the melanisation may also reduce the loss of infectivity of SBV in the larvae. Therefore it is possible that activation of AMP expression and suppression of melanisation could increase ability of SBV to be transmitted horizontally via cannibalization route. We observed no changes of AMPs and the melanisation pathway genes expression in the orally infected larvae with high levels of DWV replication alone. In the injected pupae, high levels of DWV

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alone did not alter expression of the tested melanisation pathway genes, but resulted in up-regulation of the AMPs, which could be contributed to the effect of DWV on the regulation of AMP expression in response to wounding. We suggest that the effects of single DWV infection on the expression of these immune-related genes could reflect evolutionary adaptations of DWV to vertical transmission. Up-regulation of AMPs is costly and suppression of melanisation may increase susceptibility to infections, therefore these changes may have negative impact on honeybee survival and, consequently, of the survival of DWV.

Title:

Evolutionarily related Sacbrood virus and Deformed wing virus evoke different transcriptional responses in the honeybee which may facilitate horizontal or vertical transmission of these viruses

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Introduction

The Western honeybee, *Apis mellifera,* is the most important managed insect pollinator worldwide. In recent decades a global decline in the number of honeybee colonies was reported, threatening security of the global food supply (Vanbergen et al., 2013), with pathogens – in particular viruses – contributing significantly to these declines. These viral pathogens are predominantly single-stranded, positive sense RNA viruses of the families *Dicistroviridae* and *Iflaviridae,* and may exhibit differing virulence levels, causing infections ranging from asymptomatic to acute and resulting in rapid insect death (McMenamin & Genersch, 2015). It is also apparent that some viruses exhibit strain differences in virulence. For example, the most widespread honeybee virus, deformed wing virus (DWV) (de Miranda & Genersch, 2010; Lanzi et al., 2006) and the very closely related variants *Varroa destructor* virus-1 (Ongus et al., 2004) and Kakugo virus (*Fujiyuki et al., 2004*), usually cause asymptomatic infections with low levels of the virus when transmitted vertically or orally. In contrast, DWV transmission by the ectoparasitic mite *Varroa destructor* – by direct injection to the honeybee haemolymph – results in the selection of highly pathogenic strains of DWV with extremely limited genetic diversity (Martin et al., 2012; Ryabov et al., 2014) which accumulate to very high levels in infected pupae and cause characteristic symptoms, including deformed wings and shortened abdomen. The doses of DWV, including its virulent strains, which are delivered orally to larvae during brood rearing (or in the laboratory) cause only asymptomatic infections and accumulate to low levels, making it possible for infected honeybees to survive to adulthood and transmit the virus horizontally or vertically (Ryabov et al., 2014; Yue & Genersch, 2005). In contrast to DWV, sacbrood virus (SBV; a related member of the *Iflaviridae*) accumulates to high levels and causes acute infections in orally inoculated honeybee larvae (Bailey, Gibbs & Woods, 1964; Ghosh et al., 1999). SBV infection has a much more pronounced impact on honeybee development than DWV; honeybee larvae with high levels of the virus have a gondola-shaped sac-like appearance with tough leathery skin and die before pupation. It is likely that SBV is transmitted from the larvae killed by SBV to in-hive worker honeybees, which subsequently transmit the virus to young larvae (Bailey, 1969).

In this study, by using RNA-Seq, we analyzed global honeybee transcriptional responses to both DWV and SBV. We further analyzed the impact of DWV and SBV on the expression of several immune related genes of the honeybee by real-time PCR (qRT-PCR). We found that different sets of genes were differentially expressed (DE) in honeybee larvae with high levels of either DWV alone or SBV and DWV combined, and that high SBV infections had a more significant impact on global gene expression in the honeybee compared to high levels of DWV, in particular on the expression of immune-related genes. We found, in both larval feeding and pupal injection experiments, that high levels of SBV were associated with up-regulation of the expression of antimicrobial peptide (AMP) genes and changes in expression of the genes involved in regulation of melanisation, which may suppress this function. Different effects of DWV and SBV on expression of the AMP and melanisation pathway genes may be an adaptation of these viruses, correspondingly, to facilitate respectively vertical and horizontal routes of transmission.

Materials & Methods

Honeybee rearing, virus preparations and inoculation

Colonies of healthy Western honeybees (*Apis mellifera*) with low managed levels of *Varroa destructor* infestation were maintained in Warwickshire, UK, and used as a source of larvae and pupae. DWV virus preparation was isolated from honeybee pupae sourced from a colony with high *Varroa* infestation levels. Virus preparations containing both SBV and DWV (SBV+DWV) were purified from larvae and pupae of the *Varroa*-infested colonies where some larvae showed typical SBV-induced symptoms. Virus isolation was carried out as described previously (Moore et al., 2011) and the virus preparations stored at -80°C prior to use. For inactivation, the virus preparations were irradiated by UV light (Simonet & Gantzer, 2006).

Artificial rearing of the honeybee larvae was carried out essentially as described previously (Aronstein & Saldivar, 2005; Vandenberg & Shimanuki, 1987). For oral inoculation newly hatched honeybee worker larvae were transferred to an artificial honeybee larval diet and maintained at +33°C. After 12 hr the larvae were orally inoculated with a single dose of the virus preparation containing SBV and DWV. Approximately 10^{10} SBV and 10^{10} DWV virus particles (SBV+DWV) were added to 50 ml of the honeybee rearing diet per bee, which was consumed within 12 hr; no virus was added to the subsequent portions of the larval food. The controls in the feeding experiment included virus-free phosphate-buffered saline (PBS), and the UV-inactivated SBV+DWV virus preparation (UV-inactivated virus, SBV+DWV). The larvae were maintained for an additional 9 days up to the late fourth instar stage. Whole-body RNA samples were extracted from individual insects at 4 days post inoculation (dpi) or 9 dpi.

For the honeybee pupa infection, worker pupae sourced at the white eye stage (12th to 13th days of development) received injections into the haemolymph using a syringe with a 0.3 mm outer diameter needle (Ryabov et al., 2014) either with 10 ml of phosphate-buffered saline (PBS), or with DWV preparations (10^6 DWV virus particles in PBS), or with the mixture of SBV and DWV (10^6 SBV and 10^6 DWV virus particles in PBS). The pupae were reared at +33°C as previously described (Ryabov et al., 2014). Whole-body RNA samples were extracted from individual pupae at 2 dpi or 5 dpi.

Gene expression analysis

Total RNA was extracted from the individual experimental honeybees with Tri-reagent (Trizol) (Ambion) according to the manufacturer's instructions. The extracted column-purified total RNA from individual honeybees was used for high-throughput sequencing of the mRNA populations by RNA-Seq. The experiment and the reads were deposited into the European Nucleotide Archive under accession number PRJEB6511 (http://www.ebi.ac.uk/ena/data/view/PRJEB6511).

Quantification of viral RNA and the honeybee transcripts were carried out by quantitative reverse transcription PCR (qRT-PCR) as described previously (Ryabov et al., 2014). In brief, the RNA samples were treated with RNA-free DNAse1 (New England BioLabs), purified using RNAeasy plant mini kit (Qiagen) and used for cDNA synthesis with random hexanucleotide primers. qRT-PCR reactions were performed using SYBR Green kit (Ambion) with the primers to viral RNA and to the honeybee transcripts (Table S1).

Bioinformatics

The RNA-Seq reads were aligned using Bowtie2 (Langmead et al., 2009) (with the least stringent alignment settings to allow detection of the sequence variants, "--very-sensitive" option) to the latest honeybee transcriptome annotation (OGS3; containing 16041 putative transcripts), as well as to a set of sequences of the known fungal and viral pathogens of the honeybees used previously (Bull et al., 2012; Ryabov et al., 2014). We used samtools idxstats to produce a summary of the number of reads aligning to the honeybee transcriptome and the DWV and SBV reference sequences (GenBank accession numbers NC_004830 and AF092924 respectively). The NGS gene expression profiles were used to identify differentially expressed (DE) genes using DESeq (Anders & Huber, 2010) and edgeR (Robinson, McCarthy & Smyth, 2010), with adjusted *p*-values and a false discovery rate (FDR) below 0.05. Drosophila homologues of the honeybee genes were identified previously (Ryabov et al., 2014) and those DE in the contrasts were used for Gene Ontology (GO) analysis (Ashburner et al., 2000) using AmiGO (Carbon et al., 2009).

Results

Oral infection of honeybee larvae with DWV and SBV

Artificially reared honeybee worker larvae were orally inoculated with virus preparations containing SBV and DWV ("SBV+DWV") and controls included UV-inactivated "SBV+DWV" virus preparation and PBS (Fig. 1A). The doses of both DWV and SBV, 10^{10} genome equivalents, were sufficient to allow replication of the viruses to high levels when ingested at the larval stage (Ryabov and Evans, unpublished). Notably, this DWV dosage was 100 times higher than a dose used in oral infection of the adult bees which did not result in establishing high levels of DWV infection (Moeckel, Gisder, & Genersch, 2011). Quantification of SBV and DWV in the experimental insects assayed at 9 dpi showed that the individuals of both control groups had low levels of both DWV and SBV (Ct values 31 to 22, and 32 to 24 respectively), while among the virus-fed insects there were individuals with high levels of either DWV or SBV, as well as those with high levels of both viruses (Ct values 8 to 14, and 9 to 15).

For comprehensive characterization of honeybee gene expression in response to high levels of DWV and SBV, we used an RNA-Seq approach. The analysis was carried out using whole-body RNA extracted from individual honeybee pupae sampled at 9 dpi. Controls included pupae with low levels of DWV and SBV (samples 1 and 2) for comparison with the three virus-infected samples; one of these (sample 3) had high level of DWV and low level of SBV, and two samples had high levels of both SBV and DWV (samples 4 and 5) (Fig. 1A, Table 1).

Approximately 10 million 101 nt reads were produced for each library (Table 1) and were aligned to the latest honeybee transcriptome annotation (OGS3) and to the sequences of known fungal and viral pathogens of the honeybees used previously (Bull et al., 2012; Ryabov et al., 2014). Apart from DWV-like viruses and SBV (GenBank accession numbers NC_004830 and AF092924 respectively) no other pathogens were detected. We observed a dramatic increase of the DWV and SBV coverage, normalized to the host actin mRNA coverage (GB44311), in the infected honeybees compared to the controls (Table 1). For example, there was an ~1000-fold increase in DWV reads in the virus-infected pupae (samples 3, 4 and 5) compared to controls (samples 1 and 2), from 0.05 to about 50 in concordance with previously reported actin-normalized levels of DWV in pupae with low (0.1 DWV genomes/actin mRNA) and high (10-100 DWV genomes/actin mRNA) levels of DWV by qRT-PCR (Moore et al., 2011; Ryabov et al., 2014). SBV levels showed over 1000-fold increase in samples 4 and 5 compared to the control samples (1 and 2) and sample 3; the ratios of SBV to actin read coverage increased from 0.04 - 0.20 to 378-573 (Table 1). The observed increase of the SBV load was similar to previously reported differences between the SBV levels in asymptomatic honeybee larvae with low SBV levels and the symptomatic larvae with high SBV (Blanchard et al., 2014).

RNA-Seq analysis reveals that high levels of DWV, and SBV with DWV co-infection, evoke different transcriptional responses in orally infected honeybee larvae

We stratified the RNA-Seq samples according to the levels of DWV and SBV (high and low) into three groups, “Control” (samples 1 and 2), “DWV” (sample 3), and “SBV+DWV” (samples 4 and 5) and, by using both DESeq (Anders & Huber, 2010) and edgeR (Robinson, McCarthy & Smyth, 2010), identified differentially expressed (DE) genes in five contrasts (Fig. 2, Table S2) to assess the effect of virus infections on the host gene expression. Potential functional consequences of DE were inferred following overrepresented Gene Ontology (GO) analysis (Ashburner et al., 2000), Table S3.

The highest numbers of DE genes were identified in the contrasts involving the “SBV+DWV” group. Of these, contrast 4 (high SBV+DWV vs control) had 1638 DE genes, which included almost all (1076 of 1088) of those identified as DE in Contrast 2 (high SBV+DWV vs. control and high DWV alone). High commonality, 697 of 824 genes, was also observed between the DE genes in Contrast 5 and Contrast 4 (high SBV+DWV vs. high DWV alone and high SBV+DWV vs. control respectively (Fig. 2). The direction of gene expression change was the same (*e.g.* genes up-regulated in Contrast 4 were also up-regulated in Contrast 5).

The number of DE genes in Contrast 3 (transcriptome changes associated with high DWV levels alone) was lower than those observed in Contrasts 2, 4 and 5, all of which involved the SBV+DWV group (Fig. 2). A very low number of genes (n=4) was identified in Contrast 1 (common response to high levels of DWV alone and high levels of SBV+DWV), and the low commonality between Contrasts 3 and 4 (n=9) strongly suggested that transcriptional responses to high levels of DWV and SBV+DWV were different (Fig. 2). Indeed, GO analysis (Table 2,

Table S3) showed that different overrepresented GO terms were associated with the DE genes in Contrast 3 (high DWV levels) compared with the genes in Contrasts 2, 4, and 5 (high levels of SBV and DWV), providing further evidence that high level replication of SBV or DWV affected different biological processes in the honeybee. When compared with the low virus level control, the insects with high DWV levels (Contrast 3) showed up-regulation of the genes involved in translation, metabolic processes, and ATP metabolism (Table 2, Table S3). Changes in honeybee gene expression associated with high levels of SBV+DWV were more pronounced when compared to those associated with high DWV levels alone. The down-regulated DE genes associated with increased levels of SBV (Contrasts 2, 4, and 5) were involved in cuticle and muscle development (Table 2, Table S3), consistent with the reported phenotypic effects of SBV infection, which include halted development and abnormal cuticle (Bailey, Gibbs & Woods, 1964). Surprisingly, despite very low commonality between Contrasts 3 and 4, a considerable proportion of DE genes in Contrast 3 (68 of 223) were also DE in Contrast 5 (Fig. 2). However, the vast majority of these (67/68) exhibited virus-dependent DE in opposing directions, *i.e.* genes up-regulated in response to high levels of DWV alone were down-regulated in response to high levels of SBV, even in the presence of high levels of DWV (Table S4). The over-represented GO terms associated with these genes indicated that high levels of DWV induced increased expression of the genes involved in ATP metabolism, whereas high levels of SBV had the opposite effect on the expression of these genes, overriding the effect of DWV on their expression (Table 2, Table S4). In respect to the genes up-regulated in response to high levels of SBV we were particularly intrigued with the over-representation of GO terms associated with immune response, *e.g.* “Immune system process”, “Defense response” (Table 2, Table S3).

Differing effects of DWV and SBV on the expression of immune-related genes

Of 381 putative immune-related genes of the honeybee identified in previous studies (Evans et al., 2006; Ryabov et al., 2014), 98 were DE among the contrasts of the RNA-Seq experiment (Fig. 2, Table S5) with 74 of these genes in contrast 2 (high SBV+DWV vs. high DWV alone and control), 94 of these DE in contrast 4 (high SBV+DWV vs. control), 57 of these genes in contrast 5 (high SBV+DWV vs. high DWV alone) (Fig. 2, Table S5). Notably, there was a high commonality with 54 between the DE immune-related genes shared between the contrasts 2, 4 and 5, all converging at the high SBV+DWV group (Fig. 2, Table S3). In particular, we observed dramatic up-regulation (30- to 1000-fold) of six antimicrobial peptide (AMP) genes (Table 3, Table S5). Expression of AMPs in insects is controlled by the Toll and the Imd signaling pathways (De Gregorio et al., 2002). Notably, in honeybees abaecin (GB47318) and hymenoptaecin (GB51223) are controlled by the Imd pathway (Schluns & Crozier, 2007), while others are likely controlled by the Toll pathway (Evans et al., 2006), implying that both pathways are activated in pupae with high SBV levels. In addition, high SBV levels also influenced expression of the Toll pathway components genes, including up-regulation of PGRP-SA (GB51741), persephone (GB55007), spatzle (GB52631) and one of the Toll receptors (GB50418), and down-regulation of two Toll receptors (GB40699 and GB43456) (Table 3). We also observed changes in expression of the genes involved in regulation of the melanisation pathway *e.g.* the simultaneous down-regulation of the prophenoloxidase activating enzyme (PPAE, GB50013), the only honeybee enzyme which proteolytically cleaves prophenoloxidase (Soderhall & Cerenius, 1998) and up-regulation of two putative serpins, the negative regulators of the proteolytic event in the melanisation and signaling pathways (NEC-like proteins, GB48820 and GB54611) (Table 3). We propose that these changes in gene expression may result in suppression of the melanisation pathway.

To further explore the possible connection between the replication of DWV and SBV and the expression of the AMPs controlled by the Toll pathway (defensin-1, GB41428), or the Imd pathway (hymenoptaecin, GB51223), and the components of the melanisation pathway (putative serpin, GB48820, and prophenoloxidase activating enzyme, GB50013), we quantified gene expression levels in orally-infected larvae (Fig. 1A) by qRT-PCR. While no increase of DWV levels was observed at 4 dpi via the oral route compared to the PBS control, the SBV levels in the virus-infected group were significantly higher than in the control, PBS-exposed insects (Fig. 3A). After 9 days post inoculation, the control insects exposed to the buffer (PBS) and to the UV-inactivated virus mixture of DWV and SBV (UV-vir) showed equally low levels of SBV and DWV (Fig. 3B). These results demonstrate that *in vitro* manipulations did not activate replication of SBV and DWV that may already have been present at low levels in experimental larvae or pupae.

As before, resulting pupae that developed from larvae fed with infectious virus were stratified according to the observed SBV and DWV levels at 9 dpi (Group “hSBV” - high SBV and low DWV levels, Group “hDWV” - high DWV and low SBV levels, and Group “hSBV/hDWV” - high levels of both tested viruses) and the expression level of honeybee immune genes of interest was quantified (Fig. 3). Both AMPs, hymenoptaecin and defensin-1, were up-regulated in Group “hSBV” insects but remained at control levels in Group “hDWV” individuals (Fig. 3D, F). The level of hymenoptaecin increased, but to a lower level in Group “hDWV/hSBV” than Group “hSBV” (Fig. 3D) whereas expression of defensin-1 was similar in these groups (Fig. 3F). It is possible that hymenoptaecin expression may be directly influenced by the level of SBV (which was lower in absolute terms in Group “hSBV/hDWV” than in Group “hSBV”). Alternatively, the elevated levels of DWV in Group “hSBV/hDWV” may suppress Imd pathway activation – which controls expression of hymenoptaecin – but not the Toll pathway-controlled defensin-1. Group “hSBV” and “hSBV/hDWV” samples had elevated expression of the putative serpin and reduced expression of PPAE compared to Group “hDWV” or controls fed PBS or UV-inactivated virus preparation (Fig. 3H, 3J), implying that altered expression of these two melanisation pathway genes could be a result of elevated SBV levels (Fig. 3B). The qRT-PCR analyses were in good agreement with the RNA-Seq data (Table 3).

Injection of honeybee pupae haemolymph with DWV and SBV

The devastating developmental consequences of high levels of SBV infection on larvae (Bailey, Gibbs & Woods, 1964) may account for the changes in immune-related gene expression. We therefore investigated gene expression changes in pupae directly inoculated by injection (Fig. 1B). We observed no pupae with high virus levels in the PBS-injected control group at 2 and 5 dpi, while high levels of DWV were observed in the DWV-injected pupae, and high levels of both SBV and DWV were present in pupae injected with the SBV+DWV virus mixture (Fig. 4A, B).

At 2 dpi there was no significant difference between the expression levels of defensin-1 and serpin (Fig. 4E, G) whereas the expression levels of hymenoptaecin were significantly higher in the SBV+DWV-injected pupae compared to DWV-injected pupae (Fig. 4C). In contrast, PPAE levels were higher in DWV-injected pupae than in those that received both viruses (Fig. 4I). At 5 dpi, PPAE was significantly down-regulated in the SBV+DWV group, while the levels of PPAE in the PBS and DWV groups were not significantly different (Fig. 4J). The same effects of high levels of DWV and SBV on expression of PPAE were observed in the larval feeding experiment (Fig. 3J). Expression levels of hymenoptaecin were significantly different between the pupae injected with PBS, DWV, or SBV+DWV groups at 5 dpi, with the highest levels observed in the SBV+DWV group and lowest in the control (PBS) group (Fig. 4D). In addition, at 5 dpi defensin-1 and serpin (GB48820) were significantly up-regulated in the DWV pupae and SBV+DWV-injected pupae compared to the PBS-injected control. There were no significant differences between the pupae groups with high levels of DWV alone and high levels of both SBV and DWV (Fig. 4F, H) at 5 dpi. Notably, high levels of DWV alone in the larval feeding experiment did not alter the expression of defensin-1 and serpin (GB48820) (Fig. 3F, H, Group “hDWV”). It is possible that high levels of DWV in the pupae infected by injection may differentially affect the expression of defensin-1 and serpin (GB48820) compared to orally infected larvae. Lourenco et al. (2013) have reported that adult bees exhibit elevated AMP levels following injection. In the absence of bacterial challenge, wounding-associated AMP expression levels decrease within 24 hours in bumblebees. Therefore, it is possible that high DWV levels prevent the post-wounding resetting of defensin-1 levels.

Discussion

Transcriptome analysis of the honeybees showed strong up-regulation of the expression of AMPs in the orally infected larvae with high levels of SBV. The pupal injection experiment further confirmed that hymenoptaecin and defensin-1 are up-regulated in the insects with high SBV levels. Of note is that high levels of DWV, a related Iflavirus, did not up-regulate AMPs in the orally infected larvae. Expression of AMPs in insects is regulated by the Toll and Imd signaling pathways and induced by recognition of the bacterial or fungal pathogen-associated molecular patterns, such as bacterial peptidoglycan (Lemaitre & Hoffmann, 2007). Our results therefore raise interesting questions including, (i) how replication of SBV, a positive strand RNA virus, activates the signaling pathways and (ii) why DWV, a related Iflavirus, does not up-regulate AMPs. Although up-regulation of expression of AMPs by RNA viruses has been reported for insects previously (this includes infections of Drosophila C virus in Drosophila (Zhu, Ding & Zhu, 2013) and dengue virus in *Aedes aegypti* (Luplertlop et al., 2011), it remains unclear in which stages virus infection may influence the Toll and Imd signaling pathways, which are normally activated by peptigoglycans of Gram-positive and Gram-negative bacteria, correspondingly (Lemaitre & Hoffmann, 2007).

Although simultaneous activation of the Toll and Imd pathways, by SBV influencing intracellular components, cannot be ruled out without further studies, an alternative hypothesis is that SBV pathogenesis indirectly results in activation of these pathways. For example, the extensive disruption of the tracheal epithelial lining and pertrophic membranes caused by SBV infection (Mussen & Furgala, 1977) may allow contamination of the haemolymph by bacteria present in the tracheal or intestinal lining. This would result in recognition of the peptidoglycans and consequent Toll and Imd pathway activation. In contrast, DWV infection does not lead to disruption of the gut epithelium (Fievet et al., 2006) and even high levels of DWV, commensurate with symptomatic infection, do not result in AMP up-regulation (Bull et al., 2012; Nazzi et al., 2012; Ryabov et al., 2014). Further molecular studies will be required to discriminate between the direct or indirect activation of Imd and Toll pathways following SBV infection. It should be noted that a simplistic explanation of elevated bacterial levels in SBV-infected pupae does not account for the observations. We quantified the total bacterial load by qRT-PCR using generic primers for bacterial 16S rRNA (Table S1) (Nadkarni et al., 2002) and observed no statistically significant differences between the bacterial loads of honeybees with low virus levels and high levels of DWV, SBV and combination within the same age and developmental stage groups (Fig. S1). However, it is possible that the elevated AMP levels in SBV-infected pupae suppress bacterial expansion so confounding simple quantification of bacterial levels.

Evolution has shaped the virulence and pathogenesis of viruses to facilitate their transmission to new hosts. We speculate that the related Iflaviruses, SBV and DWV, induce different responses in their host that suit their principal or historical route of transmission. We further suggest that the different effects on the expression of the immune-related genes in the honeybee in response to SBV and DWV may have been selected during evolution of SBV and DWV and have made these viruses evolutionarily adjusted to the principal routes of their transmission. DWV, in the absence of the *Varroa* mite vectoring, could be transmitted both vertically through queens and drones, and orally (de Miranda & Genersch, 2010; Yue & Genersch, 2005). DWV infection of the honeybee larvae does not halt development and does not cause early death at the larval stage, which suggests that honeybee survival is essential for DWV transmission and that this virus has evolved to minimize negative impact on its honeybee host. (Fujiyuki et al., 2004; Ryabov et al., 2014). On the other hand, horizontal oral transmission is considered to be a principal route for SBV, which causes acute infections at the larval stage leading to death of infected insects, and the spread of SBV is likely to involve cannibalization of the diseased larvae (Schmickl & Crailsheim, 2001; Woyke J, 1977). Therefore, the observed suppression of the melanisation pathway in the SBV-infected larvae, which could be a consequence of the combined down-regulation of PPAE and up-regulation of serpin (GB48820), may be beneficial for SBV transmission because melanisation contributes to resistance to viruses and could decrease infectivity of SBV virus particles in the larvae and decrease efficiency of its horizontal transmission (Fig. 5), for example, similarly to the suppression of Semliki virus by phenoloxidase cascade in mosquito (Rodriguez-Andres et al., 2012). Suppression of melanisation in the long-term, may reduce resistance to pathogens and negatively impact on honeybee survival. Therefore such suppression of melanisation may have a negative impact on DWV, which relies more on vertical transmission; it is beneficial for DWV-infected honeybees to have functional melanisation pathways (Fig. 5).

There is a possibility that up-regulation of AMP expression may prevent bacterial growth and possible degradation of SBV particles in diseased larvae and pupae. Therefore this would increase chances of SBV transmission when diseased larvae and pupae are removed and/or cannibalized as part of the social immune response (Evans & Spivak, 2010) (Fig. 5). On the other hand, activation of immune pathways, which result in up-regulation of AMP production is costly (Moret & Schmid-Hempel, 2000) and therefore could negatively impact on honeybee survival and ultimately on DWV transmission (Fig. 5).

Conclusions

Our results indicate that SBV and DWV, evolutionarily related RNA viruses, evoke different transcriptional responses in their honeybee host, including effects on the expression of immune-related genes. Honeybee larvae with high levels of SBV replication, showed concerted up-regulated expression of antimicrobial peptides (AMPs) and down-regulated expression of the prophenoloxidase-activating enzyme (PPAE) together with up-regulation of the expression of a putative serpin, which could lead to the suppression of the melanisation pathway. The same effect was observed in the individuals with high levels of both SBV and DWV, but high levels of DWV alone did not affect expression of the AMPs and the genes involved in the regulation of melanisation. The effects of high SBV replication levels on expression of these immune genes were unlikely to be the consequences SBV-induced developmental changes, because some of them were observed in honeybees infected with SBV by injection at the pupal stage. It is possible that different impacts of SBV and DWV on the expression of immune-related genes may be an adaptation to horizontal and vertical transmission routes, the principal transmission routes of SBV and DWV respectively. These findings could be used to further investigate the role of AMPs and melanisation in virus-host interaction and transmission of insect viruses, including economically important viruses of the honeybee.

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Table 1(on next page)

Table 1

Summary of the NGS libraries of the larval oral inoculation experiment.

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Table 1. Summary of the NGS libraries of the larval oral inoculation experiment.

Sample ID	**Treatment group**	**ENA sample accession**	**Total reads**	***A. mellifera* OGS3, mRNA reads**	**Total DWV reads (Aligned to GenBank accession number NC_004830)**	**DWV to actin mRNA (GB44311) coverage ratio**	**Total SBV reads (Aligned to GenBank accession number AF092924)**	**SBV to actin mRNA (GB44311) coverage ratio**
1	Control	SAMEA2591288	9691343	6842703	7555	0.047	28541	0.201
2	Control	SAMEA2591289	10630145	6204592	6210	0.049	4009	0.036
3	DWV	SAMEA2591290	9785423	3352681	3240468	55.263	6179	0.120
4	SBV+DWV	SAMEA2591291	10069125	645114	736640	34.841	7021099	378.684
5	SBV+DWV	SAMEA2591292	10257560	604367	887349	54.627	8171254	573.641

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Table 2(on next page)

Table 2

Gene ontology (GO) Biological Process (BP) terms associated with the up-regulated and down-regulated differentially expressed genes in the honeybees of the larval feeding NGS experiments (only the top 10 over-represented GO PB terms with the lowest *p*-values are shown).

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Table 2. Gene ontology (GO) Biological Process (BP) terms associated with the upregulated and downregulated differentially expressed genes in the honeybees of the larval feeding NGS experiments (only the top 10 over-represented GO PB terms with the lowest p-values are shown).

Contrast 3
Upregulated DE genes

GO Term	P-value	Sample frequency	Background frequency
GO:0006412 translation	3.31E-05	31/186 (16.7%)	787/14580 (5.4%)
GO:0044237 cellular metabolic process	1.87E-04	96/186 (51.6%)	4811/14580 (33.0%)
GO:0009161 ribonucleoside monophosphate metabolic process	2.16E-04	12/186 (6.5%)	132/14580 (0.9%)
GO:0009123 nucleoside monophosphate metabolic process	2.35E-04	12/186 (6.5%)	133/14580 (0.9%)
GO:0046034 ATP metabolic process	3.67E-04	11/186 (5.9%)	113/14580 (0.8%)
GO:0032543 mitochondrial translation	6.05E-04	6/186 (3.2%)	23/14580 (0.2%)
GO:0009167 purine ribonucleoside monophosphate metabolic process	1.11E-03	11/186 (5.9%)	126/14580 (0.9%)
GO:0009126 purine nucleoside monophosphate metabolic process	1.11E-03	11/186 (5.9%)	126/14580 (0.9%)
GO:0010467 gene expression	4.08E-03	56/186 (30.1%)	2397/14580 (16.4%)
GO:0044249 cellular biosynthetic process	5.38E-03	56/186 (30.1%)	2418/14580 (16.6%)

Contrast 3
Downregulated DE genes

none

Commonality between Contrasts 2, 4, and 5
Upregulated DE genes

GO Term	P-value	Sample frequency	Background frequency
GO:0050896 response to stimulus	1.11E-10	104/263 (39.5%)	2855/14580 (19.6%)
GO:0006950 response to stress	3.34E-10	57/263 (21.7%)	1084/14580 (7.4%)

GO:0002376 immune system process	1.04E-09	33/263 (12.5%)	405/14580 (2.8%)
GO:0006952 defense response	9.74E-08	29/263 (11.0%)	373/14580 (2.6%)
GO:0044699 single-organism process	6.35E-07	194/263 (73.8%)	8031/14580 (55.1%)
GO:0006955 immune response	2.61E-06	24/263 (9.1%)	298/14580 (2.0%)
GO:0065007 biological regulation	5.27E-06	109/263 (41.4%)	3621/14580 (24.8%)
GO:0044763 single-organism cellular process	7.96E-06	154/263 (58.6%)	5930/14580 (40.7%)
GO:0045087 innate immune response	6.92E-05	16/263 (6.1%)	157/14580 (1.1%)
GO:0044707 single-multicellular organism process	2.72E-04	115/263 (43.7%)	4170/14580 (28.6%)

Commonality between Contrasts 2, 4, and 5
Downregulated DE genes

GO Term	P-value	Sample frequency	Background frequency
GO:0042335 cuticle development	3.78E-12	27/242 (11.2%)	234/14580 (1.6%)
GO:0040003 chitin-based cuticle development	7.15E-11	23/242 (9.5%)	182/14580 (1.2%)
GO:0030239 myofibril assembly	8.95E-10	12/242 (5.0%)	37/14580 (0.3%)
GO:0055002 striated muscle cell development	6.24E-08	12/242 (5.0%)	51/14580 (0.3%)
GO:0055001 muscle cell development	6.24E-08	12/242 (5.0%)	51/14580 (0.3%)
GO:0031032 actomyosin structure organization	2.26E-07	13/242 (5.4%)	70/14580 (0.5%)
GO:0006030 chitin metabolic process	3.65E-07	16/242 (6.6%)	122/14580 (0.8%)
GO:1901071 glucosamine-containing compound metabolic process	1.07E-06	16/242 (6.6%)	131/14580 (0.9%)
GO:0006040 amino sugar metabolic process	1.21E-06	16/242 (6.6%)	132/14580 (0.9%)
GO:0006022 aminoglycan metabolic process	5.98E-06	16/242 (6.6%)	147/14580 (1.0%)

Table 3(on next page)

Table 3

Differential expression (DE) of the putative honeybee antimicrobial peptides (AMPs), melanisation, Toll, and Imd pathway genes in the larval feeding experiment. Fold change values (log_2 transformed) are shown only for the genes DE in the contrast. Expression of the genes marked with * was quantified by qRT-PCR. DE genes were identified by both DESeq and edgeR analyses, with adjusted $p<0.05$ and false discovery rate, FDR<0.05 respectively.

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Table 3. Differential expression (DE) of the putative honeybee antimicrobial peptides (AMPs), melanisation, Toll, and Imd pathway genes in the larval feeding experiment. Fold change values ($\log_2$ transformed) are shown only for the genes DE in the contrast. Expression of the genes marked with * was quantified by qRT-PCR. DE genes were identified by both DESeq and edgeR analyses, with adjusted *p*<0.05 and false discovery rate, FDR <0.05 respectively.

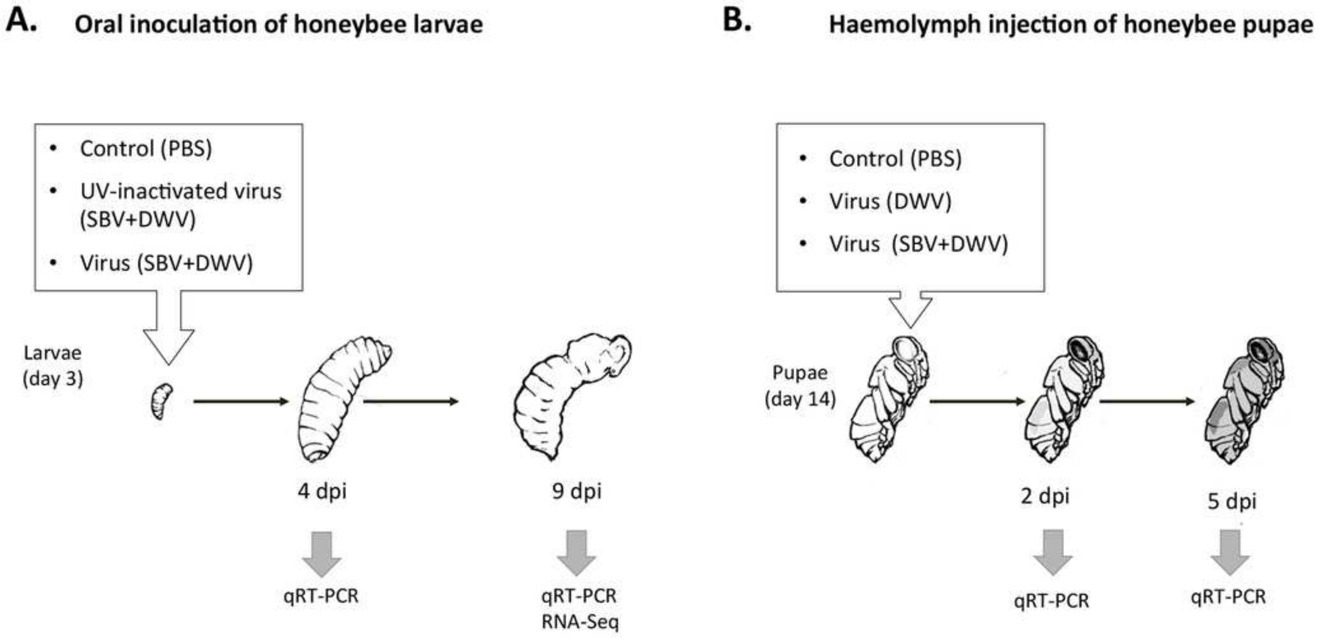
Honeybee gene (OGS3 ID)	Drosophila ortholog (Flybase ID)	Gene name / description	Pathway, group	Fold change (log2 transformed)			
				Contrast 2	Contrast 3	Contrast 4	Contrast 5
				High SBV+DWV vs. high DWV and control	High DWV vs. control	High SBV+DWV vs. control	High SBV+DWV vs. high DWV
GB41428 *	FBgn0010385	Defensin-1	AMP	9.203	.	9.328	8.738
GB47318	FBgn0032835	Abaecin	AMP	6.474	.	6.143	10.455
GB47546		Apidaecin	AMP	5.322	.	5.423	4.925
GB47618	FBgn0010385	Defensin-2	AMP	10.171	.	9.828	10.730
GB51223 *	FBgn0014002	Hymenoptaecin	AMP	7.894	.	8.057	7.410
GB53576	FBgn0261922	Apisimin	AMP	.	.	2.738	.
GB50013 *	FBgn0036891	Prophenoloxidase-activating enzyme (PPAE)	Melanisation	-2.612	.	-2.791	.
GB48820 *	FBgn0028985	Serpin (NEC LIKE)	Toll / Melanisation	4.681	.	4.867	4.151
GB54611	FBgn0028984	Serpin (NEC LIKE)	Toll / Melanisation	2.092	.	2.027	2.359
GB40699	FBgn0029114	Tollo (Receptor)	Toll	.	.	-1.187	.
GB43456	FBgn0034476	Toll-7 (Receptor)	Toll	-1.681	.	-1.780	.
GB49441	FBgn0003450	persephone-Serine protease	Toll	4.182	.	4.134	4.365
GB54611	FBgn0028984	NEC-like	Toll	2.092	.	2.027	2.359
GB55007	FBgn0030051	persephone-Serine Protease Immune Response Integrator	Toll	2.067	.	1.975	.

GB44055	FBgn0000250	cactus (NF-kappa-B inhibitor)	Toll	.	.	2.372	2.457
GB50418	FBgn0262473	Toll-1 (Receptor)	Toll	2.073	.	2.104	1.962
GB51741	FBgn0030310	Peptidoglycan recognition protein SA	Toll	2.070	.	2.056	2.119
GB52631	FBgn0003495	spatzle	Toll	3.224	.	3.284	3.012
GB51498	FBgn0033402	Myd88	Toll	.	1.549	nd	.
GB48707	FBgn0024222	immune response deficient 5	Toll	.	1.340	nd	.
GB42500	FBgn0035976	PGRP-LC	Imd	1.515	.	1.462	1.723
GB45648	FBgn0013983	imd	Imd	.	.	1.240	.

1

Figure 1

Schematic representation of experimental infection of honeybees with SBV and DWV, (A) larval oral inoculation, and (B) pupal haemolymph injection.



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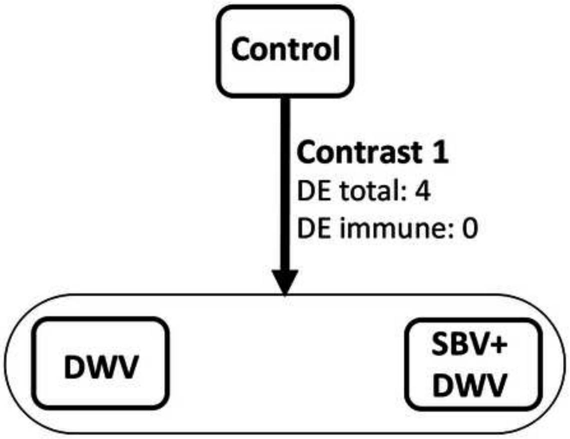
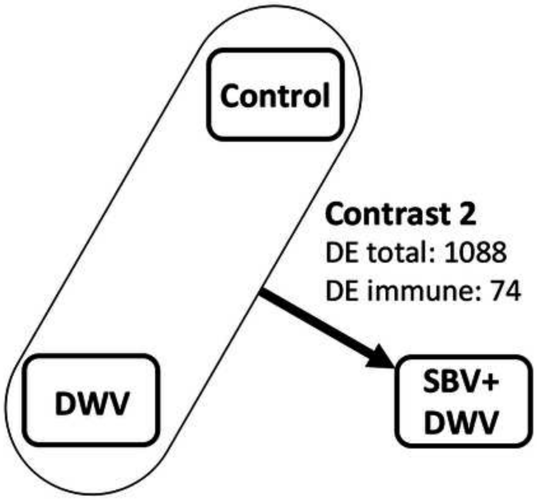
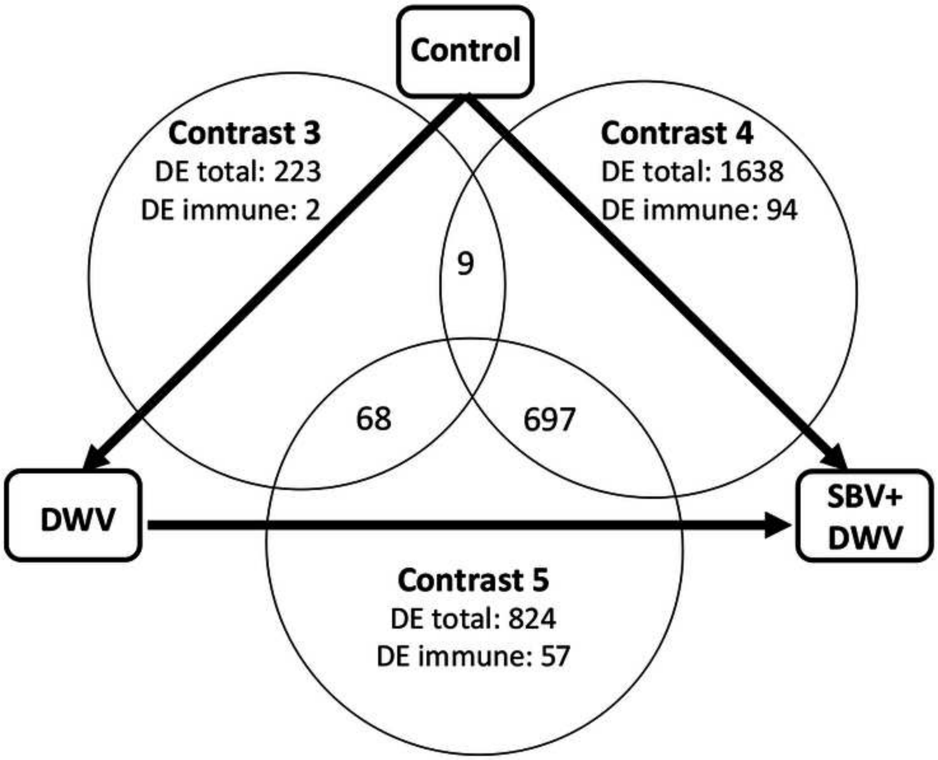
2

Figure 2

Effect of virus infection on global honeybee gene expression, RNA-Seq experiment: experimental groups and contrasts. Arrows indicate direction of the contrasts (head against tail). The numbers of differentially expressed (DE) honeybee genes and of DE immune-related genes are shown for each of the contrasts.


Control
Contrast 1
DE total: 4
DE immune: 0
DWV
SBV+
DWV

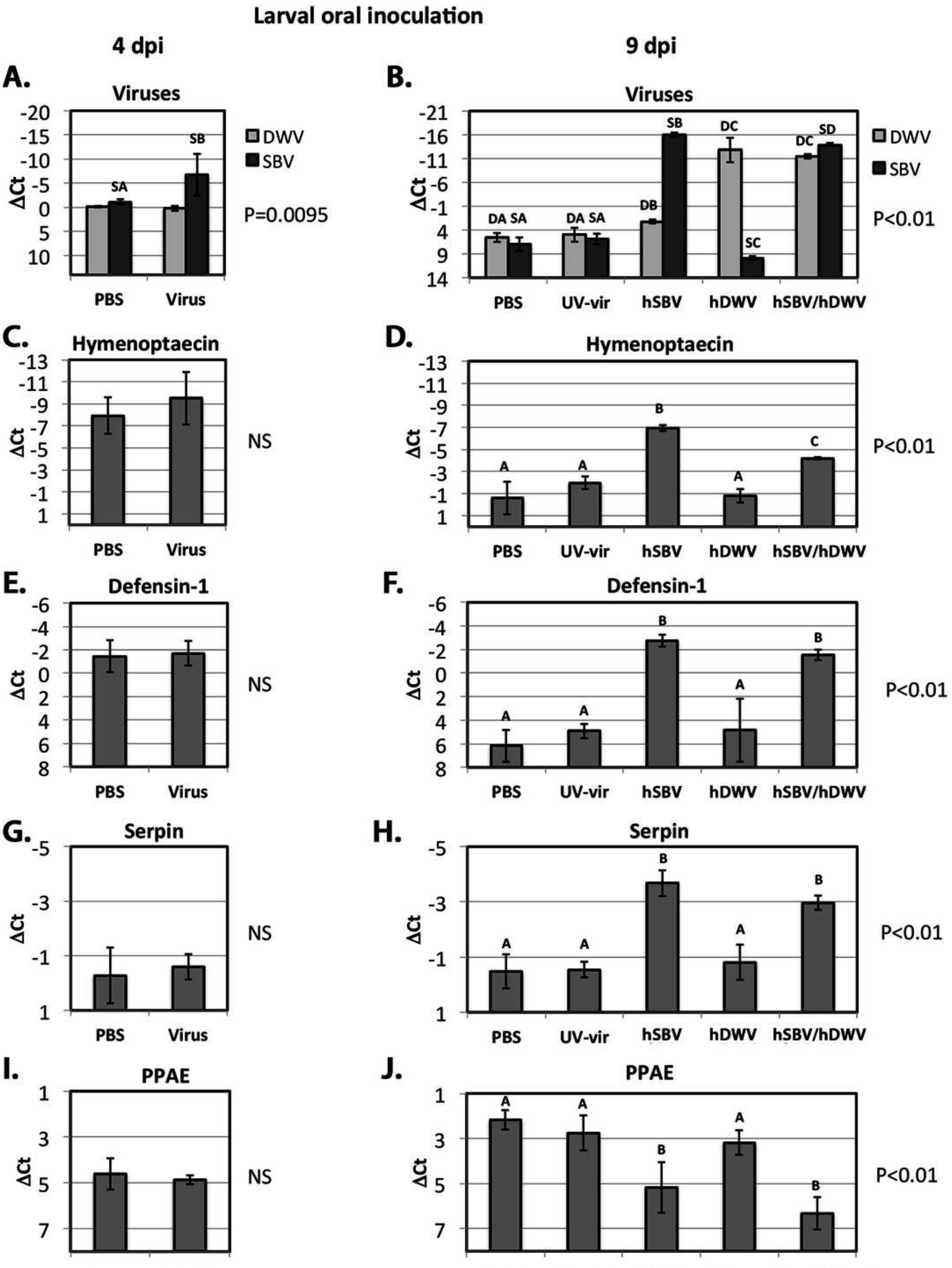

Control
Contrast 2
DE total: 1088
DE immune: 74
DWV
SBV+
DWV


Control
Contrast 3
DE total: 223
DE immune: 2
Contrast 4
DE total: 1638
DE immune: 94
9
68
697
DWV
SBV+
DWV
Contrast 5
DE total: 824
DE immune: 57

3

Figure 3

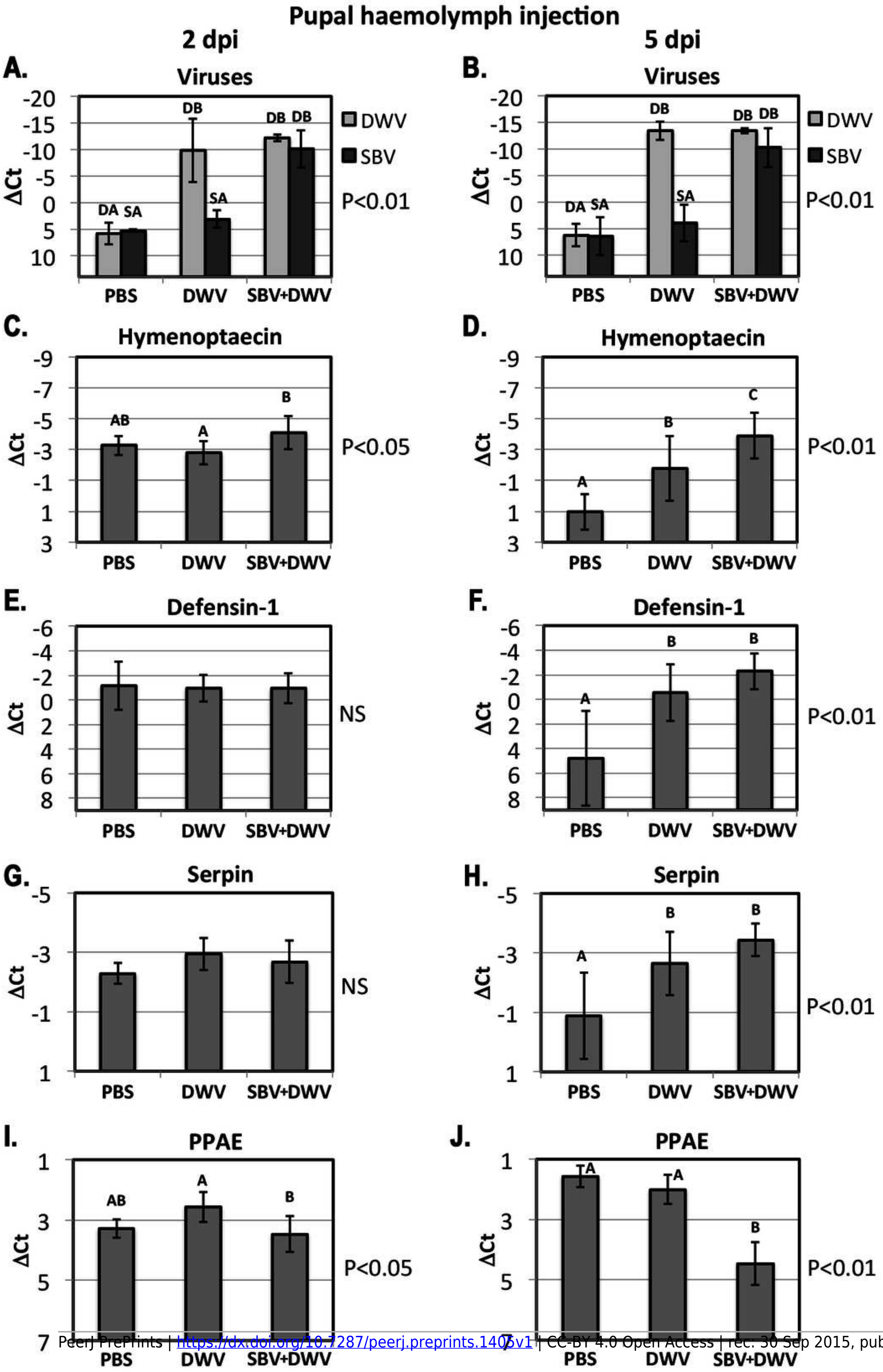
Oral infection. The relative levels of SBV and DWV genomic RNAs (A, B), and the AMPs: Imd pathway-controlled hymenoptaecin, GB51223 (C, D) and Toll pathway-controlled defensin-1, GB41428 (E, F), putative serpin, GB48820 (G, H), and prophenoloxidase activating enzyme, PPAE, GB50013 (I, J). Transcripts were quantified by qRT-PCR. Bars show mean ΔCt values, which were calculated by subtracting Ct values for Rp49 (GB47740) from the Ct values of the target genes, and standard deviation (SD). Letters above the bars indicate statistically significantly different groups using t-test comparisons, *p*<0.01.


Larval oral inoculation
4 dpi
9 dpi
A.
Viruses
ΔCt
DWV
SBV
SA
SB
P=0.0095
PBS
Virus
B.
Viruses
ΔCt
DWV
SBV
DA SA
DA SA
DB
SB
DC
SC
DC
SD
P<0.01
PBS
UV-vir
hSBV
hDWV
hSBV/hDWV
C.
Hymenoptaecin
ΔCt
NS
PBS
Virus
D.
Hymenoptaecin
ΔCt
A
A
B
A
C
P<0.01
E.
Defensin-1
ΔCt
NS
F.
Defensin-1
ΔCt
A
A
B
A
B
P<0.01
G.
Serpin
ΔCt
NS
H.
Serpin
ΔCt
A
A
B
A
B
P<0.01
I.
PPAE
ΔCt
NS
J.
PPAE
ΔCt
A
A
B
A
B
P<0.01

4

Figure 4

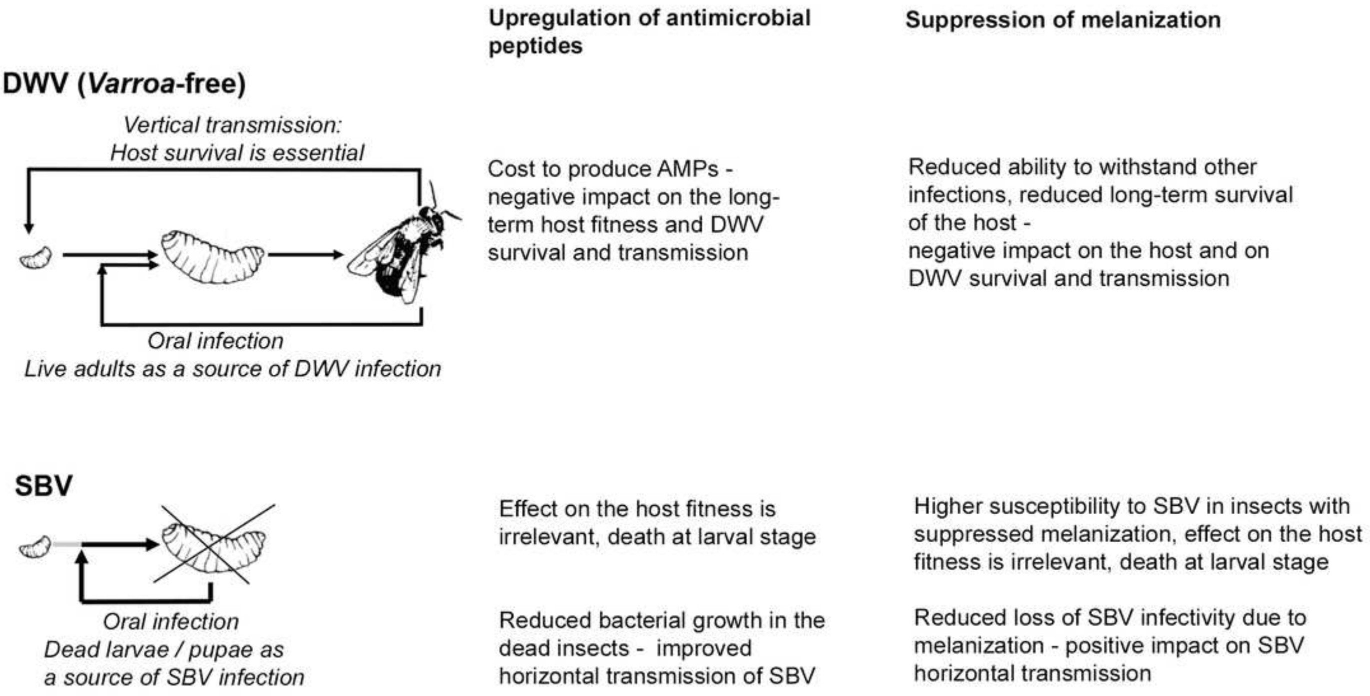
Pupal injection. The relative levels of SBV and DWV genomic RNAs (A, B), and the AMPs: Imd pathway-controlled hymenoptaecin, GB51223 (C, D) and Toll pathway-controlled defensin-1, GB41428 (E, F), putative serpin, GB48820 (G, H), and prophenoloxidase activating enzyme, PPAE, GB50013 (I, J) transcripts were quantified by qRT-PCR. Bars show mean ΔCt values, which were calculated by subtracting Ct values for Rp49 (GB47740) from the Ct values of the target genes, and standard deviation (SD). Letters above the bars indicate statistically significant different groups using t-test comparisons, $p<0.01$. NS denotes “not significant”.


Pupal haemolymph injection
2 dpi
5 dpi
A.
Viruses
B.
Viruses
ΔCt
DWV
SBV
DA SA
DB
SA
DB DB
P<0.01
PBS
DWV
SBV+DWV
C.
Hymenoptaecin
AB
A
B
P<0.05
D.
Hymenoptaecin
A
B
C
P<0.01
E.
Defensin-1
NS
F.
Defensin-1
A
B
B
P<0.01
G.
Serpin
NS
H.
Serpin
A
B
B
P<0.01
I.
PPAE
AB
A
B
P<0.05
J.
PPAE
A
A
B
P<0.01

5

Figure 5

Schematic representation of the impacts of SBV and DWV infections on the melanisation pathway, AMP production, host survival and viral transmission.



	Upregulation of antimicrobial peptides	**Suppression of melanization**
DWV (*Varroa*-free)	Cost to produce AMPs - negative impact on the long-term host fitness and DWV survival and transmission	Reduced ability to withstand other infections, reduced long-term survival of the host - negative impact on the host and on DWV survival and transmission
SBV	Effect on the host fitness is irrelevant, death at larval stage Reduced bacterial growth in the dead insects - improved horizontal transmission of SBV	Higher susceptibility to SBV in insects with suppressed melanization, effect on the host fitness is irrelevant, death at larval stage Reduced loss of SBV infectivity due to melanization - positive impact on SBV horizontal transmission