

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 I flavivirus 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

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.

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6

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26 Introduction

27 The Western honeybee, *Apis mellifera*, is the most important managed insect pollinator
28 worldwide. In recent decades a global decline in the number of honeybee colonies was reported,
29 threatening security of the global food supply (Vanbergen et al., 2013), with pathogens – in
30 particular viruses – contributing significantly to these declines. These viral pathogens are
31 predominantly single-stranded, positive sense RNA viruses of the families *Dicistroviridae* and
32 *Iflaviridae*, and may exhibit differing virulence levels, causing infections ranging from
33 asymptomatic to acute and resulting in rapid insect death (McMenamin & Genersch, 2015). It is
34 also apparent that some viruses exhibit strain differences in virulence. For example, the most
35 widespread honeybee virus, deformed wing virus (DWV) (de Miranda & Genersch, 2010; Lanzi
36 et al., 2006) and the very closely related variants *Varroa destructor* virus-1 (Ongus et al., 2004)
37 and Kakugo virus (*Fujiyuki et al., 2004*), usually cause asymptomatic infections with low levels
38 of the virus when transmitted vertically or orally. In contrast, DWV transmission by the
39 ectoparasitic mite *Varroa destructor* – by direct injection to the honeybee haemolymph – results
40 in the selection of highly pathogenic strains of DWV with extremely limited genetic diversity
41 (Martin et al., 2012; Ryabov et al., 2014) which accumulate to very high levels in infected pupae
42 and cause characteristic symptoms, including deformed wings and shortened abdomen. The
43 doses of DWV, including its virulent strains, which are delivered orally to larvae during brood
44 rearing (or in the laboratory) cause only asymptomatic infections and accumulate to low levels,
45 making it possible for infected honeybees to survive to adulthood and transmit the virus
46 horizontally or vertically (Ryabov et al., 2014; Yue & Genersch, 2005). In contrast to DWV,
47 sacbrood virus (SBV; a related member of the *Iflaviridae*) accumulates to high levels and causes
48 acute infections in orally inoculated honeybee larvae (Bailey, Gibbs & Woods, 1964; Ghosh et

49 al., 1999). SBV infection has a much more pronounced impact on honeybee development than
50 DWV; honeybee larvae with high levels of the virus have a gondola-shaped sac-like appearance
51 with tough leathery skin and die before pupation. It is likely that SBV is transmitted from the
52 larvae killed by SBV to in-hive worker honeybees, which subsequently transmit the virus to
53 young larvae (Bailey, 1969).

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

67

68 **Materials & Methods**

69 **Honeybee rearing, virus preparations and inoculation**

70 Colonies of healthy Western honeybees (*Apis mellifera*) with low managed levels of
71 *Varroa destructor* infestation were maintained in Warwickshire, UK, and used as a source of

72 larvae and pupae. DWV virus preparation was isolated from honeybee pupae sourced from a
73 colony with high *Varroa* infestation levels. Virus preparations containing both SBV and DWV
74 (SBV+DWV) were purified from larvae and pupae of the *Varroa*-infested colonies where some
75 larvae showed typical SBV-induced symptoms. Virus isolation was carried out as described
76 previously (Moore et al., 2011) and the virus preparations stored at -80°C prior to use. For
77 inactivation, the virus preparations were irradiated by UV light (Simonet & Gantzer, 2006).

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

89 For the honeybee pupa infection, worker pupae sourced at the white eye stage (12th to
90 13th days of development) received injections into the haemolymph using a syringe with a 0.3
91 mm outer diameter needle (Ryabov et al., 2014) either with 10 ml of phosphate-buffered saline
92 (PBS), or with DWV preparations (10^6 DWV virus particles in PBS), or with the mixture of SBV
93 and DWV (10^6 SBV and 10^6 DWV virus particles in PBS). The pupae were reared at +33°C as

94 previously described (Ryabov et al., 2014). Whole-body RNA samples were extracted from
95 individual pupae at 2 dpi or 5 dpi.

96

97 **Gene expression analysis**

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

104 Quantification of viral RNA and the honeybee transcripts were carried out by quantitative
105 reverse transcription PCR (qRT-PCR) as described previously (Ryabov et al., 2014). In brief, the
106 RNA samples were treated with RNA-free DNase1 (New England BioLabs), purified using
107 RNAeasy plant mini kit (Qiagen) and used for cDNA synthesis with random hexanucleotide
108 primers. qRT-PCR reactions were performed using SYBR Green kit (Ambion) with the primers
109 to viral RNA and to the honeybee transcripts (Table S1).

110

111 **Bioinformatics**

112 The RNA-Seq reads were aligned using Bowtie2 (Langmead et al., 2009) (with the least
113 stringent alignment settings to allow detection of the sequence variants, "--very-sensitive"
114 option) to the latest honeybee transcriptome annotation (OGS3; containing 16041 putative
115 transcripts), as well as to a set of sequences of the known fungal and viral pathogens of the
116 honeybees used previously (Bull et al., 2012; Ryabov et al., 2014). We used samtools idxstats to

117 produce a summary of the number of reads aligning to the honeybee transcriptome and the DWV
118 and SBV reference sequences (GenBank accession numbers NC_004830 and AF092924
119 respectively). The NGS gene expression profiles were used to identify differentially expressed
120 (DE) genes using DESeq (Anders & Huber, 2010) and edgeR (Robinson, McCarthy & Smyth,
121 2010), with adjusted *p*-values and a false discovery rate (FDR) below 0.05. *Drosophila*
122 homologues of the honeybee genes were identified previously (Ryabov et al., 2014) and those
123 DE in the contrasts were used for Gene Ontology (GO) analysis (Ashburner et al., 2000) using
124 AmiGO (Carbon et al., 2009).

125

126 **Results**

127 **Oral infection of honeybee larvae with DWV and SBV**

128 Artificially reared honeybee worker larvae were orally inoculated with virus preparations
129 containing SBV and DWV (“SBV+DWV”) and controls included UV-inactivated
130 “SBV+DWV” virus preparation and PBS (Fig. 1A). The doses of both DWV and SBV, 10^{10}
131 genome equivalents, were sufficient to allow replication of the viruses to high levels when
132 ingested at the larval stage (Ryabov and Evans, unpublished). Notably, this DWV dosage was
133 100 times higher than a dose used in oral infection of the adult bees which did not result in
134 establishing high levels of DWV infection (Moeckel, Gisder, & Genersch, 2011). Quantification
135 of SBV and DWV in the experimental insects assayed at 9 dpi showed that the individuals of
136 both control groups had low levels of both DWV and SBV (Ct values 31 to 22, and 32 to 24
137 respectively), while among the virus-fed insects there were individuals with high levels of either
138 DWV or SBV, as well as those with high levels of both viruses (Ct values 8 to 14, and 9 to 15).

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

145 Approximately 10 million 101 nt reads were produced for each library (Table 1) and were
146 aligned to the latest honeybee transcriptome annotation (OGS3) and to the sequences of known
147 fungal and viral pathogens of the honeybees used previously (Bull et al., 2012; Ryabov et al.,
148 2014). Apart from DWV-like viruses and SBV (GenBank accession numbers NC_004830 and
149 AF092924 respectively) no other pathogens were detected. We observed a dramatic increase of
150 the DWV and SBV coverage, normalized to the host actin mRNA coverage (GB44311), in the
151 infected honeybees compared to the controls (Table 1). For example, there was an ~1000-fold
152 increase in DWV reads in the virus-infected pupae (samples 3, 4 and 5) compared to controls
153 (samples 1 and 2), from 0.05 to about 50 in concordance with previously reported actin-
154 normalized levels of DWV in pupae with low (0.1 DWV genomes/actin mRNA) and high (10-
155 100 DWV genomes/actin mRNA) levels of DWV by qRT-PCR (Moore et al., 2011; Ryabov et
156 al., 2014). SBV levels showed over 1000-fold increase in samples 4 and 5 compared to the
157 control samples (1 and 2) and sample 3; the ratios of SBV to actin read coverage increased from
158 0.04 - 0.20 to 378-573 (Table 1). The observed increase of the SBV load was similar to
159 previously reported differences between the SBV levels in asymptomatic honeybee larvae with
160 low SBV levels and the symptomatic larvae with high SBV (Blanchard et al., 2014).

161

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

164 We stratified the RNA-Seq samples according to the levels of DWV and SBV (high and low)
165 into three groups, “Control” (samples 1 and 2), “DWV” (sample 3), and “SBV+DWV” (samples
166 4 and 5) and, by using both DESeq (Anders & Huber, 2010) and edgeR (Robinson, McCarthy &
167 Smyth, 2010), identified differentially expressed (DE) genes in five contrasts (Fig. 2, Table S2)
168 to assess the effect of virus infections on the host gene expression. Potential functional
169 consequences of DE were inferred following overrepresented Gene Ontology (GO) analysis
170 (Ashburner et al., 2000), Table S3.

171 The highest numbers of DE genes were identified in the contrasts involving the
172 “SBV+DWV” group. Of these, contrast 4 (high SBV+DWV vs control) had 1638 DE genes,
173 which included almost all (1076 of 1088) of those identified as DE in Contrast 2 (high
174 SBV+DWV vs. control and high DWV alone). High commonality, 697 of 824 genes, was also
175 observed between the DE genes in Contrast 5 and Contrast 4 (high SBV+DWV vs. high DWV
176 alone and high SBV+DWV vs. control respectively (Fig. 2). The direction of gene expression
177 change was the same (*e.g.* genes up-regulated in Contrast 4 were also up-regulated in Contrast
178 5).

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

185 Table S3) showed that different overrepresented GO terms were associated with the DE genes in
186 Contrast 3 (high DWV levels) compared with the genes in Contrasts 2, 4, and 5 (high levels of
187 SBV and DWV), providing further evidence that high level replication of SBV or DWV affected
188 different biological processes in the honeybee. When compared with the low virus level control,
189 the insects with high DWV levels (Contrast 3) showed up-regulation of the genes involved in
190 translation, metabolic processes, and ATP metabolism (Table 2, Table S3). Changes in
191 honeybee gene expression associated with high levels of SBV+DWV were more pronounced
192 when compared to those associated with high DWV levels alone. The down-regulated DE genes
193 associated with increased levels of SBV (Contrasts 2, 4, and 5) were involved in cuticle and
194 muscle development (Table 2, Table S3), consistent with the reported phenotypic effects of SBV
195 infection, which include halted development and abnormal cuticle (Bailey, Gibbs & Woods,
196 1964). Surprisingly, despite very low commonality between Contrasts 3 and 4, a considerable
197 proportion of DE genes in Contrast 3 (68 of 223) were also DE in Contrast 5 (Fig. 2). However,
198 the vast majority of these (67/68) exhibited virus-dependent DE in opposing directions, *i.e.* genes
199 up-regulated in response to high levels of DWV alone were down-regulated in response to high
200 levels of SBV, even in the presence of high levels of DWV (Table S4). The over-represented GO
201 terms associated with these genes indicated that high levels of DWV induced increased
202 expression of the genes involved in ATP metabolism, whereas high levels of SBV had the
203 opposite effect on the expression of these genes, overriding the effect of DWV on their
204 expression (Table 2, Table S4). In respect to the genes up-regulated in response to high levels of
205 SBV we were particularly intrigued with the over-representation of GO terms associated with
206 immune response, *e.g.* “Immune system process”, “Defense response” (Table 2, Table S3).
207

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

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

230 like proteins, GB48820 and GB54611) (Table 3). We propose that these changes in gene
231 expression may result in suppression of the melanisation pathway.

232 To further explore the possible connection between the replication of DWV and SBV and
233 the expression of the AMPs controlled by the Toll pathway (defensin-1, GB41428), or the Imd
234 pathway (hymenoptaecin, GB51223), and the components of the melanisation pathway (putative
235 serpin, GB48820, and prophenoloxidase activating enzyme, GB50013), we quantified gene
236 expression levels in orally-infected larvae (Fig. 1A) by qRT-PCR. While no increase of DWV
237 levels was observed at 4 dpi via the oral route compared to the PBS control, the SBV levels in
238 the virus-infected group were significantly higher than in the control, PBS-exposed insects (Fig.
239 3A). After 9 days post inoculation, the control insects exposed to the buffer (PBS) and to the
240 UV-inactivated virus mixture of DWV and SBV (UV-vir) showed equally low levels of SBV and
241 DWV (Fig. 3B). These results demonstrate that *in vitro* manipulations did not activate replication
242 of SBV and DWV that may already have been present at low levels in experimental larvae or
243 pupae.

244 As before, resulting pupae that developed from larvae fed with infectious virus were
245 stratified according to the observed SBV and DWV levels at 9 dpi (Group “hSBV” - high SBV
246 and low DWV levels, Group “hDWV” - high DWV and low SBV levels, and Group
247 “hSBV/hDWV” - high levels of both tested viruses) and the expression level of honeybee
248 immune genes of interest was quantified (Fig. 3). Both AMPs, hymenoptaecin and defensin-1,
249 were up-regulated in Group “hSBV” insects but remained at control levels in Group “hDWV”
250 individuals (Fig. 3D, F). The level of hymenoptaecin increased, but to a lower level in Group
251 “hDWV/hSBV” than Group “hSBV” (Fig. 3D) whereas expression of defensin-1 was similar in
252 these groups (Fig. 3F). It is possible that hymenoptaecin expression may be directly influenced

253 by the level of SBV (which was lower in absolute terms in Group “hSBV/hDWV” than in Group
254 “hSBV”). Alternatively, the elevated levels of DWV in Group “hSBV/hDWV” may suppress
255 Imd pathway activation – which controls expression of hymenoptaecin – but not the Toll
256 pathway-controlled defensin-1. Group “hSBV” and “hSBV/hDWV” samples had elevated
257 expression of the putative serpin and reduced expression of PPAE compared to Group “hDWV”
258 or controls fed PBS or UV-inactivated virus preparation (Fig. 3H, 3J), implying that altered
259 expression of these two melanisation pathway genes could be a result of elevated SBV levels
260 (Fig. 3B). The qRT-PCR analyses were in good agreement with the RNA-Seq data (Table 3).

261

262

263 **Injection of honeybee pupae haemolymph with DWV and SBV**

264 The devastating developmental consequences of high levels of SBV infection on larvae
265 (Bailey, Gibbs & Woods, 1964) may account for the changes in immune-related gene expression.
266 We therefore investigated gene expression changes in pupae directly inoculated by injection
267 (Fig. 1B). We observed no pupae with high virus levels in the PBS-injected control group at 2
268 and 5 dpi, while high levels of DWV were observed in the DWV-injected pupae, and high levels
269 of both SBV and DWV were present in pupae injected with the SBV+DWV virus mixture (Fig.
270 4A, B).

271 At 2 dpi there was no significant difference between the expression levels of defensin-1
272 and serpin (Fig. 4E, G) whereas the expression levels of hymenoptaecin were significantly
273 higher in the SBV+DWV-injected pupae compared to DWV-injected pupae (Fig. 4C). In
274 contrast, PPAE levels were higher in DWV-injected pupae than in those that received both
275 viruses (Fig. 4I). At 5 dpi, PPAE was significantly down-regulated in the SBV+DWV group,

276 while the levels of PPAE in the PBS and DWV groups were not significantly different (Fig. 4J).
277 The same effects of high levels of DWV and SBV on expression of PPAE were observed in the
278 larval feeding experiment (Fig. 3J). Expression levels of hymenoptaecin were significantly
279 different between the pupae injected with PBS, DWV, or SBV+DWV groups at 5 dpi, with the
280 highest levels observed in the SBV+DWV group and lowest in the control (PBS) group (Fig.
281 4D). In addition, at 5 dpi defensin-1 and serpin (GB48820) were significantly up-regulated in
282 the DWV pupae and SBV+DWV-injected pupae compared to the PBS-injected control. There
283 were no significant differences between the pupae groups with high levels of DWV alone and
284 high levels of both SBV and DWV (Fig. 4F, H) at 5 dpi. Notably, high levels of DWV alone in
285 the larval feeding experiment did not alter the expression of defensin-1 and serpin (GB48820)
286 (Fig. 3F, H, Group “hDWV”). It is possible that high levels of DWV in the pupae infected by
287 injection may differentially affect the expression of defensin-1 and serpin (GB48820) compared
288 to orally infected larvae. Lourenco et al. (2013) have reported that adult bees exhibit elevated
289 AMP levels following injection. In the absence of bacterial challenge, wounding-associated
290 AMP expression levels decrease within 24 hours in bumblebees. Therefore, it is possible that
291 high DWV levels prevent the post-wounding resetting of defensin-1 levels.

292

293 Discussion

294 Transcriptome analysis of the honeybees showed strong up-regulation of the expression
295 of AMPs in the orally infected larvae with high levels of SBV. The pupal injection experiment
296 further confirmed that hymenoptaecin and defensin-1 are up-regulated in the insects with high
297 SBV levels. Of note is that high levels of DWV, a related Iflavirus, did not up-regulate AMPs in
298 the orally infected larvae. Expression of AMPs in insects is regulated by the Toll and Imd

299 signaling pathways and induced by recognition of the bacterial or fungal pathogen-associated
300 molecular patterns, such as bacterial peptidoglycan (Lemaitre & Hoffmann, 2007). Our results
301 therefore raise interesting questions including, (i) how replication of SBV, a positive strand RNA
302 virus, activates the signaling pathways and (ii) why DWV, a related I flavivirus, does not up-
303 regulate AMPs. Although up-regulation of expression of AMPs by RNA viruses has been
304 reported for insects previously (this includes infections of Drosophila C virus in Drosophila
305 (Zhu, Ding & Zhu, 2013) and dengue virus in *Aedes aegypti* (Luplertlop et al., 2011), it remains
306 unclear in which stages virus infection may influence the Toll and Imd signaling pathways,
307 which are normally activated by peptidoglycans of Gram-positive and Gram-negative bacteria,
308 correspondingly (Lemaitre & Hoffmann, 2007).

309 Although simultaneous activation of the Toll and Imd pathways, by SBV influencing
310 intracellular components, cannot be ruled out without further studies, an alternative hypothesis is
311 that SBV pathogenesis indirectly results in activation of these pathways. For example, the
312 extensive disruption of the tracheal epithelial lining and peritrophic membranes caused by SBV
313 infection (Mussen & Furgala, 1977) may allow contamination of the haemolymph by bacteria
314 present in the tracheal or intestinal lining. This would result in recognition of the peptidoglycans
315 and consequent Toll and Imd pathway activation. In contrast, DWV infection does not lead to
316 disruption of the gut epithelium (Fievet et al., 2006) and even high levels of DWV,
317 commensurate with symptomatic infection, do not result in AMP up-regulation (Bull et al., 2012;
318 Nazzi et al., 2012; Ryabov et al., 2014). Further molecular studies will be required to
319 discriminate between the direct or indirect activation of Imd and Toll pathways following SBV
320 infection. It should be noted that a simplistic explanation of elevated bacterial levels in SBV-
321 infected pupae does not account for the observations. We quantified the total bacterial load by

322 qRT-PCR using generic primers for bacterial 16S rRNA (Table S1) (Nadkarni et al., 2002) and
323 observed no statistically significant differences between the bacterial loads of honeybees with
324 low virus levels and high levels of DWV, SBV and combination within the same age and
325 developmental stage groups (Fig. S1). However, it is possible that the elevated AMP levels in
326 SBV-infected pupae suppress bacterial expansion so confounding simple quantification of
327 bacterial levels.

328 Evolution has shaped the virulence and pathogenesis of viruses to facilitate their
329 transmission to new hosts. We speculate that the related Iflaviruses, SBV and DWV, induce
330 different responses in their host that suit their principal or historical route of transmission. We
331 further suggest that the different effects on the expression of the immune-related genes in the
332 honeybee in response to SBV and DWV may have been selected during evolution of SBV and
333 DWV and have made these viruses evolutionarily adjusted to the principal routes of their
334 transmission. DWV, in the absence of the *Varroa* mite vectoring, could be transmitted both
335 vertically through queens and drones, and orally (de Miranda & Genersch, 2010; Yue &
336 Genersch, 2005). DWV infection of the honeybee larvae does not halt development and does not
337 cause early death at the larval stage, which suggests that honeybee survival is essential for DWV
338 transmission and that this virus has evolved to minimize negative impact on its honeybee host.
339 (Fujiyuki et al., 2004; Ryabov et al., 2014). On the other hand, horizontal oral transmission is
340 considered to be a principal route for SBV, which causes acute infections at the larval stage
341 leading to death of infected insects, and the spread of SBV is likely to involve cannibalization of
342 the diseased larvae (Schmickl & Crailsheim, 2001; Woyke J, 1977). Therefore, the observed
343 suppression of the melanisation pathway in the SBV-infected larvae, which could be a
344 consequence of the combined down-regulation of PPAE and up-regulation of serpin (GB48820),

345 may be beneficial for SBV transmission because melanisation contributes to resistance to viruses
346 and could decrease infectivity of SBV virus particles in the larvae and decrease efficiency of its
347 horizontal transmission (Fig. 5), for example, similarly to the suppression of Semliki virus by
348 phenoloxidase cascade in mosquito (Rodriguez-Andres et al., 2012). Suppression of melanisation
349 in the long-term, may reduce resistance to pathogens and negatively impact on honeybee
350 survival. Therefore such suppression of melanisation may have a negative impact on DWV,
351 which relies more on vertical transmission; it is beneficial for DWV-infected honeybees to have
352 functional melanisation pathways (Fig. 5).

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

360

361 **Conclusions**

362 Our results indicate that SBV and DWV, evolutionarily related RNA viruses, evoke
363 different transcriptional responses in their honeybee host, including effects on the expression of
364 immune-related genes. Honeybee larvae with high levels of SBV replication, showed concerted
365 up-regulated expression of antimicrobial peptides (AMPs) and down-regulated expression of the
366 prophenoloxidase-activating enzyme (PPAE) together with up-regulation of the expression of a
367 putative serpin, which could lead to the suppression of the melanisation pathway. The same

368 effect was observed in the individuals with high levels of both SBV and DWV, but high levels of
369 DWV alone did not affect expression of the AMPs and the genes involved in the regulation of
370 melanisation. The effects of high SBV replication levels on expression of these immune genes
371 were unlikely to be the consequences SBV-induced developmental changes, because some of
372 them were observed in honeybees infected with SBV by injection at the pupal stage. It is
373 possible that different impacts of SBV and DWV on the expression of immune-related genes
374 may be an adaptation to horizontal and vertical transmission routes, the principal transmission
375 routes of SBV and DWV respectively. These findings could be used to further investigate the
376 role of AMPs and melanisation in virus-host interaction and transmission of insect viruses,
377 including economically important viruses of the honeybee.

378

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386

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Table 1 (on next page)

Table 1

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

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

Sample ID	Treatment group	ENA sample accession	Total reads	<i>A. mellifera</i> 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 BP terms with the lowest p -values are shown).

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.

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 (\log_2 transformed)			
				Contrast 2 High SBV+DWV vs. high DWV and control	Contrast 3 High DWV vs. control	Contrast 4 High SBV+DWV vs. control	Contrast 5 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	.

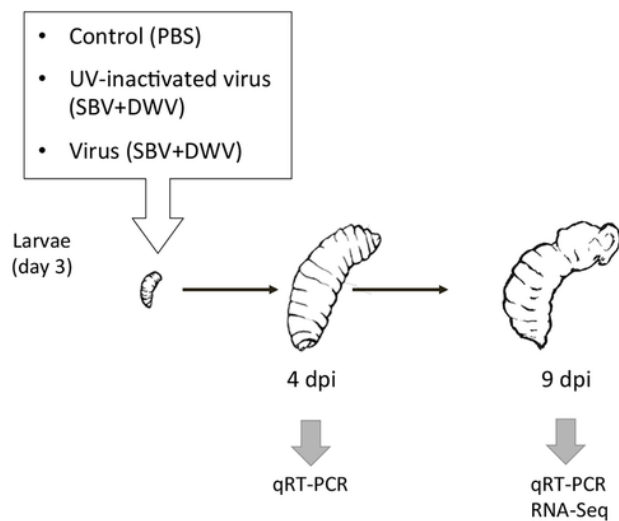
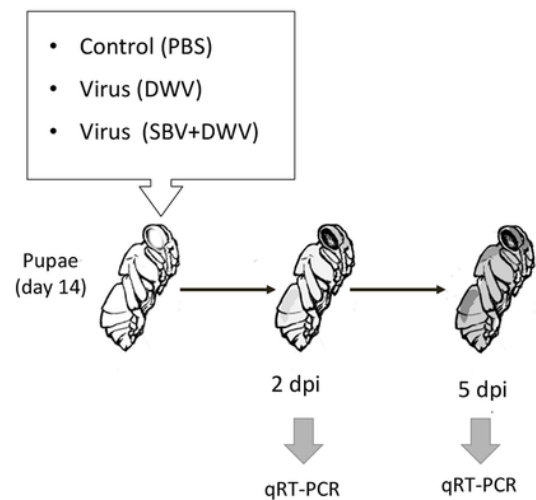
GB44055	FBgn0000250	cactus (NF-kappa-B inhibitor)	Toll	.	.	2.372	2.457
GB50418	FBgn0262473	Toll-1 (Receptor)	Toll	2.073	.	2.104	1.962
GB51741	FBgn0030310	Peptidoglycan recognition protein SA	Toll	2.070	.	2.056	2.119
GB52631	FBgn0003495	spatzle	Toll	3.224	.	3.284	3.012
GB51498	FBgn0033402	Myd88	Toll	.	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	.

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Figure 1

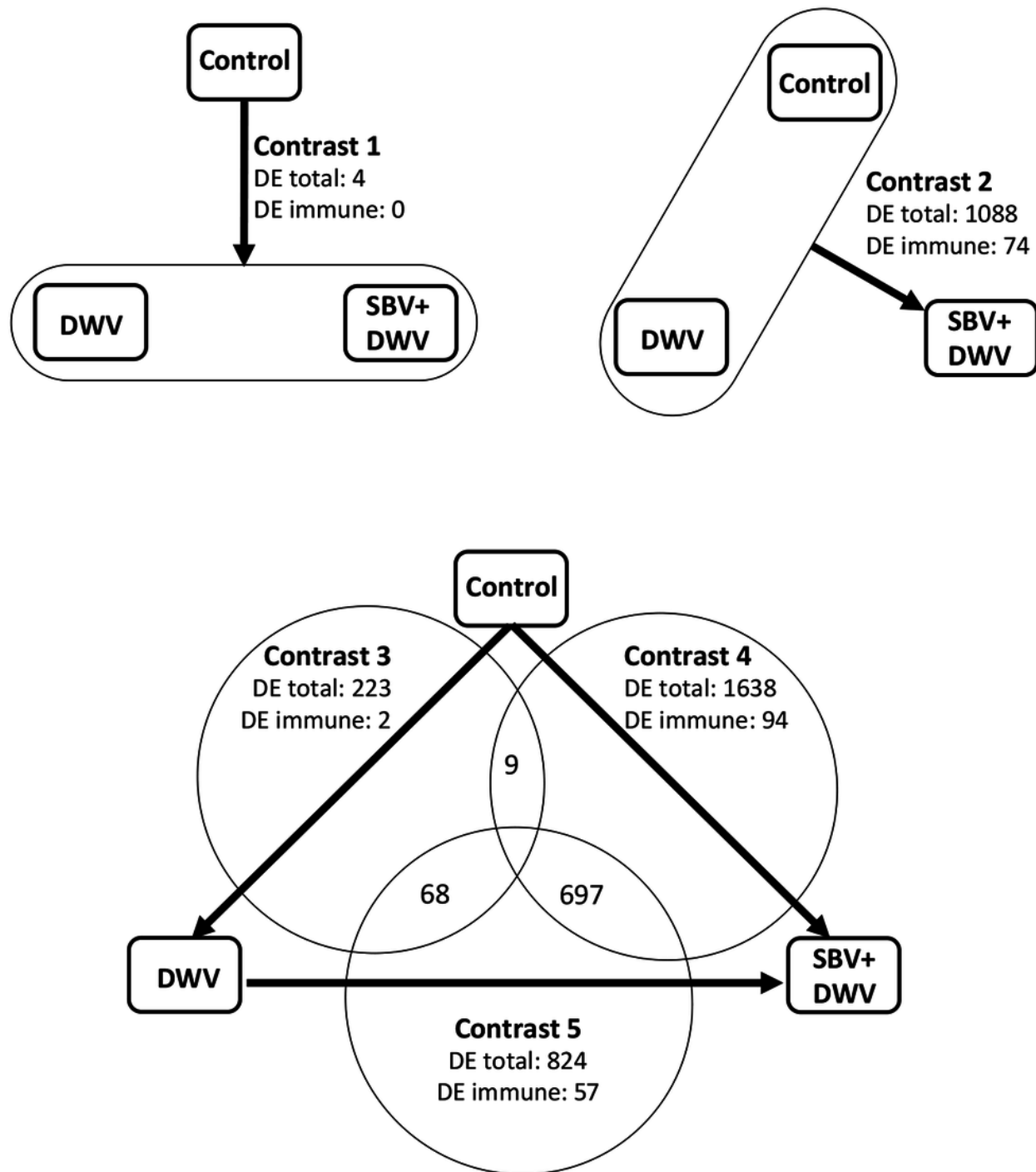
Schematic representation of experimental infection of honeybees with SBV and DWV, (A) larval oral inoculation, and (B) pupal haemolymph injection.

A. Oral inoculation of honeybee larvae**B. Haemolymph injection of honeybee pupae**

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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.

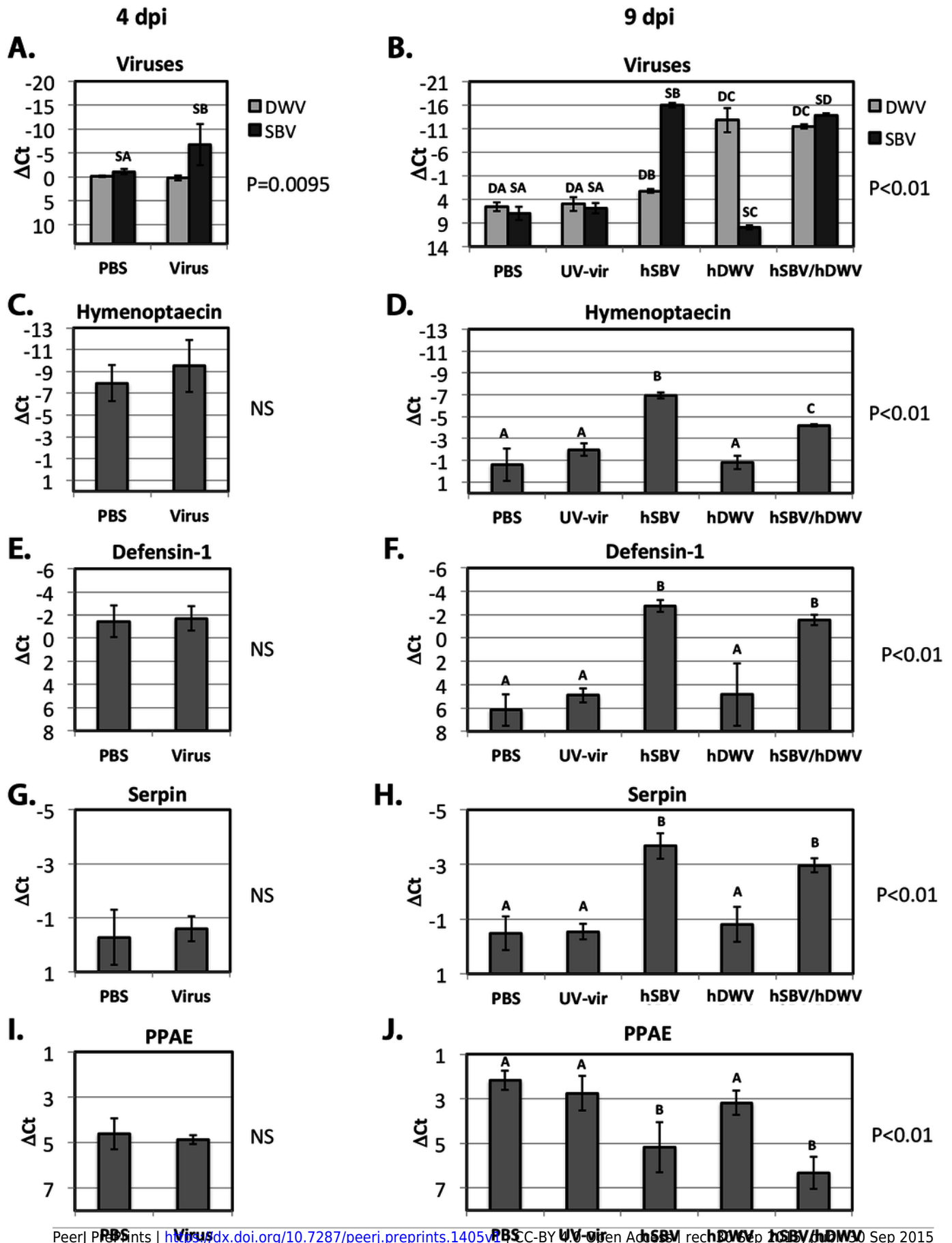


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Figure 3

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 Δ Ct 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

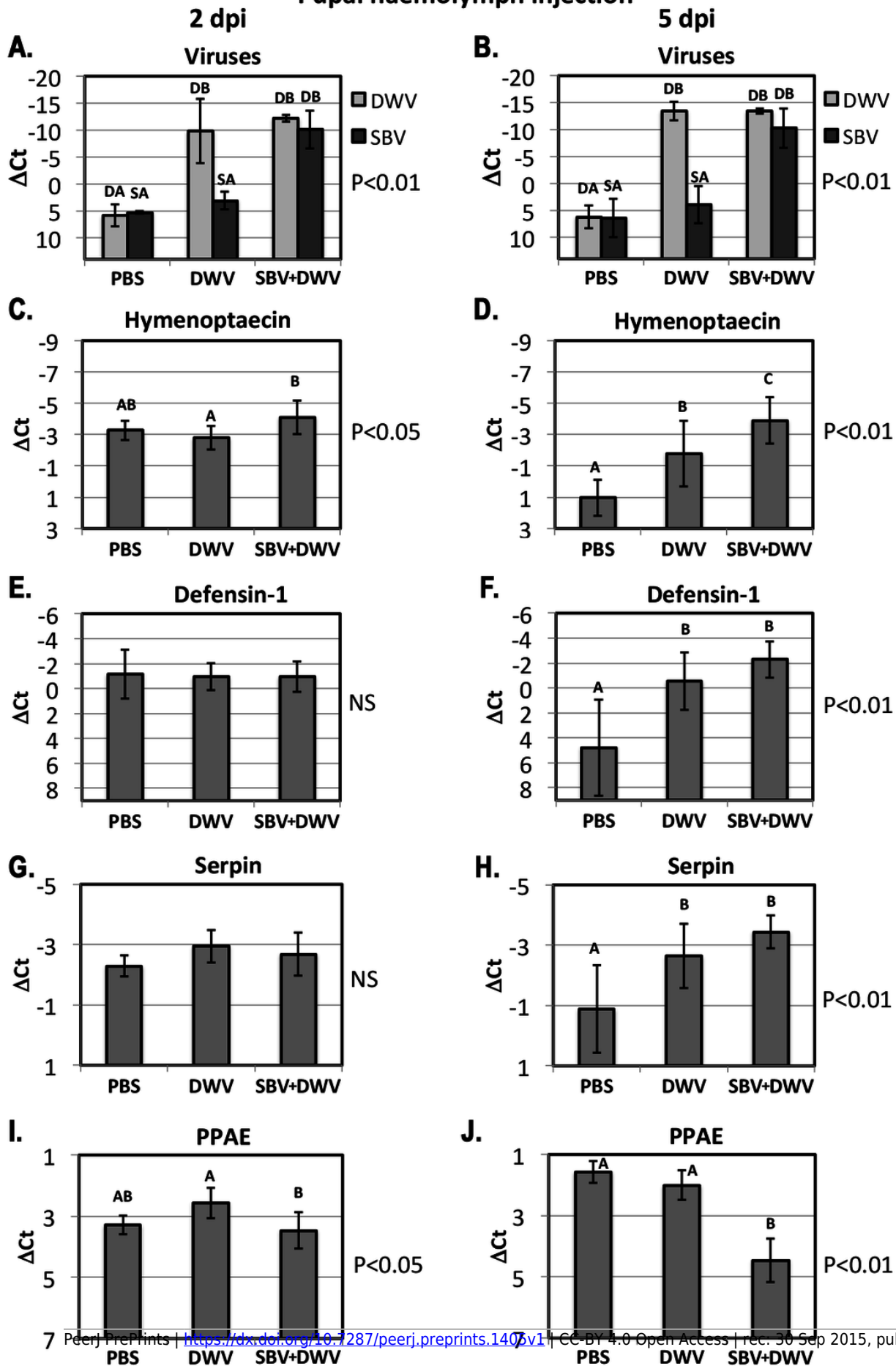


4

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 Δ Ct 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



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Figure 5

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

