Deformed Wing Virus spillover from honey bees to bumble bees: a reverse genetic study.

Running title: Deformed Wing Virus spillover in bumble bees.

Olesya N Gusachenko[1], Luke Woodford[1], Katharin Balbirnie-Cumming[2], Eugene V Ryabov[3], David J Evans[1]

[1]Evans laboratory, Biomedical Sciences Research Complex, University of St. Andrews, North Haugh, St. Andrews, UK; [2]Centre for Inflammation Research, Queen`s Medical Research Institute, University of Edinburgh, Edinburgh, UK; [3]USDA-ARS Bee Research Laboratory, Beltsville Agricultural Research Center, Beltsville, MD 20705, USA.

Abstract

Deformed wing virus (DWV) is a persistent pathogen of European honey bees and the major contributor to overwintering colony losses. The prevalence of DWV in honey bees has led to significant concerns about spillover of the virus to other pollinating species. Bumble bees are both a major group of wild and commercially-reared pollinators. Several studies have reported pathogen spillover of DWV from honey bees to bumble bees, but evidence of a true sustained viral infection has yet to be demonstrated. Here we investigate the infectivity and transmission of DWV in bumble bees using the buff-tailed bumble bee *Bombus terrestris* as a model. We apply a reverse genetics approach combined with controlled laboratory conditions to detect and monitor DWV infection. A novel reverse genetics system for three representative DWV variants, including the two master variants of DWV - type A and B - was used. Our results directly confirm DWV replication in bumble bees but also demonstrate striking resistance to infection by certain routes. Bumble bees may support DWV replication but it is not clear how infection could occur under natural environmental conditions.

Introduction

Deformed wing virus (DWV) is a widely established pathogen of the European honey bee, *Apis mellifera*. In synergistic action with its vector — the parasitic mite *Varroa destructor* — it has had a devastating impact on the health of honey bee colonies globally. As the primary managed insect pollinator, honey bees are of high ecological and economic value and contribute an estimated 30-50% of mobile pollination activity [1, 2]. Two-thirds of all colonies in the USA (~1.6 million hives) are transported to California in February/March for almond pollination [3]. Inevitably, transporting bees also transports their pathogens. This, coupled with the local pathogen density associated with ~50 000 bees in a single hive, has raised concerns about pathogen spillover from managed honey bees to other pollinators [4]. DWV was found as a frequent component of pollen pellets [5] and is also present in bee faeces [6], suggesting honey bee foragers and colonies could facilitate horizontal virus transmission to the wider pollinator community. Significantly, DWV RNA has been detected in many insects sharing the environment with managed honey bees, including Asian bee species and wild bees, cockroaches, ants, wasps, and bumble bees [4, 5, 7–19]. Due to their extended activity at lower temperatures (compared to honey bees) bumble bees are considered particularly important pollinators in temperate and subarctic climates [20, 21] and are the only other insect managed for commercial-scale pollination [21]. As a consequence, the potentially negative impact of extensive honey bee management and failing pathogen control on co-located *Bombus* species has received significant attention.

Following a report that DWV was detected in symptomatic *Bombus terrestris* and *Bombus pascuorum* individuals with deformed wings [7] there have been several studies of DWV prevalence in a wide range of *Bombus* species [4, 5, 11, 12, 22–24]. In *Varroa*-infested honey bee colonies DWV levels can exceed 10^{11} genome copies per bee [25], with considerable potential for environmental contamination. DWV-positive *Bombus sp*. have been shown to correlate to areas with high DWV prevalence in *Apis* [4, 23, 24]. The majority of screening

studies have used RT-PCR for DWV detecting both positive and negative strand DWV RNA in environmental bumble bee samples (reviewed in [26]). However, the near ubiquitous presence of managed hives, the honey bee – and consequently pathogen – density around hives, and the foraging range of *Apis* mean that DWV is likely widespread [5]. Although detailed analysis of DWV replication requires detection of the negative strand intermediate of replication it should be noted that this alone is not formal proof that the virus is replicating. There remains the possibility that negative strands are present as carry-over in *Apis*-derived cellular material ingested by bumble bees.

The name DWV is currently attributed to an evolving complex of closely related viruses, which includes DWV-A [27], Kakugo virus [28], Varroa destructor virus-1 (VDV-1; also referred to as DWV-B [29–31]) and a range of recombinants between DWV-A and -B [32–35]. All viruses exhibit at least 84% identity at the nucleotide level and 95% identity at the protein level [32, 35, 36]. The sensitivity of current diagnostic methods means DWV detection in environmental samples is regularly reported, with different DWV variants identified in *Bombus* [4]. Far less frequent are studies investigating potential routes of transmission from *Apis* to *Bombus*, or the subsequent replication of DWV in bumble bees. Due to the absence of a suitable cell line for *in vitro* propagation, laboratory-based assays have been limited to the application of field-sourced virus. Infectivity of DWV obtained from field honey bee samples was tested via inoculations of adult *Bombus terrestris* [4, 22]. It was reported that a DWV complex containing both DWV-A and -B is infectious when fed at high concentrations - 10^9 GE of virus per bee [4].

We have used a reverse genetic (RG) approach to generate near-clonal populations of genetically tagged DWV-A, -B and a B/A hybrid after transfection of honey bees with *in vitro* transcribed RNA. A similar system has recently been reported for DWV-A [37–39]. Using RG-derived DWV inocula we address the question of DWV pathogenesis and likely transmission routes in *Bombus terrestris* at both the individual and colony level. Using this strategy we

provide direct evidence of DWV replication in this pollinator via virus feeding and injection. Importantly, adult *Bombus terrestris* appear resistant to productive infection by DWV orally and do not exhibit the developmental defects characteristic of DWV infection and replication in honey bees.

Results

Infectivity of DWV RNA and virus in injected honey bee and bumble bee pupae

In order to test infectivity of DWV variants in controlled laboratory experiments we have developed RG systems for DWV-A and -B master variants. DWV cDNAs were built using gene synthesis based upon published sequences [27, 40, 41] and that of a recombinant DWV reported earlier [25]. The RG constructs design is similar to a recently reported system for DWV-A [37] with a full-length DWV cDNA placed under transcriptional control of a T7 promoter. RNA generated *in vitro* is directly injected into honey bee pupae from which infectious virus is recovered. To allow discrimination from any endogenous DWV we engineered synonymous mutations creating new restriction sites, allowing the unambiguous identification of the RG genomes. The following nomenclature was used for the generated viruses: “VDD” - type A DWV, “VVV” - type B, “VVD” - B/A recombinant (Figure 1A).

In temperate climates honey bee brood is only available for ~50% of the year and *in vivo* research is of necessity seasonal. Although the DWV IRES retains partial activity in at least one cell line of non-honey bee origin (*Lymanthria dispar* LD652Y cells) [42], attempted infection of those cells with DWV or transfection of *in vitro*-generated DWV RNA did not result in virus replication (OG, unpublished data). This impediment to DWV studies prompted us to investigate the recovery of clonal stocks of DWV in bumble bees (*Bombus terrestris audax*) injected with viral RNA. Commercially farmed bumble bee colonies are available year-round and, in our preliminary studies, are free of DWV RNA (data not shown). Four of six RNA-injected bumble bee pupae tested positive for DWV, while all honey bee pupae injected with full-length RNA

were shown to be DWV-positive (Figure 1B, C). The RG origin of DWV in injected samples was confirmed by restriction digest of PCR products using endonucleases specific for the introduced genetic tag (Figure 1B,C - restriction digest). The remaining tissue from RNA-injected samples was used to prepare crude DWV stocks. RT-qPCR analysis showed that the *Apis*-derived stocks contained between 10^7-10^9 genome equivalents (GE) of DWV/µl, while *Bombus*-derived inoculum contained 3.75×10^5 GE/µl. For comparison, DWV levels in mock-injected *Apis* pupae did not exceed 10^5 GE DWV/µg of RNA (representing the endogenous viral load) and *Bombus* pupae had no detectable DWV sequences in mock-inoculated samples.

DWV extracted from *Bombus* was infectious when re-inoculated by injection to *Bombus* pupae. We observed no differences in the infectivity of *Apis*- or *Bombus*-derived DWV (Figure S1) and, at the genome level, no obvious signs of adaptation following comparison of the parental cDNA sequence with NGS data from the second passage of *Bombus*-derived DWV (Figure S2).

In honey bees, both DWV-A and -B are pathogenic when inoculated, though recent studies have produced contradictory results when comparing their relative virulence [29, 41, 43]. We therefore tested infectivity of DWV types A, B and a B/A hybrid in bumble bee brood and adults. As our primary interest was to investigate the potential for DWV spillover from infected honey bees we used DWV inoculates prepared from RNA-injected honey bee pupae for all further experiments.

White-eyed (1 day post molting) or purple-eyed (5.5 days) bumble bee pupae were injected with 10^3 or 10^6 GE of each DWV variant, and virus levels quantified 48 hours post-inoculation. In all cases we observed a 10^2-10^4 increase in total DWV load compared to the inoculated amount, providing clear evidence for replication (Figure 2A). More DWV accumulated in older pupae but this was only statistically significant for 10^6 GE of the VVV variant (Tukey's multiple comparisons test, P=0.03).

<u>*Bombus* larvae can be infected with DWV *per os*</u>

124 Since bumble bee pupae are not parasitised by *Varroa* naturally-infected pupae must acquire
125 the virus by prior exposure of larvae. We therefore investigated virus infection after feeding
126 DWV to 1st and 2nd instar *Bombus* larvae (age 1 and 2 on Figure 2B respectively). Each larva
127 individually received a single dose of diet containing 10^8 GE of DWV on the first day of the
128 experiment. DWV was detectable in all fed larvae on the 5th day post inoculation, although virus
129 levels were comparable to the amount administered in the diet. When analysed ~50% of fed
130 larvae had detectable levels of DWV negative strand RNA, absent from the inocula and
131 indicative of virus replication.

132 Investigation of colony scale transmission of DWV and adult infection *per os*

133 In honey bees, horizontal transmission of DWV occurs *per os* in larvae or adults, or when
134 *Varroa* feed on pupae and adults. Whilst we show here that DWV can replicate in *Bombus sp.*
135 pupae after direct injection, and in virus fed larvae, the route by which adult bumble bees could
136 become infected remains unclear. We reasoned that two likely routes would be via direct oral
137 exposure of adult *Bombus* to virus in the environment or indirectly following larval infection with
138 virus carried by adult bees.

139 Groups of adult bumble bees received 10^7 or 10^8 DWV GE per bee via feeding with virus-
140 supplemented sucrose solution. All control group bees remained viable during the experiment,
141 and only one dead bee was found in each of the virus fed groups. Bees were analyzed 7 days
142 after DWV feeding when none of the virus-fed samples tested positive for DWV (Figure 3).
143 According to qPCR analysis of DWV-fed bees no sample contained greater than 10^5 GE DWV
144 per 1 μg of total RNA. Average RNA yield per bee did not exceed 50 μg and therefore the level
145 of virus detected was 100-200-fold lower than the amount received. Hence, despite evidence
146 that bumble bee larvae displayed susceptibility to DWV infection *per os*, feeding of adult bumble
147 bees up to 10^8 GE DWV per bee did not lead to infection or detectable replication.

We extended this study to investigate whole-nest inoculation with DWV. Three individual bumble bee nests were fed for 4-6 weeks with a sucrose solution supplemented with $2x10^8$ GE/adult bee/day of VVD, VVV or VDD DWV. All brood (pooled egg samples, 30 larvae, 47 pupae) and 30 adults from virus-exposed nests were screened for DWV using end-point RT-PCR assay and showed no positive results (data not shown).

Direct inoculation of adult bumble bees

With no evidence that adult bumble bees could be infected when fed a DWV-supplemented diet, or that they were able to transfer virus to larvae, we performed direct virus injections of adult bumble bees to determine whether adults could support DWV replication. Two groups of adult bumble bees were intra-abdominally injected with 10^4 or 10^8 DWV GE per bee. Envisaging a possible impact of injection on viability, an additional group of bumble bees was injected with PBS solution only. 20-30% of injected bees died before termination of the experiment in each group. The remaining bumble bees were screened for DWV RNA 8 days post-inoculation. End-point PCR analysis indicated that 40% and 100% were DWV positive in the 10^4-injected and 10^8-injected groups respectively (Figure 3A), with qPCR analysis showing that the virus levels in these groups ranged between 2.74×10^4-1.39×10^7 and 4.82×10^6-2.13×10^8 GE/µg of total RNA respectively. Accumulation of DWV (-)RNA in DWV-positive samples was confirmed by strand-specific RT-PCR assay (Figure 3B). This demonstrates that DWV can replicate in adult *Bombus terrestris* after direct injection at 10^8 GE per bee. We therefore proceeded to investigate if there were any pathogenic consequences of virus infection and replication.

Pathogenicity of DWV in *Bombus terrestris*

DWV produces characteristic pathogenicity in honey bees and similar symptoms have been reported in bumble bees [7]. We injected 43 white-eyed bumble bee pupae with 10^6 GE of DWV and maintained them through development in an incubator. In parallel, a group of 37 similarly-aged pupae were injected with PBS. All fully-developed bumble bees could be classified into

one of three groups: normal phenotype, discolored with normal wings and non-viables, which did not eclose (Figure 4). Analysis by qPCR demonstrated that all DWV-inoculated bumble bees contained high levels of virus (8.8×10^{8}-2.2×10^{10} GE/µg of RNA) with no significant differences in viral load between the three phenotypic groups (ANOVA, P=0.21) (Figure 4). DWV levels were 1-2 $\log_{10}$ greater than in pupae analysed 48 hours post-inoculation (Figure 2A) reflecting the additional time the virus had to replicate. This further supports the conclusion that pupae can be infected with DWV by direct inoculation. Strikingly, none of the eclosed bumble bees showed any signs of wing deformities that are characteristic of DWV infection of honey bees. Inspection of PBS-injected pupae showed that they could be separated into the same phenotypical groups; normal, discoloured and dead. There was no statistical difference between the proportions in each group of virus- or PBS-injected bumble bees (ANOVA, P>0.999). Upon analysis, none of the mock inoculated group samples showed evidence of DWV infection. In parallel, control injection of honey bee pupae with the same virus preparation caused characteristic wing deformities in eclosed bees (Figure S4).

Discussion

The term “pathogen spillover” describes the transmission of a pathogen from a reservoir host to a different species in a shared environment [44]. To achieve this, several conditions must be fulfilled; the reservoir host must be sufficiently abundant to guarantee exposure, pathogen prevalence must be high enough to ensure direct or environmental transmission, and the recipient host must be susceptible to infection via direct or indirect transmission [45].

Managed honey bee colonies are near-ubiquitous in many environments, including both rural and urban locations. On agricultural crops that require commercial pollination very high colony densities are achieved through migratory beekeeping. The combination of colony movements following the introduction of *Varroa destructor* resulted in the near-global distribution of the ectoparasite, with the concomitant spread of a range of honey bee viruses that are detrimental

198 to colony survival [29]. Most important of these viruses is DWV which likely accounts for the
199 majority of overwintering colony losses [46, 47]. DWV causes overt and lethal developmental
200 defects after pupal inoculation and reduces the longevity of bees that do successfully emerge.
201 Colonies that collapse due to high *Varroa*/DWV levels may be robbed-out by other insects
202 including bumble bees, ants and wasps.

203 In addition to the cocktail of *Varroa* and DWV, honey bees are increasingly subject to stresses
204 through a combination of limited dietary variation, repeated transportation to new pollination
205 sites and exposure to agrochemicals, all of which are associated with increased susceptibility to
206 pathogens and potential colony failure [48]. As a consequence, honey bees readily fulfill two of
207 the requirements needed for pathogen spillover *i.e.* abundance in the environment and
208 pathogen prevalence. In addition, due to the extensive foraging range of honey bees and the
209 excretion of viable DWV in faeces [6], a high density of hives ensures widespread
210 environmental contamination.

211 Notwithstanding the likely exposure of other species to the pathogen-laden environment,
212 spillover also requires susceptibility of the species exposed and pathogen infectivity via a
213 relevant transmission route. We exploited commercially available colonies of *Bombus terrestris*
214 to determine if and how they could support infection by DWV. The buff-tailed bumble bee is a
215 relevant model system in which to explore pathogen spillover; it is naturally found in the same
216 environment as honey bees [4], and there are reports of DWV infection of wild-caught *B.*
217 *terrestris* and several related species [4, 5, 11, 12, 22–24].

218 We developed a RG system for the two prototype strains of DWV (type A and B) and a hybrid
219 previously reported to predominate in *Varroa*-infested colonies [32]. The synthesized virus
220 genomes contain silent restriction sites that unambiguously distinguish the three types and –
221 since they are unique to the RG cDNAs – allow discrimination from endogenous DWV. Direct
222 inoculation of honey bee pupae with *in vitro* generated RNA resulted in DWV replication (Figure

223 2). Virus purified from these pupae caused symptomatic infection when reinoculated into honey
224 bee pupae and accumulated to 10^{10} GE/µg of RNA (Figure S4), a level similar to that observed
225 in *Varroa*-exposed pupae [25].

226 Using a similar strategy we demonstrated that bumble bee pupae could be infected when
227 inoculated with *in vitro* generated DWV RNA. The resulting virus was purified, shown to be
228 infectious for naïve bumble bee pupae when re-injected and deep sequencing of the virus
229 population indicated no significant sequence changes from the originating cDNA (Figure S2).
230 Infected pupae reached up to ~10^9 GE of DWV/µg of RNA two days post-injection (Figure 2A)
231 and accumulation of the negative strand DWV RNA was confirmed by strand-specific qPCR
232 (Figure S3). Therefore DWV is infectious for both honey bee and bumble bee pupae and
233 adaptive changes are unlikely to create a bottleneck in any potential transmission between the
234 species.

235 Direct recovery and amplification of DWV in bumble bees offers advantages to virologists and
236 entomologists attempting to determine the significance of the limited differences between the
237 reported strains. Since essentially all honey bee pupae are infected with DWV, perhaps with the
238 exclusion of those from Australia [49], it is impossible to obtain truly clonal virus preparations.
239 By recovering virus after RNA inoculation in bumble bees pure populations of DWV strains can
240 be prepared for subsequent studies of virus pathogenesis and evolution.

241 Since *Varroa* does not parasitise bumble bee pupae it is difficult to rationalise direct injection as
242 a potential transmission route. Therefore, considering robbing by bumble bees of collapsing
243 honey bee colonies and the likely widespread DWV contamination in environments with large
244 numbers of honey bees, we reasoned that oral transmission was a more likely route for virus
245 acquisition. We therefore investigated oral susceptibility of larvae and adult bumble bees, fed
246 directly or by extended exposure of bumble bee nests to virus-supplemented diet.

247 Direct feeding with 10^8 GE of DWV enabled the detection of the negative strand of DWV, absent

248 from the input virus preparation and indicative of virus replication, in ~50% of larvae (Figure 2).
249 In contrast, DWV fed adult bumble bees, or larvae and pupae harvested from nests
250 supplemented with diet containing 10^8 GE of DWV per bee, failed to provide any evidence for
251 virus acquisition and transmission *per os*. Therefore, whilst larvae exhibit some susceptibility to
252 infection by orally acquired DWV, it is clear that the threshold for infection may be high and that
253 it cannot be achieved by extended feeding by adult bees throughout larval development.

254 Although we found no evidence for oral infection of adult bumble bees with DWV, we were able
255 to demonstrate the presence of DWV in adult *Bombus* after direct virus injections. Adult bumble
256 bees are able to support replication of DWV though, as with pupal inoculation with RNA, there
257 may be a threshold (exceeded at 10^8 GE) needed to achieve 100% infection.

258 In this study we allowed pupae to complete development and scored them phenotypically upon
259 eclosion. Bumble bee pupae are susceptible to handling and survival rates (~50%) were similar
260 in virus- or mock-inoculated samples (Figure 4). Amongst the three phenotypically-distinct
261 groups there was no difference in viral load in virus inoculated samples. Strikingly, the same
262 three groups and the proportion of each were present in the mock-inoculated samples. No wing
263 deformities were detected in eclosed bumble bees from either group.

264 It has previously been reported that DWV-positive field isolates of both *B. terrestris* and *B.*
265 *pascuorum* have been identified with wing deformities characteristic of those seen in DWV-
266 infected honey bees [7]. However, these studies did not demonstrate DWV replication or
267 reproduce symptoms by DWV inoculation. In honey bees apart from DWV infection, wing
268 malformation can be caused during development by injury, hormonal disorders, intoxication or
269 absence of cocoon [37]. While laboratory inoculations represent the gold standard for virus
270 infectivity assays this approach probably does not recapitulate an environmentally meaningful
271 infection route. Compared to their independent effect a combination of stressors is suggested to
272 introduce a greater threat to wild pollinators in their native environment [50]. For example,

exposure to clothianidin - a neonicotinoid insecticide - was found to have a negative impact on honey bee immune status and promote DWV infection [51]. In *Bombus terrestris* condition mediated virulence of Slow bee paralysis virus upon starvation has been demonstrated [52].

This study uses a reverse genetic approach to investigate pathogen spillover from honey bees to bumble bees. Whilst clear evidence is obtained to confirm DWV replication in bumble bee larvae, pupae and adults, we were unable to demonstrate a compelling route by which transmission would likely occur in the natural environment. The levels of virus required to orally infect bumble bee larvae are significantly higher than have been reported in environmental pollen samples [53]. Indeed, the levels required are likely higher than larvae are ever exposed to. In contrast, adult bumble bees may experience very high virus levels in collapsing honey bee colonies while robbing. However, adult bumble bees feeding on virus-supplemented syrup remain uninfected and – importantly – are unable to transmit infectious virus to their developing larvae. Further studies will be required to determine whether the gut environment of adult bumble bees is sufficiently hostile to DWV that the virus is inactivated *e.g* by gut proteases, or if there are other factors that restrict infection and replication of DWV in bumble bees.

Previous reports strongly underline the haplotype identity of DWV between honey bee and bumble bee populations [4, 24]. Published data show that majority of the virus is located in the gut, which can be interpreted either as a restriction of infection to the digestive tract or as presence of DWV in the ingested material [22]. The results obtained from this study provides a strong indication that oral acquisition of virus from a contaminated environment does not represent an effective DWV transmission route between *Apis* and *Bombus*. Bumble bees are known to carry their own mite parasites, with *Apicystis bombi* and *Crithidia bombi* found in honey bee collected pollen [54]. However, these mites do not parasitise honey bees and lack feeding behaviour similar to Varroa or the capacity for virus vectoring. Therefore, a route for productive DWV spillover to bumble bees from infected honey bees remains to be determined.

We propose that ubiquitous detection of DWV RNA in geographically related bumble bees primarily reflects the carryover material originating from virus prevalence in the abundant honey bee population, whereas incidence of pathogen spillover is limited to the cases of conditioned infection. Further studies on defining the stressors causing DWV infectivity in bumble bees are required in order to estimate the actual impact of DWV on this important group of pollinators.

Materials and Methods

Honey bees

All honey bee (*Apis mellifera*) brood were collected from the University of St Andrews research apiary. All hives used for sampling were routinely treated for *Varroa* with an appropriate miticide. Pupae were extracted from the comb and maintained in the incubator set at 34.5°C with 90% relative humidity.

Bumble bees

Bumble bees *(Bombus terrestris audax*; Biobest, Belgium) were maintained in the laboratory in isolated nests supplemented with feeders containing 50% aqueous sucrose solution. Nests were regularly fed with bee pollen from a DWV-free region (Saxonbee, Australia). Separately treated adult bumble bees were maintained in cages at RT and fed *ad libidum* on pollen/syrup. Pupae and larvae were extracted from the brood cells and transferred into the incubator set at 30.5°C with 90% relative humidity. Larvae were fed a diet consisting of 25% (v:v) ground pollen and 25% sucrose solution (w:v) in H_2O.

RG system and *in vitro* RNA synthesis

All RG constructs in this study were based on a cDNA clone of a recombinant DWV variant VDV-1_{VVD} (GenBank HM067438.1). The initial pVVD construct was made using a similar protocol described for previously published reverse genetics systems of DWV [37, 39]. Briefly, the full-length cDNA was cloned in a pBR-derived plasmid vector, flanked at the 5' end by a T7

322 promoter and hammerhead ribozyme [55], and at the 3' end by a *Pme* I restriction site following
323 the poly-A tail. VDD (DWV-A) and VVV (DWV-B) constructs were built by substituting parts of
324 pVVD, with the DWV-A capsid-coding and DWV-B non-structural protein-coding cDNA
325 sequences based on published data [27, 40, 41] and produced by custom gene synthesis (IDT,
326 Leuven, Belgium). Unique synonymous restriction sites were introduced into each cDNA
327 sequence. All resulting plasmids were verified by Sanger sequencing.

328 RNA was generated *in vitro* from linearised templates, using the T7 RiboMAX™ Express Large
329 Scale RNA Production System (Promega). Full length and truncated templates were linearised
330 with *Pme* I or *Nru* I (nt 9231) respectively. Truncated RNA transcripts were polyadenylated
331 using the poly(A) Tailing Kit (Thermo Fisher Scientific) to ensure their stability. After purification,
332 RNA transcripts were eluted in RNAse free H_2O, their integrity confirmed by gel electrophoresis,
333 and stored at -80°C.

334 RNA and virus injections

335 RNA and virus injections of 5 and 10 µl were performed with insulin syringes (BD Micro Fine
336 Plus, 1 ml, 30 G) into honey bee and bumble bee pupae respectively. Up to 10 µg (equivalent to
337 1.7×10^{12} of DWV RNA copies) of *in vitro* transcribed RNA was injected individually into pupae.
338 Truncated VVD transcript injections were used as a negative control. All RNA-injected pupae
339 were analyzed at 72 h post injection. Injections of pupae or low temperature-immobilized adult
340 bees with virus stocks diluted in PBS were performed using the same technique as for RNA
341 transcripts.

342 Virus stocks

343 DWV stocks were prepared from pupae injected with *in vitro* transcribed RNA. Homogenized
344 tissue was diluted with sterile PBS in 1:1 (w:v) ratio and centrifuged at 13 000 g, 4°C for 10 min.
345 Supernatant was filter sterilized with 0.22 µM filter (PES, Millipore) and treated with RNase A to

346 destroy all unencapsidated RNA. RNA was extracted from 100 µl of the virus stock using
347 RNeasy kit (Qiagen) and analyzed by RT-qPCR. Apart from the virus inoculum used for testing
348 the infectivity of the *Bombus*-derived DWV, all virus stocks were prepared from honey bee
349 pupae.

350 Oral inoculation and bumble bee nest feeding

351 Individual bumble bee larvae were placed into 96 well plates containing 2 µl of pollen/syrup
352 mixed with DWV inoculum. DWV feeding was delivered once on the first day of the experiment.
353 Fresh diet without virus was added individually after virus-containing food had been consumed.
354 On the fifth day larvae were snap-frozen in liquid nitrogen and stored at -80°C.

355 Adult bumble bees were fed with virus in groups of 20 on the first and on the second day of the
356 experiment. Total amount of DWV supplied with each of the two virus feedings was 2.2×10^{9} and
357 2.2×10^{8} DWV GE for 10^{8} and 10^{7} groups respectively. Bumble bees were snap-frozen in liquid
358 nitrogen and analyzed for the presence of DWV RNA 7 days after DWV feeding.

359 For the colony-scale inoculations three bumble bee nests each containing a mated queen and
360 120-125 adult bees were used. Prior to the start of the experiment all brood was removed from
361 the nests. Virus feeding was delivered daily by replacing the nest feeder with a tube containing
362 2 ml of sucrose solution supplemented with DWV, previously confirmed to be infectious for
363 bumble bee pupae by direct injection. Each nest received a daily dose of the virus
364 corresponding to 2.4×10^{10} DWV GE. The virus-containing solution was fully consumed (<3-4 h)
365 before replacement with the regular sucrose feeder. DWV feeding continued for 4-6 weeks and
366 stopped when the first group of larvae developed during the virus-feeding period approached
367 pupation. Upon termination of the experiment all brood (eggs, larvae and pupae) and 10 adult
368 workers were sampled from each nest and tested for the presence of DWV.

369 RNA extraction, reverse transcription and PCR

370 Samples were homogenized with a Precellys Evolution homogenizer (Bertin Instruments,
371 France). Total RNA was extracted using the GeneJet RNA Purification Kit (Thermo Scientific).
372 cDNA was prepared with qScript cDNA Synthesis Kit (Quanta Biosciences) from 1 µg of total
373 RNA following the manufacturer's protocol. Detection of DWV and actin mRNA, used as an
374 internal RNA quality control, was carried out by end-point PCR with *Taq* DNA polymerase (New
375 England Biolabs) and 2 µl of cDNA. DWV_RTPCR primers were designed to detect all three
376 DWV variants under study (Table S1). To amplify the regions containing restriction site tags in
377 VDD and VVV Kpn2I_F/R and PflFI_F/R primer pairs were used respectively. PCR cycling
378 conditions were 30 cycles of 95°C (15 seconds), 55°C (15 seconds), 68°C (2 minutes) with an
379 initial 95°C step (30 seconds) and a final extension at 68°C (5 minutes). PCR samples were
380 analyzed on a 1% agarose gel and when required, PCR products were subjected to restriction
381 digest prior to loading on the gel.

382 The quantification of DWV genome copies was performed by SYBR-Green Real-Time
383 Quantitative PCR (qPCR) using Luna Universal qPCR master mix (New England Biolabs), 0.25
384 µM forward and reverse DWV_qPCR primers and 2 µl of cDNA. The following thermal profile
385 was used: 1 min at 95°C, followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C
386 with a final post-amplification melting curve analysis step. DWV titres were calculated by
387 comparison of the resulting Ct value to the standard curve generated from a serial dilution of the
388 VVD cDNA qPCR standard prepared by reverse transcribing the RNA transcript.

389 <u>Negative strand assay</u>

390 Strand-specific detection of DWV RNA was performed as described earlier [25]. Briefly, 1 µg of
391 total RNA was used in reverse transcription reaction carried out with Superscript III reverse
392 transcriptase (Invitrogen) and the adapter extended primer designed to anneal to the negative
393 strand RNA of DWV. The PCR step was carried out by *Taq* DNA polymerase (NEB) using

forward primer identical to the adapter sequence (Table S1) and DWV_RTPCR_R primer. PCR was run for 35 cycles in the same conditions as described above.

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Conflicts of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. **Reverse genetics (RG) system for three DWV variants. A**. Diagram showing identical regions between genomic RNA sequences of master DWV variants (DWV-A - GenBank AJ489744 and DWV-B - GenBank AY251269.2) and three RG clones (VVV, VDD and VVD) derived from the cloned cDNA of the recombinant DWV - VVD (GenBank HM067438.1); identical sequence regions are shown in black, restriction sites tags introduced into cDNA of each variant are indicated in italics, DWV genomic RNA organization is presented below to help interpretation. **B**. Detection of RG DWV RNA by RT-PCR in honey bee pupae injected with *in vitro* synthesized RNA transcripts: "VDD", "VVV", and "VVD" - PCR products from pupae injected with corresponding full-length RNA, "VVDtr" - PCR from pupae, injected with truncated VVD RNA, "+" and "-" - PCR controls, "M" - molecular weight DNA marker; restriction digest - verification of the RG origin of detected DWV by the digest of the PCR

573 products at artificially introduced restriction sites. **C.** Detection of RG DWV in bumble bee
574 pupae injected with VVD RNA and with virus stock obtained from RNA-injected pupae -
575 "VVDvir"; "VVD RNAtr" - RT-PCR from pupae injected with truncated RNA, "Mock" - PBS-
576 injected pupae, "+" - positive PCR control for DWV, "M" - molecular weight DNA marker; RG
577 origin of the PCR products for all DWV-positive samples was confirmed by digest with *HpaI*
578 restriction enzyme, amplification of the actin mRNA product was used as an indicator of RNA
579 integrity and loading control.

580 **Figure 2. Inoculation of bumble bee pupae and larvae with RG-DWV. A.** RT-qPCR analysis
581 of DWV accumulation in bumble bee pupae injected with VVV, VDD and VVD DWV. Pupae
582 were injected at white-eyed - "we" - and purple-eyed - "pe" - stages and analyzed 48 h post
583 injection. Each value corresponds to an individual sample analyzed, black lines show mean
584 ±SD. **B.** Detection of DWV RNA in bumble bee larvae from two different age groups fed with 10^8
585 GE VVD DWV: qPCR analysis of DWV levels in individual larvae samples, black-circled values
586 correspond to samples which produced a positive result in (-)RNA assay; each value
587 corresponds to an individual sample analyzed, error bars show mean ±SD. DWV (-)RNA assay -
588 strand-specific RT-PCR products run in 1% agarose gel, "+" - positive PCR control for DWV,
589 "M" - molecular weight DNA marker, V - PCR from the DWV inoculum used for larvae feeding.

590 **Figure 3**. **Detection of DWV in adult bumble bees. A**. Detection of DWV RNA by RT-PCR in
591 adult bumble bees after inoculation with VVD DWV via feeding or injections (10 samples from
592 each group shown): "Mock" – non-treated bumble bees from the same colony, "PBS" - PBS-
593 injected group (no virus), "10^7 and 10^8 fed" - bumble bees fed with sucrose solution containing
594 10^7 or 10^8 DWV GE of DWV per bee, "10^4 and 10^8 injected" - bumble bees injected with 10^4 or
595 10^8 DWV GE of DWV respectively. **B.** Detection of the DWV (-)RNA strand in adult bumble bees
596 injected with 10^8 DWV GE. "+" and "-" - positive and negative PCR controls, "M" - molecular
597 weight DNA marker. Detection of the bumble bee actin RNA was used to assay the quality of

extracted RNA. RG origin of the PCR products for all DWV-positive samples was confirmed by restriction enzyme digest.

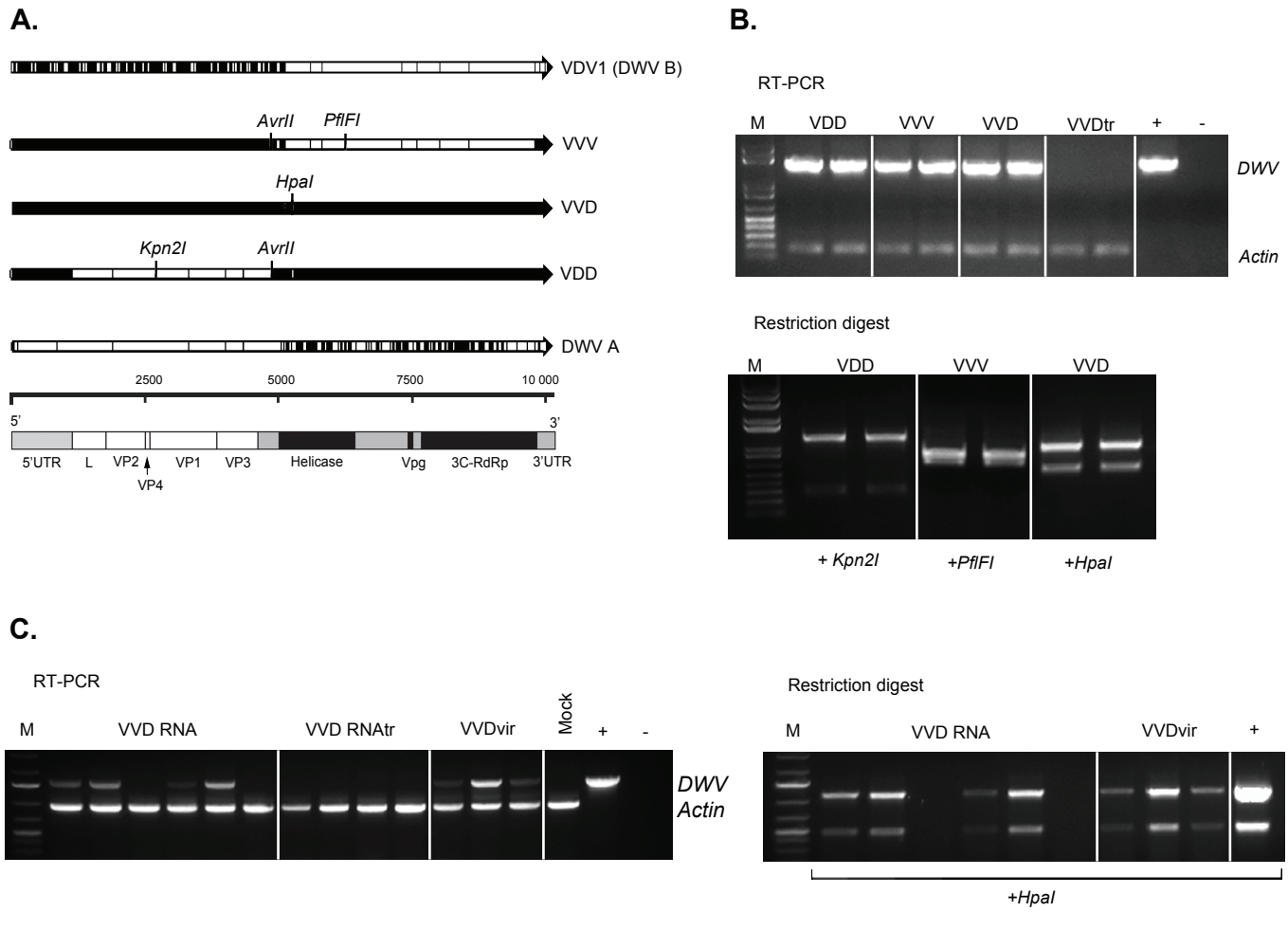
Figure 4. Morbidity of DWV in bumble bees. A. RT-qPCR analysis of DWV level in developed bumble bees injected with 10^6 GE of DWV at white-eyed pupa stage. Individual values for each sample are shown with dots, lines represent mean ±SD. **B.** Percentage of visually normal ("*norm"*), discolored ("*disc")* and non-viable ("*nv")* bumble bees developed from pupae in VVD-injected (n=43) and PBS-injected ("Mock", n=37). **C.** Phenotype of bumble bees developed from pupae in the incubator.

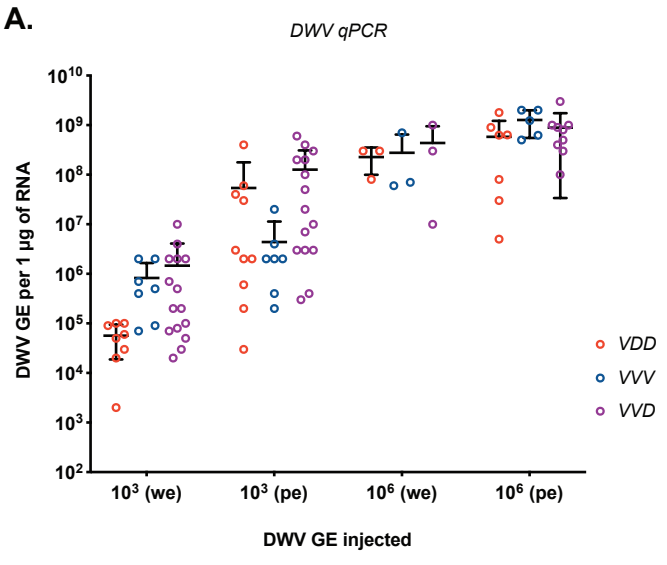
Figure S1. Infectivity of *Bombus* and *Apis*-derived DWV. qPCR analysis of DWV accumulation in bumble bee (BP) and honey bee (HP) pupae injected with VVD DWV inoculum prepared from pupae transfected with *in vitro* synthesized RNA. "*Apis*" and "*Bombus*" DWV stocks were prepared from honey bee and bumble bee pupae tissues respectively. Each value corresponds to an individual sample analyzed, error bars show mean ±SD. After injection bees were incubated for 48 and 24 h for bumble bee and honey bee pupae respectively before analysis.

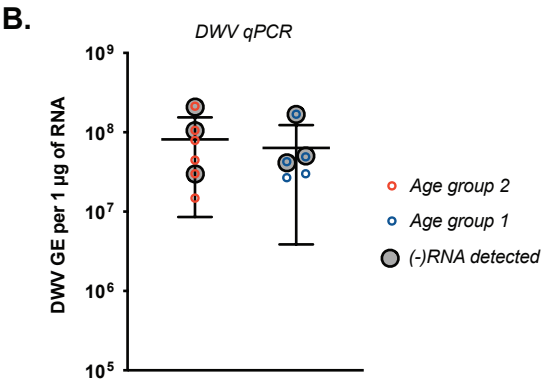
Figure S2. Phylogenetic analysis of injected virus variation in first generation honey bees and second generation bumble bees. DWV isolated from the injected bees was sequenced using Illumina and subsequently analysed by Shorah [56, 57] to identify any adaptive sequence changes which may have been undertaken in the bumble bee samples. The phylogenetic tree shows the top matches all align identically to the original injected variant (VVD9 RG construct), indicating no adaptive change has taken place in the new host. Reference sequences for 'Type A' (AY292384.1) and 'Type B' (CVUB01000001.1) included. HAPX indicates the variant found in each Illumina sample and the final number indicates the proportion of the virus population each sample comprises (1.00 = 100%).

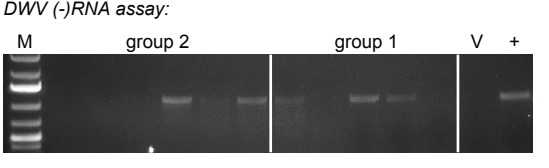
Figure S3. Detection of the DWV (-)RNA strand in bumble bee and honey bee pupae. RT-PCR assay for (-)RNA of DWV in pupae injected with full-length ("full") and truncated ("tr.") VVD RNA, VVD virus inoculum ("VVDvir") or PBS (negative control). RNA-injected pupae were analyzed 3 days post-injection. Virus-injected pupae were analyzed 48 and 24 h post-injection for bumble bees and honey bees respectively. "+" and "-" - positive and negative PCR controls, "M" - molecular weight DNA marker. RG origin of the PCR products for all DWV-positive samples was confirmed by restriction enzyme digest.

Figure S4. Development of VVD DWV injected honey bee pupae. Appearance of deformities in virus-injected pupae upon eclosion (shown on the right) and qPCR analysis of DWV RNA accumulation in honey bees with deformed wings developed from honey bee pupae injected with 10^4 GE of DWV at white-eyed pupa stage. Each value corresponds to an individual sample analyzed, black lines show mean ±SD.

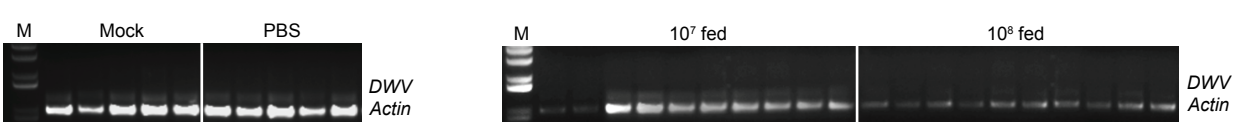

A.
VDV1 (DWV B)
AvrII
PflFI
VVV
HpaI
VVD
Kpn2I
AvrII
VDD
DWV A
2500
5000
7500
10 000
5'
3'
5'UTR
L
VP2
VP4
VP1
VP3
Helicase
Vpg
3C-RdRp
3'UTR
B.
RT-PCR
M
VDD
VVV
VVD
VVDtr
+
-
DWV
Actin
Restriction digest
M
VDD
VVV
VVD
+ Kpn2I
+PflFI
+HpaI
C.
RT-PCR
M
VVD RNA
VVD RNAtr
VVDvir
Mock
+
-
DWV
Actin
Restriction digest
M
VVD RNA
VVDvir
+
+HpaI


A.
DWV qPCR
DWV GE per 1 µg of RNA
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{3} (we)
10^{3} (pe)
10^{6} (we)
10^{6} (pe)
DWV GE injected
VDD
VVV
VVD


B.
DWV qPCR
DWV GE per 1 µg of RNA
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
Age group 2
Age group 1
(-)RNA detected


DWV (-)RNA assay:
M
group 2
group 1
V
+

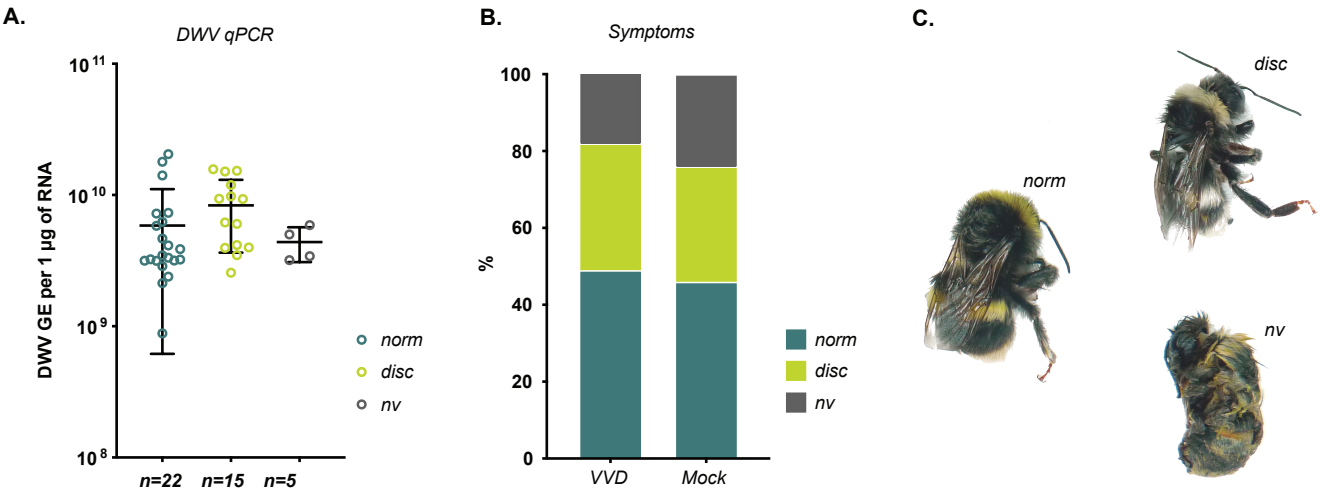
A.


M
Mock
PBS
DWV
Actin
M
10^7 fed
10^8 fed
DWV
Actin

M
10^4 injected
10^8 injected
+
-
DWV
Actin

B.

M
10^8 injected
+
-
-


A.
DWV qPCR
DWV GE per 1 µg of RNA
10 11
10 10
10 9
10 8
n=22
n=15
n=5
norm
disc
nv
B.
Symptoms
%
100
80
60
40
20
0
VVD
Mock
norm
disc
nv
C.
norm
disc
nv