In vivo evolution of viral virulence: switching of deformed wing virus between hosts results in virulence changes and sequence shifts

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Summary

The health of the Western honey bee is threatened by a global epidemic of deformed wing virus (DWV) infections driven by the ectoparasitic mite *Varroa destructor* acting as mechanical and biological virus vector. Three different variants of DWV, DWV-A, -B, and -C exist. Virulence differences between these variants and their relation to *V. destructor* are still controversially discussed. We performed laboratory experiments to analyze the virulence of DWV directly isolated from crippled bees (DWV_{P0}) or after one additional passage in bee pupae (DWV_{P1}). We demonstrated that DWV_{P0} was more virulent than DWV_{P1} for pupae, when pupal mortality was taken as virulence marker, and for adult bees, when neurotropism and cognitive impairment were taken as virulence markers. Phylogenetic analysis supported that DWV exists as quasispecies and showed that DWV_{P0} clustered with DWV-B and DWV_{P1} with DWV-A when the phylogeny was based on the master sequences of the RNA-dependent RNA polymerase but not so when it was based on the VP3 region master sequences. We propose that switching of DWV between the bee and the mite host is accompanied by changes in viral sequence, tissue tropism, and virulence and that the RNA-dependent RNA polymerase is involved in determining host range and virulence.

Introduction

The Western honey bee *Apis mellifera* is the dominantly used insect species for commercial pollination in agriculture and hence, plays a crucial role in global human food supply (Aizen et al., 2008; Aizen and Harder, 2009; Garibaldi et al., 2013). Therefore, diseases threatening the productivity or even survival of *A. mellifera* are of major concern for human society. Among the numerous infectious pathogens and infesting parasites (Genersch, 2010) posing a serious threat to honey bee health and survival are the ectoparasitic mite *Varroa destructor* (for a recent review see (Rosenkranz et al., 2010)) and deformed wing virus (DWV) (for a recent review see (de Miranda and Genersch, 2010)). Both, the *Varroa* mite and DWV have been implicated as causative agents in heavy colony losses mainly during the winter season all over the world (Martin, 2001; vanEngelsdorp et al., 2007; Highfield et al., 2009; Carreck et al., 2010; Genersch et al., 2010; Guzmán-Novoa et al., 2010; Le Conte et al., 2010; McMahon et al., 2016; Natsopoulou et al., 2017).

DWV is a single- and plus-stranded RNA virus which belongs to the family *Iflaviridae* in the order *Picornavirales* and was first described in 1991 as pathogen of *A. mellifera* (Bailey and Ball, 1991). Meanwhile numerous studies have demonstrated that DWV is rather not a bee-specific but a multi-host virus, able to infect a wide range of insect pollinators (for a recent review: (Gisder and Genersch, 2017) and references therein). Furthermore, the host range of DWV was shown to go beyond the insects: DWV is also able to replicate in the arachnid *V. destructor*, hence, to truly infect this ectoparasitic mite infesting honey bees (Ongus et al., 2004; Yue and Genersch, 2005; Gisder et al., 2009; Campbell et al., 2016). However, the original host of DWV has clearly been identified as the honey bee *A. mellifera* from where it shifted to *V. destructor* (Wilfert et al., 2016).

RNA viruses are known to exist as quasispecies. The basis of viral quasispecies is a replication process governed by RNA-dependent RNA-polymerases lacking proof reading and repair functions (Holland et al., 1992; Steinhauer et al., 1992) and supporting RNA recombination via a so-called copy-choice mechanism (Lai, 1992). Consequently, viral replication is error-prone with an extraordinary high mutation rate which results in mutations accumulating in the genomes of the viral progeny (Holland et al., 1992; Domingo and Holland, 1997). Viral quasispecies form dynamic mutant clouds, each one characterized by a predominant master sequence surrounded by an infinite number of virus mutants thus resulting in high sequence complexity (Holland et al., 1992; Domingo and Holland, 1997; Domingo, 2002; Lauring and Andino, 2010). The mutant clouds' composition and master sequence may vary between infected individuals and is subject to constant change because of the continuous lack of proof reading and repair and the continuous generation of recombinants during replication. The only restriction to the sequence space that can be occupied by the mutant cloud of a certain virus quasispecies is the feature "infectivity to the host" or more general the fitness of the generated viruses (Domingo and Holland, 1997; Sierra et al., 2000). Therefore, a multi-host virus like DWV may exist with several, host-adapted subclouds, each one occupying a different region of the common DWV sequence space and characterized by a different master sequence but still belonging to the same quasispecies (Domingo and Holland, 1997).

In the absence of *V. destructor*, DWV is transmitted horizontally (Yue and Genersch, 2005; Chen et al., 2006; Gisder et al., 2009) as well as vertically (Yue et al., 2007; de Miranda and Fries, 2008). These transmission routes mainly result in asymptomatic, covert infections (de Miranda and Genersch, 2010; Forsgren et al., 2012). With the advent and spread of *V. destructor* in the global honey bee

population, vectorial transmission of DWV by the mite became established as an additional viral transmission route resulting in the occurrence of overt DWV infections: Symptoms of an overt infection include infection of the brain (Fujiyuki et al., 2004; Yue and Genersch, 2005; Fujiyuki et al., 2009; Möckel et al., 2011; Zioni et al., 2011), ataxia, bloated abdomen, discoloration, and most importantly crippled wings in newly emerging bees (DWV syndrome, for a recent review see (de Miranda and Genersch, 2010)).

Such overt DWV infections in bees are intimately linked (i) to *V. destructor* having parasitized the developing pupa (Ball, 1983; Ball, 1989; Ball, 1993; Hung et al., 1995; Bowen-Walker et al., 1999; Carreck et al., 2010), (ii) to DWV being vectorially transmitted to the developing pupa by the parasitizing mite (Bowen-Walker et al., 1999; Martin, 2001; Yue and Genersch, 2005; Gisder et al., 2009; Möckel et al., 2011; Schöning et al., 2012; Wilfert et al., 2016), (iii) to DWV replication in the mite prior to virus transmission (Yue and Genersch, 2005; Gisder et al., 2009; Schöning et al., 2012; Ryabov et al., 2014; Campbell et al., 2016), hence, to the mite acting as intermediary host, and (iv) to the transmission of DWV variants with increased virulence (Gisder et al., 2009; Ryabov et al., 2014; McMahon et al., 2016; Natsopoulou et al., 2017).

The existence of at least two variants of DWV, which differ in their genomic sequence and presumably also in their host range or host preference, had already become evident with the isolation and characterization of *Varroa destructor* virus-1 (VDV-1), a DWV-variant replicating in *V. destructor* (Ongus et al., 2004) but also found in crippled bees and replicating in the head of honey bees (Zioni et al., 2011). The strong association between DWV replication in parasitizing mites and the occurrence of symptomatic infections (Yue and Genersch, 2005; Gisder et al., 2009; Schöning et al., 2012) had right away suggested differences in virulence for honey

bees between the mite-associated VDV-1 variant of DWV (Ongus et al., 2004) and the originally described DWV variant (Lanzi et al., 2006; Fujiyuki et al., 2009). Subsequent studies confirmed the existence of at least three major DWV genotypes, now called DWV-A ("classical" DWV), DWV-B (VDV-1 related, replicating in mites), and DWV-C (Martin et al., 2012; Mordecai et al., 2016; Wilfert et al., 2016). These variants are described to differ in virulence and associated pathology as well as in their relation to *V. destructor* although it is still controversially discussed which variant is the more virulent one and how these variants are related to the mite (Yue and Genersch, 2005; Gisder et al., 2009; Moore et al., 2011; Martin et al., 2012; Schöning et al., 2012; Ryabov et al., 2014; McMahon et al., 2016; Mordecai et al., 2016; Natsopoulou et al., 2017).

Nevertheless, it is now widely accepted that *V. destructor* plays a crucial role in the emergence, epidemiology, and virulence of the different variants of DWV. Some of the studies also showed that DWV replication in *V. destructor* prior to vectorial transmission of the virus to pupae correlated with increased viral virulence for honey bees (Yue and Genersch, 2005; Gisder et al., 2009; Schöning et al., 2012; Ryabov et al., 2014). These results suggested that viral replication in *V. destructor*, hence, when the mite does not just act as mechanical vector but acts as a biological vector and an intermediary host, might select for more virulent DWV variants. This prompted us to hypothesize that changes in viral virulence might occur when DWV moves between bee and mite hosts. To understand if shifting between Insecta and Acari hosts could influence viral virulence, we performed laboratory infection assays using DWV directly isolated from crippled bees and, hence, closely associated with replication in mites (DWV_{P0}) and DWV propagated from DWV_{P0} in honey bee pupae (DWV_{P1}). We compared the lethality of DWV_{P0} and DWV_{P1} for pupae and adult bees as well as the tissue tropism of both isolates when injected into naïve adult bees. We

then analyzed the cognitive capacity of bees infected with DWV_{P0} and DWV_{P1}. Finally, we looked for differences in the sequence space occupied by DWV_{P0} and DWV_{P1} to gain a better understanding of the complex triangular relationship between DWV, mites, and bees.

Results

Crippled bees as source for DWV_{P0}

DWV replication in the mite prior to mite-vectored virus transmission to bee pupae has been shown to be a prerequisite for the manifestation of overt DWV infections (crippled wings, infection of the brain) in the emerging bee (Yue and Genersch, 2005; Gisder et al., 2009; Schöning et al., 2012). This link between DWV replication in mites and the emergence of crippled bees prompted us to analyze whether crippled bees can serve as source for DWV variants closely associated with viral replication in mites. To this end, we determined the DWV master sequences present in individual overtly and covertly infected bees and in the mites that had parasitized these bees during development. The DWV leader-protein (L-protein) gene master sequence was used as proxy for the entire DWV master sequence infecting an individual because the L-protein region is the less conserved and hence, the most variable region of the DWV-genome (Lanzi et al., 2006; de Miranda and Genersch, 2010) and, therefore, identical L-protein gene sequences should indicate closely related isolates.

We isolated total RNA from head (h), thorax (t), and abdomen (a) of emerging symptomatic bees (n = 13; crippled wings, virus detection in the head) and covertly infected bees (n = 7; no obvious symptoms and no virus detection in the head) as well as from those mites (n = 36) that had been parasitizing these bees during pupal development. Using strand-specific RT-PCR (Yue and Genersch, 2005; Gisder et al.,

2009; Schöning et al., 2012) we analyzed the mites for the plus- and minus-RNA strands of DWV thus determining whether the collected mites carried a DWV infection, hence, served as intermediary host for DWV. We again confirmed our previous results (Yue and Genersch, 2005; Gisder et al., 2009; Schöning et al., 2012) that “DWV replication in mites” is linked to “health status of the emerging bee”: all covertly infected, asymptomatic bees (hb, healthy bee; n=7; Fig 1A) had been parasitized as pupae by mites not harboring replicating DWV (hb/m, mite having parasitized a healthy bee; n=16) while all symptomatic bees (crb, crippled bee; n=13; Fig 1B) had been parasitized as pupae by mites (crb/m, mite having parasitized a crippled bee; n=20) which were all infected by DWV, hence, contained replicating DWV.

Subsequently, a region of the L-protein gene of DWV (514 bp; pos. 1203 – pos. 1716) was amplified and sequenced in all DWV-positive samples. Although all bees and mites originated from the same heavily mite infested colony, we identified a total of 72 polymorphic positions within the sequenced region of the L-protein gene and obtained 26 different L-protein gene sequences from the 66 analyzed individuals (bees and mites; Fig 1). Phylogenetic analyses (Tamura-Nei genetic distance model, (Tamura and Nei, 1993)) of the DWV L-protein gene master sequences of the seven covertly infected bees revealed that these master sequences differed from each other in four (hb1 – hb4) of these seven bees and their parasitizing mites (Fig 1A). In three covertly infected bees hb1 – hb3, even the master sequences detected in the thorax (hb-t) and abdomen (hb-a) were different from each other (Fig 1A). The DWV L-protein gene master sequences isolated from the three covertly infected bees hb4, hb6, and hb7 were identical (Fig 1A).

In contrast, analysis of the symptomatic bees revealed that the DWV L-protein gene master sequences were always identical for each bee in all three body parts (h,

t, a; Fig 1B) although they again differed between the bees (crb1 - crb13; Fig 1B). More importantly, however, was that the DWV L-protein gene master sequence in each crippled bee was identical to the ones determined in the mites which had been parasitizing the respective bee during pupal development (Fig 1B). Hence, each bee and the mites, that had parasitized this bee during pupal development, shared the same DWV L-protein gene master sequence although the master sequences between the symptomatic bees differed (Fig 1B). These results showed that DWV isolated from a crippled bee is still closely related to the virus variant present in the mites that parasitized this bee. Because these mites all harbored replicating DWV, we decided to use crippled bees as source for DWV closely associated with viral replication in the mite host (DWV_{P0}).

To mimic the movement of DWV between the Acari and Insecta hosts, we then propagated DWV_{P0} in bee pupae by infecting virus-free naïve pupae with DWV_{P0} and re-isolating the virus, now designated DWV_{P1}, from the emerging adult bees. Hence, these DWV_{P1} isolates were generated from DWV_{P0} by one additional passage in the bee host.

Lethality of DWV_{P0} and DWV_{P1} for bees

We first assessed the virulence of DWV_{P0} and DWV_{P1} for bee pupae by performing injection bioassays with naïve, virus-free white-eyed pupae. Injecting a dosage of 2E+05 DWV particles per pupa resulted in 65.01% ± 9.93% (mean ± SEM) total mortality after ten days for DWV_{P0}, while injecting DWV_{P1} caused only 32.64% ± 7.98% (mean ± SEM) mortality within the same time frame (Fig 2A). Statistical analysis (One-Way ANOVA and Tukey's multiple comparison test) revealed that this difference was significant (p-value < 0.01) as was the difference between infected groups and control groups (DWV_{P0} vs. mock-infected pupae or non-manipulated

pupae p-value < 0.0001 and DWV_{P1} vs. mock-infected pupae or non-manipulated pupae p-value < 0.05). Mortality in the two control groups (non-manipulated, mock-infected) did not differ significantly (p-value = 0.9707) suggesting that injecting buffer did not have any detrimental effect on the virus-free pupae in our experiments (Fig 2A). Therefore, although both DWV isolates were virulent and caused increased pupal mortality, DWV_{P0} was significantly more virulent than DWV_{P1} when pupal bee mortality was chosen as virulence marker. This result suggested that DWV_{P1} showed an attenuated phenotype already after one passage in bees when compared to $DWV_{P0.}$

We next analyzed the lethality of DWV_{P0} and DWV_{P1} for adult bees. Newly emerged worker bees were infected with DWV_{P0} and DWV_{P1} with two different dosages (2E+05 and 2E+09 DWV virus equivalents (ve) per bee) in injection bioassays. Mortality of the bees was monitored over 27 days. Mortality in the two control groups (non-manipulated, mock-infected) did not differ significantly (Two-Way ANOVA, column factor p-value = 0.52, df=2). Bee mortality significantly increased following injection of 2E+05 DWV particles per adult honey bee compared to the mock-infected control group (column factor p-value < 0.0001, df=2) between day 19 to day 24 post infection (Tukey´s multiple comparison test as *post hoc* test, p-value < 0.05) (Fig 2B). Injection of 2E+09 DWV particles per bee further increased bee mortality in comparison to the mock-infected control group (column factor p-value = 0.0008, df=2). The difference was statistically significant from day 6 - 24 post infection (p-value < 0.05) (Fig 3B). However, no significant difference in mortality could be demonstrated between the DWV_{P0} and DWV_{P1} infected groups at both dosages tested (2E+05: column factor p-value = 0.78, df=1; 2E+09: column factor p-value = 0.07, df=1) (Fig 2B). Therefore, DWV_{P0} and DWV_{P1} did not differ in virulence when adult bee mortality was chosen as virulence marker suggesting that the

attenuated phenotype observed at the level of pupal mortality did not exist at the level of adult bee mortality.

Tissue tropism of DWV_{P0} and DWV_{P1} in adult bees

A hallmark of overt DWV infections is the DWV infection of the brain while covertly infected bees do rather not show brain infections (Yue and Genersch, 2005; Genersch et al., 2010; Möckel et al., 2011). Therefore, we next studied the tissue tropism of DWV_{P0} and DWV_{P1}. We infected DWV-free adult bees with 2E+09 virus particles (DWV_{P0} or DWV_{P1}) per bee, a dosage that had been shown to be sufficient to induce brain infections when DWV isolated from crippled bees was injected into naïve bees (Möckel et al., 2011). We then analyzed the proportion of virus infected bees as well as the distribution of the virus in the bees' bodies (head, thorax, abdomen). Both DWV_{P0} and DWV_{P1} were equally (Mann-Whitney U test, p-value = 0.38) infective and resulted in 100% ± 0% (mean ± SD) for DWV_{P0}-infected and 98.72 ± 2.22 % (mean ± SD) for DWV_{P1}-infected bees as determined through DWV detection in thoraces (Fig 3A). Therefore, DWV_{P0} and DWV_{P1} did not differ in virulence for adult bees in respect to viral infectivity.

However, a statistically significant difference in tissue tropism was demonstrated (Mann-Whitney U test, p-value = 0.036). While in DWV_{P0} infected bees, 89.19 ± 7.25% (mean ± SD) of the bees tested positive for DWV in total RNA isolated from the bees' heads, this was the case for only 30.83 ± 15.88% (mean ± SD) of the bees infected with DWV_{P1} (Fig. 3A). To confirm that bees which tested positive for DWV in total RNA isolated from the bees' heads were indeed suffering from an infection of the brain, we performed fluorescence *in situ*-hybridization (FISH) analysis. Positive signals for DWV were detected in in the brain in the somata region of the mushroom bodies', in the lobula, the inner and outer chiasma, and the medulla indicating a

generalized infection of the brain (Fig. 3B). Therefore, DWV_{P0} and DWV_{P1} differed in tissue tropism with DWV_{P0} having a more pronounced neurotropism than DWV_{P1}.

Effects of DWV_{P0} and DWV_{P1} on the cognitive capacity of adult bees

The observed differences in neurotropism between DWV_{P0} and DWV_{P1} prompted us to analyze the cognitive capacity of adult bees infected with these two different DWV variants and to choose "learning impairment" as read-out for sublethal pathological effects of DWV infections in adult bees. Adult bees infected with DWV via injection of 2E+09 DWV_{P0} or DWV_{P1} particles per bee were tested for learning performance and memory retention by olfactory conditioning the proboscis extension response (PER) (Felsenberg et al., 2011) using carnation as odorant and sucrose as reward. We tested a total of (i) 119 bees infected with DWV_{P0} (all of them tested positive for DWV in head, thorax, and abdomen) together with 114 and 153 bees in the mock-infected control and non-manipulated control groups, respectively, and of (ii) 159 bees infected with DWV_{P1} (positive for DWV in RNA from thorax and abdomen) of which 52 tested positive for DWV in RNA from head, together with 144 and 149 bees in the mock-infected control and non-manipulated control groups, respectively.

When adult bees experimentally infected with DWV_{P0} were analyzed for learning performance and memory retention in the above described conditioning paradigm, the proportion of bees able to learn and remember the association between odor and sucrose reward was almost 40% lower in the infected group than in both control groups (Fig 4A). This reduction in learning performance was statistically significant for the second (Two-Way ANOVA followed by Tukey´s multiple comparison test: DWV_{P0} infected vs. mock-infected control, p-value = 0.0012; DWV_{P1} infected vs. non-manipulated control, p-value = 0.0007) and third (DWV_{P0} infected vs. mock-infected control and DWV_{P1} infected vs. non-manipulated control, p-value <

0.0001) learning trial during acquisition as well as for the memory retention test (DWV_{P0} infected vs. mock-infected control and DWV_{P1} infected vs. non-manipulated control, p-value < 0.0001). The learning performance in the two control groups did not differ significantly during acquisition (p-value = 0.97 second trial, p-value = 0.86 third trial) or when memory retention was tested (p-value = 0.96).

However, in the experiments where bees infected with DWV_{P1} were conditioned (Fig 4B), no difference between the control groups (non-manipulated, mock infected) and the group of DWV_{P1} infected bees was evident (Fig 4B). Because only about 30% of the adult bees infected with DWV_{P1} suffered from a brain infection (see above), we differentiated four experimental groups for statistical analysis: all DWV_{P1} infected bees, DWV_{P1} infected bees which tested positive for DWV in the head, mock-infected control bees, and non-manipulated control bees. Statistical analysis revealed no significant difference between these four experimental groups during both, acquisition phase and memory retention test (p-values > 0.05 for all tests). These results demonstrate that DWV_{P0} and DWV_{P1} clearly differed in virulence for adult bees: DWV_{P0} was more virulent than DWV_{P1} when “learning impairment” was chosen as virulence marker suggesting that for this virulence marker, DWV_{P1} again showed an attenuated phenotype already after one passage in bees when compared to DWV_{P0}.

Sequence changes between DWV_{P0} and DWV_{P1}

The results from the biological assays presented so far indicated that DWV_{P0} was more virulent than DWV_{P1} for infected pupae and adult bees. Hence, the laboratory infection assays followed by functional tests revealed that one passage in bees was sufficient for an attenuated DWV phenotype to evolve. Therefore, we next analyzed whether these observed pathobiological differences were accompanied by changes

in the DWV master sequences. Illumina MiSeq paired-end sequencing was carried out on three independent DWV_{P0} and three independent DWV_{P1} isolates used throughout this study. Each DWV_{P1} isolate I – III was generated from the corresponding DWV_{P0} I – III isolate, hence was "evolutionary separated" from DWV_{P0} by one passage in bee pupae. De novo assembly of the DWV reads yielded the master sequences of DWV_{P0} I – III (I: 9593 bp, II: 10157 bp, III: 10132 bp) and DWV_{P1} I – III (I: 9828 bp, II: 10136, III: 10132 bp). We compared the sequences of the paired isolates DWV_{P0} I / DWV_{P1} I, DWV_{P0} II / DWV_{P1} II, and DWV_{P0} III / DWV_{P1} III and found that they differed in 987, 813, and 779 nucleotides, respectively. Phylogenetic analysis of the master sequences was performed by using the Tamura-Nei genetic distance model (Tamura and Nei, 1993) and by including several DWV variants whose phylogenetic relationship has been resolved by the same model recently (Mordecai et al., 2016): (i) the classical variant DWV-A represented by four different sequences (GenBank Accession numbers NC_004830.2, NC_005876.1, JQ_413340, CENC01000001.1), (ii) the VDV-1-related variant DWV-B represented by two different sequences (GenBank Accession numbers NC_0064941.1 and KC_78622.1), and (iii) the recently described DWV-C variant (CEND01000001.1). We confirmed the phylogenetic relationship of these previously sequenced DWV variants (Fig. 5A) by showing that the four DWV-A variants formed one cluster, the two DWV-B variants formed another cluster and both were separated from the DWV-C branch (Mordecai et al., 2016). Adding the DWV_{P0} and DWV_{P1} master sequences to this tree revealed that DWV_{P0} I – III as well as DWV_{P1} II and III could be attributed to the DWV-B cluster while DWV_{P1} I grouped together with the DWV-A variants (Fig. 5A). Although the overall phylogenetic divergence within the DWV-B branch was low, clear sequence shifts from DWV_{P0} II to DWV_{P1} II and from DWV_{P0} III to DWV_{P1} III were evident, with DWV_{P0} II and III being more closely related to the previously

sequenced DWV-B variants than DWV_{P1} II and III. Most interestingly, however, was the fact that the master sequence DWV_{P1} I, derived from DWV_{P0} I by only one additional passage in bee pupae, clustered with the DWV-A variants although the master sequence DWV_{P0} I clustered with the DWV-B variants, (Fig 5A).

In order to analyze the sequence shifts in more detail, we concentrated on the conserved genomic regions coding for the viral capsid protein VP3 (pos. 2634-3838) at the 5'-end of the genome and the RNA-dependent RNA-polymerase (RdRp, pos. 8016-8522) at the 3'-end of the genome. Both regions have been used in previous studies and gave valuable insight into DWV epidemiology (Wilfert et al., 2016) and sequence diversity within the DWV quasispecies (Mordecai et al., 2016). Therefore, we included the VP3 and RdRp gene sequences of the previously sequenced DWV variants A, B, and C in our phylogenetic analysis.

The VP3 gene sequences of DWV_{P0} I – III and DWV_{P1} I – III were all slightly different from each other but all grouped within the DWV-B cluster (Fig 5B). It is evident from the tree shown in Fig 5B that the DWV_{P0} and DWV_{P1} sequences within each pair ($DWV_{P0/P1}$ I, $DWV_{P0/P1}$ II, $DWV_{P0/P1}$ III) were more closely related to each other than the DWV_{P0} and DWV_{P1} sequences between the pairs.

The RdRp gene master sequences showed greater variability and were distributed over the DWV-A and DWV-B clusters. The three DWV_{P0} isolates grouped with the respective sequences of the DWV-B variants. However, the RdRp gene sequences of the DWV_{P1} isolates clustered with the DWV-A variants (Fig 5C) clearly suggesting a sequence shift within the RdRp gene from the mite-associated DWV-B variant to the bee-associated DWV-A variant through one round of virus propagation in the bee host.

Discussion

With the spread of *Varroa destructor* in the global honey bee population, the mite became established as virus vector and DWV variants with increased virulence for honey bees emerged. We here present our data suggesting that switching of DWV between the bee and the mite host is accompanied by changes in viral sequence, tissue tropism, and virulence. Two characteristics of DWV, which are still controversially discussed in the community, are crucial for interpreting our data and understanding their impact: (i) DWV exists as quasispecies and (ii) DWV can replicate in mites.

Does DWV exist as quasispecies?

RNA viruses like DWV in general are known to exist as quasispecies forming mutant clouds (Holland et al., 1992; Lauring and Andino, 2010). It has recently been suggested that DWV is such a quasispecies (Mordecai et al., 2016) and that the diversity of circulating DWV master sequences decreases in the presence of *V. destructor* acting as virus vector (Martin et al., 2012; Ryabov et al., 2014). This observation points to *V. destructor* acting as evolutionary bottleneck for the DWV quasispecies. However, the quasispecies concept for DWV was questioned recently and DWV-A and DWV-B were considered fixed DWV genotypes because their genomic sequences did not form an interconnected mutant cloud (McMahon et al., 2016) as demanded for a viral quasispecies.

We here provide further evidence that DWV exists as quasispecies and that DWV-A and -B indeed form interconnected mutant clouds. Our data on the master sequence heterogeneity of the L-protein gene fragment sequenced from mites and bees originating from one colony, showed high sequence complexity between individuals and, for covertly infected bees, even within infected individuals between different body parts. Especially the latter is rather typical for RNA virus quasispecies

because different tissues in an infected individual often represent bottlenecks to the intrahost viral spread thus reducing quasispecies complexity by selection and subsequent enrichment of favored subsets (Georgescu et al., 1997; Hovi et al., 2004; Bull et al., 2011). Hence, the detection of different L-protein gene master sequences between and within infected bees clearly argued for DWV existing as a quasispecies.

This view was further substantiated by our phylogenetic analyses of the whole genome master sequences of DWV_{P0} I – III and DWV_{P1} I – III in comparison to previously sequenced and phylogenetically analyzed DWV variants (Mordecai et al., 2016). The whole genome master sequences of the DWV strains isolated from crippled bees (DWV_{P0}) or after one additional passage in bee pupae (DWV_{P1}) differed considerably and were found on both main branches of the phylogenetic tree. There is obviously considerable dynamic within the DWV mutant cloud because we observed one case in which the DWV master sequence shifted from the DWV-B cluster to the DWV-A cluster upon switching from the mite host to the bee host within one bee passage. Therefore, at least in this case DWV_{P0} and DWV_{P1} formed an interconnected mutant cloud, as requested for a viral quasispecies, which spanned from the DWV-B cluster to the DWV-A cluster. Based on our results, we conclude that DWV exists as quasispecies and circulating mutant clouds can be described by approximation by their respective master sequences.

Does DWV replicate in mites?

Sequence diversity dynamics in viral quasispecies result from error-prone viral replication in host cells. Hence, DWV replication in mites is a necessary prerequisite for changes in DWV sequence diversity to occur in mites and, therefore, the interpretation of our results strongly depends on the ability of DWV to replicate in mites. However, this topic is still fiercely and controversially discussed among bee

scientists, although actually, the replication of DWV in mites had already been convincingly demonstrated more than a decade ago: detection of the negative strand RNA of VDV-1, which is indicative of viral replication, and electron microscopic pictures of paracrystalline arrays of VDV-1 particles in mite tissue provided direct evidence for virus replication in mites (Ongus et al., 2004). Quite recently, the mite's synganglion was identified as the tissue supporting VDV-1 replication (Campbell et al., 2016). VDV-1 though originally isolated from mites (Ongus et al., 2004) has been shown by phylogenetic analyses to be a variant of DWV. This variant is now called DWV-B (Martin et al., 2012; Mordecai et al., 2016). There is a general mathematical rule saying that if two values are equal to a third one, they are equal to each other. Hence, because VDV-1 replicates in mites and VDV-1 is DWV-B, it has to be concluded that at least the DWV-variant now called DWV-B is able to replicate in mites. The principal ability of DWV to replicate in mites is also confirmed by other independent studies which all repeatedly demonstrated the detection of the negative RNA strand of DWV in mites and showed that these mites that acted as intermediary hosts for DWV caused overt DWV infections in the developing pupae (Yue and Genersch, 2005; Gisder et al., 2009; Schöning et al., 2012; Ryabov et al., 2014). This relationship was again substantiated in the study at hand.

The strong association between the occurrence of overtly infected bees and DWV transmitted to pupae by mites that acted as intermediary hosts for DWV (Yue and Genersch, 2005; Gisder et al., 2009; Schöning et al., 2012) may provide an explanation why certain studies failed to detect DWV replication in mites: even in highly mite infested colonies, only the minority of parasitized pupae emerge as crippled bees, hence, only the minority of mites harbor replicating DWV and act as intermediary hosts for DWV. Instead, most mite-parasitized pupae still develop into bees which emerge without showing obvious symptoms, hence, the parasitizing

mites just either act as mechanical vectors of DWV or are even DWV-free (Yue and Genersch, 2005). Given that in a non-collapsing colony the majority of mites will not harbor replicating DWV, it is not surprising that no evidence for DWV replication in mites was found when only four mites were analyzed (Santillán-Galicia et al., 2008) or when phoretic mites collected from healthy adult bees were used for analysis (Erban et al., 2015). These results were correct for the analyzed mites, but the studies suffered from not having analyzed the right mites. Furthermore, sound results on the pathobiology and virulence of different DWV variants will rather not be achieved when samples are stratified according to the mite infestation levels of the source colonies (Ryabov et al., 2014) because the proportion of mites acting as biological vectors is not necessarily related to the mite infestation level and differs from colony to colony and over time (Yue and Genersch, 2005). For studies on DWV pathobiology and virulence it is vital to differentiate the mites according to whether or not they harbor replicating virus, hence, whether they parasitized a pupa that emerged as crippled bee (viral replication in the mite) or as healthy looking bee not suffering from a brain infection (no viral replication) (Schöning et al., 2012).

Virulence determinants of DWV

It was recently proposed that at least three distinct master variants of DWV, DWV-A, -B, and -C (Martin et al., 2012; Mordecai et al., 2016) exist. DWV-A is the classical DWV variant isolated from bees (Fujiyuki et al., 2006; Lanzi et al., 2006), while DWV-B comprises variants with sequence similarity to VDV-1, hence, to the DWV-variant originally isolated as a virus that replicates in the mite *V. destructor* (Ongus et al., 2004). DWV-B also comprises recombinants between VDV-1 and DWV-A (Martin et al., 2012). Finally, DWV-C is a recently described viral variant that can be phylogenetically distinguished from type A and type B (Mordecai et al., 2016).

The virulence of DWV-A and DWV-B for honey bees and their relation to *V. destructor* are controversially discussed. According to Martin and co-workers, the classical DWV variant (Fujiyuki et al., 2004; Lanzi et al., 2006), now named DWV-A, is associated with *V. destructor* as well as with overt, mite-triggered DWV-infections in bees (Martin et al., 2012). In contrast, DWV-B comprises variants with sequence similarity to VDV-1 and hybrids/recombinants between VDV-1 and DWV (Martin et al., 2012) and is described to not cause symptomatic infections (Martin et al., 2012) but to rather protect the bees from the presumably virulent DWV-A variants (Mordecai et al., 2016). It was concluded that a colony is prone to collapse if DWV-A dominates (Martin et al., 2012) but will remain healthy if DWV-B dominates (Mordecai et al., 2016).

This conclusion was contradictory to previous results which had already linked DWV-B with overt DWV infections and colony collapse (Yue and Genersch, 2005; Gisder et al., 2009; Genersch et al., 2010; Schöning et al., 2012) although at that time, the term DWV-B had not been coined yet. Compelling evidence linked DWV-replication in mites to overt DWV-infections in individual bees (Yue and Genersch, 2005; Gisder et al., 2009; Schöning et al., 2012; Ryabov et al., 2014) and overt DWV-infections in individual bees to colony losses (Genersch et al., 2010; Dainat et al., 2012; Dainat and Neumann, 2013). Therefore, DWV-B as the variant replicating in mites is the virulent variant of DWV and is associated with elevated colony losses over winter. This view has been confirmed recently by laboratory experiments as well as a systematic field survey (McMahon et al., 2016) and quantitative field measures of intra-colony dynamics of DWV (Natsopoulou et al., 2017) which both substantiated that DWV-B is more virulent than DWV-A and that colonies infected with DWV-B collapse sooner than colonies infected with DWV-A .

We here present evidence that DWV virulence differences do exist at different levels (pupae/adults, mortality/sublethal effects) and that they can be experimentally assessed and quantified. Our experiments were performed with two different DWV variants, the virulent variant DWV_{P0} and the less virulent DWV_{P1}. Because the attenuated DWV_{P1} was generated from DWV_{P0} by propagation in bee pupae, DWV_{P1} can be considered having evolved from highly virulent DWV_{P0} by only one passage in bees. Although this sounds surprising, similar behaviors of RNA virus quasispecies have already been reported. For instance, foot-and-mouth disease virus (FMDV), pathogenic for a wide variety of cloven-hoofed domesticated and wild animal species, developed an attenuated phenotype after one passage in mice (Sanz-Ramos et al., 2008).

The clustering of the whole genome master sequences of the virulent DWV_{P0} I – III isolates with DWV-B confirmed that the VDV-1 related DWV-B variants are highly virulent for bees. However, the whole genome master sequences of two of the less virulent DWV_{P1} isolates also grouped with DWV-B while only the third one clustered with DWV-A. Hence, the observed virulence differences between DWV_{P0} and DWV_{P1} did not co-segregate with the DWV-A/B clustering when the phylogeny was based on the whole genome master sequences. Hence, this phylogenetic analysis did not help to easily answer the question whether DWV-A or DWV-B is more virulent but rather suggested considerable virulence differences within DWV-A and -B. This result is consistent with a recent study that did also not find consistent differences between the entire DWV genomes from crippled or asymptomatic bees (Brettell et al., 2017). However, the cited study might also have suffered from the fact that it cannot be excluded that the analyzed asymptomatic bees also included bees which only looked healthy but were actually suffering from a DWV-B infection of the brain, hence, carried the virulent DWV-B variant.

The mutation rate of RNA viruses is extraordinary high and, when combined with selective pressure, can quickly result in phenotypic changes (Domingo, 2002; Manrubia et al., 2005; Lauring and Andino, 2010). Still, it cannot be expected that one passage in bees (the "distance" between DWV_{P0} and DWV_{P1}) is sufficient to change the entire genomic sequence of DWV in a way that the differentially virulent variants already belong to different phylogenetic clusters. Following the quasispecies concept for RNA viruses, "infectivity to a certain host" or more general the "fitness" of the generated viruses determine the sequence space that can be occupied by the mutant cloud (Domingo and Holland, 1997; Sierra et al., 2000). Therefore, it might not be absolutely necessary that the entire DWV whole genome master sequences shift from one cluster to the other to obtain differences in DWV infectivity to bees or mites or in DWV virulence. It might be sufficient if crucial viral virulence determinants are affected by sequence shifts thus providing the mutant cloud with a fitness benefit in a new (host) environment.

Viral host range, cell tropism, and virulence can depend on amino acid substitutions at structural or nonstructural viral proteins. A likely candidate structural gene for such a host and virulence determinant was the viral capsid protein VP3 because for instance mutations in the VP3 protein of poliovirus were shown to be associated with changes in neurovirulence and development or loss of attenuation (Kew et al., 1980; Bouchard et al., 1995; Cherkasova et al., 2002). Moreover, for DWV it was recently shown that only virus variants containing at least the capsid protein region were found replicating in *V. destructor* (Ryabov et al., 2014). However, the VP3 gene master sequences of DWV_{P0} and DWV_{P1} clustered with DWV-B and, hence, virulence differences did again not co-segregate with the DWV-A/B clustering.

In contrast, the master sequences of the DWV RdRp gene showed a different pattern: the three DWV_{P0} variants clustered with the previously sequenced and

analyzed DWV-B RdRp gene master sequences while the three DWV_{P1} variants grouped with the DWV-A RdRp gene master sequences. Hence, restricting the phylogenetic analysis to the RdRp gene master sequences resulted in a co-segregation of virulence differences and affiliation to the DWV-A and DWV-B cluster. The obtained pattern was consistent with previous results demonstrating that DWV-B is more virulent than DWV-A (Yue and Genersch, 2005; Gisder et al., 2009; Genersch et al., 2010; Zioni et al., 2011; Schöning et al., 2012; Ryabov et al., 2014; McMahon et al., 2016; Natsopoulou et al., 2017). These results point to the RdRp as possible determinant for DWV host range and virulence. This result is not totally surprising because the RdRp has already been described as nonstructural viral protein being critical for the adaptation of avian influenza A viruses to mammals (Subbarao et al., 1993; Gabriel et al., 2005; Yamada et al., 2010) and substitutions in the RdRp resulting in enhanced fidelity of replication were related to the development of attenuation in FMDV (Rai et al., 2017; Li et al., 2018) and poliovirus (Pfeiffer and Kirkegaard, 2005). Therefore, we propose that changes in the RdRp gene and protein sequence of DWV are involved in determining host range and virulence of this virus. Further studies are necessary to elucidate the different replication strategies of DWV in arachnid and insect cells.

Conclusion

We demonstrated that crippled bees harbored DWV mutants clouds (called DWV_{P0}) that were closely related to the clouds present in the mites that parasitized these bees during pupal development and acted as intermediary hosts for DWV. Biological assays showed that DWV_{P0} was highly virulent for pupae and adult bees. We also demonstrated a shift towards decreased viral virulence when DWV_{P0} underwent an additional passage in pupae giving rise to DWV_{P1}. This attenuation

was accompanied by sequence shifts in the RdRp gene master sequences from DWV-B (VDV-1-like) to “classical” DWV-A. Therefore, a virulent DWV mutant cloud (DWV_{P0}) associated through crippled bees to viral replication in the mite developed back into a less virulent mutant cloud (DWV_{P1}) when DWV was passaged in bees suggesting that the host switch from mites to bees is accompanied by changes in viral virulence and sequence shifts. Although not experimentally tested so far, we propose that a host switch of DWV from bees to mites is accompanied by increase in viral virulence, changes in tissue tropism, and sequence shifts because this is what happened with DWV since *V. destructor* became established as a virus vector in the honey bee population (Genersch et al., 2010; Moore et al., 2011; Martin et al., 2012; Ryabov et al., 2014; McMahon et al., 2016; Wilfert et al., 2016; Natsopoulou et al., 2017). Therefore, *V. destructor* is not only driving the global epidemic of DWV in the honey bee population (Wilfert et al., 2016), but it might also drive the increase in DWV virulence by replicative selection of variants which are more virulent for honey bees.

Experimental procedures

Bee Material

All bee and mite material used in this study originated from *A. mellifera* colonies of the institute’s apiary which consists of more than 300 colonies distributed at variable locations in the vicinity of Hohen Neuendorf, Germany. Crippled bees were collected from several independent, mite-infested colonies and used for isolation of DWV_{P0}. For propagating DWV_{P0} in bee pupae in order to obtain DWV_{P1}, pupae were collected from mite- and virus-free colonies generated, identified, and maintained as already described (Möckel et al., 2011). Over the study period (2013-2017), a total of 10

colonies served as sources of virus-free pupae. All experiments involving bees were conducted during the bee seasons, hence, between May and August each year.

Collection and analysis of emerging honey bees and their associated mites

Emerging bees or bees about to emerge were sampled together with their parasitizing mites from brood frames of one mite infested colony as already described (Gisder et al., 2009). As soon as an emerging bee started to open the brood cell, the cap was removed with forceps and the honey bee was carefully collected as well as all associated mites within this brood cell. The bees and mites were stored at -80°C until further analysis. For RNA-extraction, all bees were separated into head (h), thorax (t), and abdomen (a) by using a new sterile scalpel for each cut to avoid cross-contaminations of viral RNA. Each body segment and every mite were transferred into an individual 1.5 ml reaction tube. Extraction of total RNA from honey bee body segments and from mites as well as qualitative RT-PCR analysis of these RNAs for the presence of DWV were performed essentially as already described (Genersch, 2005; Yue and Genersch, 2005). Replication of DWV in honey bee body segments and in mites was analyzed via an optimized, strand-specific two-step RT-PCR protocol essentially as described (Yue and Genersch, 2005; Gisder et al., 2009).

Partial sequencing of the DWV-L-protein gene

A 514 bp-fragment of the DWV-L-protein gene (position 1203–1716; positions refer to GenBank Accession no. NC_004830.2) was amplified from DWV-positive samples via RT-PCR performed according to the standard protocol of the OneStep RT-PCR Kit from Qiagen (Hilden, Germany) by using the following primers: forward primer DWV-L1203-F (5´-GCACCTCGTACATGGGAAGT-3´) and reverse primer DWV-

L1716-R (5´-ACTCATAATCGCGCTGTTTT-3´). The following temperature scheme was used for RT-PCR: 30 min at 50 °C, 15 min at 95 °C followed by 4 cycles with 1 min at 94 °C, 1 min at 65 °C, 1 min at 72°C, 4 cycles with 1 min at 94°C, 1 min at 62.5°C, 1 min at 72°C, 4 cycles with 1 min at 94°C, 1 min at 60 °C, 1 min at 72 °C, 4 cycles with 1 min at 94 °C, 1 min at 57.5 °C, 1 min at 72 °C, 5 cycles with 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, 5 cycles with 1 min at 94 °C, 1 min at 52.5 °C, 1 min at 72 °C and 10 cycles with 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C including a final elongation step for 10 min at 72 °C. PCR products (5 µl per reaction) were analyzed on a 1% agarose gel (Biozym, Hess. Oldendorf, Germany). The agarose gel was stained with ethidium bromide and visualized by UV light. All PCR-products were purified with the QIAquick PCR Purification Kit from Qiagen (Hilden, Germany) according to the standard protocol. The nucleotide sequences of the obtained amplicons were sequenced by Eurofins Genomics (Ebersberg, Germany, https://www.eurofinsgenomics.eu/). Further sequence- and phylogenetic-analysis was performed with Geneious 9.1.8 software package. Multiple sequence alignment was performed using the Geneious ClustalW alignment tool. Phylogenetic trees of the aligned sequences were generated with Geneious tree builder using neighbor-joining analysis, the Tamura-Nei genetic distance model (Tamura et al., 2007), and 100 bootstrap replicates.

Preparation of DWV suspensions

DWV_{P0} was isolated from naturally occurring, crippled bees collected from several independent, heavily mite infested colonies. For each preparation of DWV_{P0}, suitable colonies were identified and 50 honey bees exhibiting crippled wings were randomly collected and crushed in potassium phosphate buffer (PPB, 0.01 M, pH 7) in a mixer mill (Retsch, Haan, Germany). Further purification of DWV_{P0}-particles was performed

essentially as recently described (Möckel et al., 2011). The number of purified DWV-equivalents was determined via RT-qPCR as already described (Gisder et al., 2009) after *in silico* analysis had confirmed that the used primers DWV-F1 (5'-CCTGCTAATCAACAAGGACCTGG-3') and DWV-B1 (5'-CAGAACCAATGTCTAACGCTAACCC-3') (Genersch, 2005) were suitable to detect the DWV-A and -B variants sequenced and deposited so far. Three independent DWV_{P0} preparations (DWV_{P0} I – III) were used in this study.

DWV_{P1} preparations were generated by propagating these DWV_{P0} I – III preparations in bee pupae. To this end, infection experiments were performed, each with 100 white-eyed pupae pooled from at least three colonies that were tested to be virus- and mite-free (Möckel et al., 2011). Pupae were injected with 2 µl of a DWV_{P0} suspension (DWV_{P0} I or II or III) in PPB (5E+04 ve (virus equivalents)/µl) and incubated for 10 days at 34°C with 60 **%** relative humidity (Möckel et al., 2011). Pupae which were fatally injured by coarse piercing or handling before or during infection and, therefore, showed melanization or died within 12 h post infection were removed and discarded. DWV was isolated from 50 emerged bees and defined as DWV_{P1}. Purification and quantification of the DWV_{P1}-particles was performed as described above for DWV_{P0}. Three independent DWV_{P1} preparations (DWV_{P1} I – III) were generated in three independent infections experiments performed with DWV_{P0} I – III. Thus, three "paired" DWV preparations were generated and used in this study: DWV_{P0} I / DWV_{P1} I, DWV_{P0} II / DWV_{P1} II, and DWV_{P0} III / DWV_{P1} III. For each pair, the "evolutionary distance" between DWV_{P0} and DWV_{P1} was the described bee passage.

All DWV-preparations were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to confirm the presence of the structural virus proteins VP1, VP2 and VP3. Absence of SBV (sacbrood virus), KBV

(Kashmir bee virus), ABPV (acute bee paralysis virus) and IAPV (Israeli acute paralysis virus) was verified in all DWV-preparations with one-step RT-PCR protocols as already described (Möckel et al., 2011). Due to differences in virus yield per preparation and resulting material constraints, not all experiments could be replicated with all three DWV_{P0} / DWV_{P1} preparations.

DWV injection bioassays with pupae and adult bees

Bees to be used for infection assays all originated from colonies which had been tested to be free of infestation with *V. destructor* and infections with *Nosema* spp., DWV, ABPV, IAPV, KBV, and SBV as already described (Möckel et al., 2011). Briefly, from candidate mite-free *A. mellifera* colonies, ten forager bees, ten white-eyed pupae, and ten eggs were collected and the absence of honey bee viruses was determined via RT-PCR as recently described (Möckel et al., 2011). For determining the absence of *Nosema* spp., abdomens of twenty forager bees were crushed in 2 ml of bi-distilled H_2O and the homogenates were microscopically analyzed for the absence of *Nosema* spp. spores (Genersch et al., 2010).

Infection of naïve, virus- and DWV-free white-eyed pupae was performed via injection bioassays as already described (Möckel et al., 2011). Briefly, a dosage of 2E+05 DWV genome equivalents suspended in 2 µl PPB were injected ventrally into individual pupae between the third and fourth abdominal segment. Control pupae were mock infected (injection of 2 µl PPB) or not manipulated at all. Five biological replicates of the injection bioassays (N=5) were performed, each one with three groups of 30 pupae (3 technical replicates; 3x30 pupae), each for DWV_{P0} infected, DWV_{P1} infected, mock infected, and non-manipulated pupae. Pupae which were fatally injured by coarse piercing or handling before or during infection and, therefore, showed melanization or died within 12 h post infection were removed from the

experiments. This resulted in a total of 344, 316, 367, and 360 pupae in the DWV_{P0}-infected, DWV_{P1}-infected, mock infected and non-manipulated groups, respectively. These injection bioassays were performed in three consecutive bee seasons with all three independent DWV_{P0}/DWV_{P1} preparations (DWV_{P0} I – III, DWV_{P1} I – III).

Injection bioassays were also used to infect naïve, DWV-free, newly emerged worker bees (Möckel et al., 2011). To obtain these bees, queens were caged on brood frames for egg laying. About 24 hours before adult bee emergence, brood frames were placed in an incubator at 34°C. The next day, worker bees were collected upon emergence. Groups of 30 bees each were individually infected by injecting either DWV_{P0} or DWV_{P1} into the thorax hemolymph. In addition, for each virus variant, two virus dosages (2E+05 ve, 2E+09 ve) suspended in 2 µl PPB were tested. Groups of 30 control bees each were either mock infected (injection of 2 µl PPB) or not manipulated at all. Following infection, the experimental groups were transferred into wooden cages (30 bees per cage) and fed *ad libitum* with 50% sucrose (w/v) supplemented with 15% commercially available pollen as protein source free of contaminating *Nosema* spp. spores or viruses (DWV, SBV, KBV IAPV, ABPV) (Möckel et al., 2011). The feeders were freshly prepared with food solution every day. All bees were kept in an incubator at 34°C and 60% RH for 27 days. Bee mortality was recorded daily. The “paired” preparation DWV_{P0} II/DWV_{P1} II was used for these experiments which were performed in triplicate resulting in 90 bees (3 x 30) tested in each group.

Tissue tropism of DWV_{P0} and DWV_{P1}

Adult bees experimentally infected with DWV_{P0} I / II (n=150) or DWV_{P1} I / II (n=90) were cut into the segments head, thorax and abdomen and the heads and thoraces

were individually transferred into 1.5 ml reaction tubes (Eppendorf, Hamburg, Germany). Total RNA was extracted by using the RNeasy-Mini Kit (Qiagen) according to the manufacturer's protocol. One-step RT-PCR was performed with the One-Step RT-PCR Kit (Qiagen) according to standard protocols as previously described (Yue and Genersch, 2005; Möckel et al., 2011). We further analyzed tissue sections from brains of experimentally DWV-infected adult honey bees with fluorescence *in situ*-hybridization (FISH) analysis to confirm the presence of DWV in brains of infected animals. DWV-specific FISH analysis was essentially performed as already described (Yue et al., 2008; Möckel et al., 2011) by using 5' FITC-labelled oligonucleotides (Eurofinsdna) as probes for the specific detection of DWV RNA. To visualize the cytoplasm of honey bee cells, 5' Texas Red®-labelled probe EUK516 (5'-ACCAGACTTGCCCTCC-3') (Amann et al., 1990) was used and cellular nuclei were stained blue with 1 µg/ml DAPI in PBS for 10 minutes.

Olfactory conditioning of DWV-infected adult bees

The learning ability of honey bees can be studied under controlled laboratory conditions by using olfactory conditioning of the proboscis extension response (PER) (Felsenberg et al., 2011). Bees to be used in olfactory conditioning experiments all originated from colonies which had been tested to be free of infestation with *V. destructor* and infections with *Nosema* spp., DWV, ABPV, IAPV, KBV, and SBV as already described (Möckel et al., 2011). For each experiment, forager honey bees were caught in the morning from the flight board and chilled on ice until they stopped moving. Immobilized bees were infected by injection of 2 µl virus suspension in PPB (1E+09 ve DWV_{P0} or DWV_{P1} /µl) into the thorax hemolymph (DWV infected group). This dosage had been shown to induce brain infections in adult bees (Möckel et al., 2011). Control bees were injected with 2 µl PPB (mock infected control group) or

were not further manipulated (non-manipulated control group). Bees were transferred into wooden cages (30 bees/cage; separated according to groups) and fed *ad libitum* with 50% sucrose (w/v) supplemented with 15% commercially available pollen as protein source free of contaminating *Nosema* spp. spores or viruses (DWV, SBV, KBV IAPV, ABPV) (Möckel et al., 2011).

All bees were kept in an incubator at 34°C and 60% RH for 48 hours. Subsequently, the bees were immobilized on ice and harnessed in plastic tubes with a strip of tape between the head and the thorax. The harnessed bees were placed on a rack and in the afternoon, they were fed to satiation with 0.88 M sucrose. The bees were kept overnight at darkness and olfactory conditioning was carried out in the next morning, i.e. 72 hours post infection. The racks with the harnessed bees were placed at light half an hour before the classical olfactory conditioning trials started. Olfactory conditioning consisted of temporal pairing carnation odor as conditioned stimulus (CS) with the presentation of sucrose (1.25 M) as unconditioned stimulus (US) as demonstrated (Felsenberg et al., 2011). The carnation odor (CS) was presented for 5 s to each bee followed by the successive presentation of sucrose to the antennae and the proboscis (US) for 4 s. Both stimuli overlapped for 2 s. Three conditioning trials were performed with each bee with an intertrial interval of 2 min. For memory retention test, the CS was presented for 5 s alone (without US) two hours after conditioning started. Bees which showed a PER during presentation of carnation odor were evaluated as positive and bees which failed responding with a PER during presentation of the CS were evaluated as negative. Bees which failed extension of proboscis by sucrose stimulation at the end of the entire experiment or which died during olfactory conditioning procedure were excluded from further analysis. After memory retention test, all surviving bees were sacrificed by storing them at -80°C for molecular virus detection with RT-PCR.

These olfactory conditioning experiments with DWV_{P0} and DWV_{P1} infected bees were performed over three bee seasons (May to August each in 2013, 2014, 2015) to prove the robustness of the results and their independence from environmental factors possibly acting on the bees. To minimize any putative influence of the bees' genetic background on learning performance, the experimental groups consisted of bees originating from different colonies of the institute's apiary. Two independent DWV_{P0} preparations (DWV_{P0} I and II) and two independent DWV_{P1} preparations (DWV_{P1} I and II) were used. A total of eight independent olfactory conditioning experiments were performed, each for DWV_{P0} and DWV_{P1} resulting in 119 DWV_{P0} infected bees and 159 DWV_{P1} infected bees tested in total. Due to the reduced neurotropism of DWV_{P1}, only 52 DWV_{P1} infected bees showed an infection of the brain. Therefore, within the group of DWV_{P1} infected bees, learning performance was evaluated for both, all infected bees (n=159) and those that showed an infection of the brain (n=52). Due to the time constraints of the experimental design (intertrial interval of 2 min), it was not possible to test more than three groups (non-manipulated, mock-infected, infected bees) in one experimental run. However, in three of the eight experiments, DWV_{P0}- and DWV_{P1} infected bees were tested in parallel.

Sequence analysis of DWV_{P0} and DWV_{P1} master sequences

The DWV master sequences of the DWV_{P0} I - III and DWV_{P1} I - III preparations were analyzed with next-generation sequencing (NGS). Total RNA of the DWV preparations were extracted as previously described (Gisder et al., 2009) and sent to LGC Genomics GmbH (Berlin, Germany) for NGS. First-strand cDNA libraries were prepared using the Illumina TruSeq DNA library preparation kit V3 followed by sequencing of 300-bp paired-end reads on an Illumina MiSeq instrument with 1

million read pairs. Illumina's CASAVA data analysis software was used for demultiplexing of all samples and Illumina TruSeq™ adapters were clipped in all reads. Reads with a final length < 20 bases were discarded. rRNA sequences were filtered using RiboPicker 0.4.3 (http://ribopicker.sourceforge.net/) and *Apis mellifera* (Amel 4.5) sequences were also filtered. De novo assembly of DWV-reads was realized with CLC Genomics Workbench v 10.1.1. All resulting DWV-contigs were further assembled with Geneious software package 9.1.8. The ´Map to Reference tool´ was used to align the contigs with the DWV (NC_004830.2) and VDV-1 (NC_006494.1) reference genomes to obtain DWV-genome master sequences as complete as possible. The relationship of the obtained DWV_{P0}- and DWV_{P1}-master sequences with the deposited sequences of DWV Type A (NC_004830.2, NC_005876.1, JQ_413340 and CENC01000001.1), DWV Type B (KC_78622.1 and NC_006494.1), DWV Type C (CEND01000001.1) (according to Mordecai et al. (2016)) was analyzed with ClustalW alignment. Formica exsecta virus 2 (Fex2) polyprotein gene (NC_023022.1) was used as outgroup (Mordecai et al., 2016). Phylogenetic trees were generated with Geneious tree builder using Tamura-Nei genetic distance model and neighbor-joining tree build method with 100 replicates and topology threshold >10%. For a more detailed look on the sequence shift of the DWV_{P0} and DWV_{P1} master sequences, the sequences of the partial capsid protein VP3 gene (2634-3838bp) and the partial RNA-dependent RNA-polymerase (RdRp) gene (8016-8522 bp) (Mordecai et al., 2016; Wilfert et al., 2016) were also analyzed with the respective partial sequences of the DWV complex and the Fex2 genes (see above).

Data analysis

To compare the infectivity and tissue tropism of the different DWV isolates two-tailed Mann-Whitney U test was performed using GraphPad PRISM software package 6.01. Mortality of infected honey bee pupae was analyzed with a One-Way ANOVA and Tukey's multiple comparison test as *post hoc* test using GraphPad PRISM software package 6.01. Mortality of infected adult honey bees was analyzed with a Two-Way ANOVA and Tukey's multiple comparison test as *post hoc* test using GraphPad PRISM software package 6.01. For identification of different learning performances during the conditioning trials or memory testing a Two-Way ANOVA with Tukey´s multiple comparison test as *post hoc* test was used (GraphPad PRISM software package 6.01).

Data availability

All data generated or analyzed during this study, except for the sequence data of the six variants of DWV (DWV_{P0} I - III, DWV_{P1} I - III), are included in this published article. The sequences of DWV_{P0} I – III and DWV_{P1} I - III have been submitted to NCBI BankIt, submission #2136078.

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Figure Legends

Fig. 1. Unrooted phylogenic trees of the master sequences of a 514 bp fragment of the DWV L-protein gene inferred by using neighbor-joining analysis (Tamura-Nei genetic distance model). (A) The DWV L-protein gene master sequences amplified from healthy bees (hb) and their mites (hb/m) revealed considerable sequence variation between bees and mites and even within a single bee. (B) The DWV L-protein gene master sequences amplified from symptomatic bees and from the mites that parasitized these bees during pupal development revealed that each crippled bee and its accompanying mites shared the same master sequence. The trees were generated by using the Tamura-Nei genetic distance model and 100 bootstrap replicates. Scale bars represent estimated phylogenetic divergence.

Fig. 2. Lethality of DWV_{P0} and DWV_{P1} for honey bee pupae and adult bees assessed by injection bioassays. (A) Mortality of white-eyed pupae experimentally infected with DWV_{P0} (n=344) and DWV_{P1} (n=316). White-eyed honey bee pupae were individually infected with 2E+05 virus equivalents (ve) of either DWV_{P0} (red) or DWV_{P1} (green) and incubated for up to ten days to allow pupal development to be completed. Control pupae (blue) were either mock-infected (n=367) or left non-manipulated (n=360). Total mortality (mean ± SEM) within ten days is shown. Statistical analysis was performed with One-way ANOVA and Tukey's multiple comparison test as *post hoc* test (not significantly different: n.s., $p \geq 0.05$; significantly different: *, $0.05 > p \geq 0.01$; **, $0.01 > p \geq 0.001$; ****, $0.0001 > p \geq 0.00001$). (B) Survival of adult honey bees experimentally infected with DWV_{P0} and DWV_{P1}. Newly emerged worker bees were individually infected with 2E+05 ve of either DWV_{P0} (red

squares) or DWV_{P1} (green diamond) as well as with 2E+09 ve of either DWV_{P0} (red crosses) or DWV_{P1} (green circles). Control bees were either mock-infected (blue triangles) or non-manipulated (not shown). Mortality was monitored over 27 days post infection. The experiments were performed in triplicate with 30 bees per group. Error bars represent standard deviations (SD). Statistical analysis was performed with Two-way ANOVA and Tukey's multiple comparison test as *post hoc* test.

Fig. 3. Tissue tropism of DWV_{P0} and DWV_{P1}. (A) Adult honey bees were infected via injection bioassays with 2E+09 viral equivalents of DWV_{P0} or DWV_{P1}. Three days post infection, bees were sacrificed and DWV infectivity and tissue tropism were examined. RT-PCR analysis revealed presence of DWV in the thoraces of 100% ± 0% (mean ± SD; DWV_{P0}) or 98.72 ± 2.22 % (mean ± SD; DWV_{P1}) and in the heads of 89.19 ± 7.25% (mean ± SD; DWV_{P0}) or 30.83 ± 15.88% (mean ± SD; DWV_{P1}) of the injected bees (n=150 for DWV_{P0}, n=90 for DWV_{P1}). This difference was statistically significant (Mann-Whitney U test, p-value = 0.036). (B) Fluorescence *in situ*-hybridization (FISH) analysis for the detection of DWV RNA (red signals) in the brain (eukaryotic ribosomal RNA in the cytoplasm, green signals; nuclei, blue staining) of experimentally infected adult bees. DWV could be detected in the somata regions of the mushroom bodies (a), in the lobula (b), the inner (c) and outer (e) chiasma and the medulla (d). Results obtained with bees experimentally infected with DWV_{P0} are shown (left and middle picture). DWV analysis was negative for all control bees (right picture). Representative pictures are shown; scale bars represent 100 µm.

Fig. 4. Olfactory conditioning experiments assessing learning performance and memory formation in adult bees experimentally infected with DWV_{P0} and DWV_{P1}. Virus-free adult bees were infected with DWV_{P0} (A) or DWV_{P1} (B) and

conditioned with three conditioning trials 72 hours post infection; memory retention was tested two hours after conditioning. (A) PER during the presentation of the conditioned stimulus (CS, odour) of bees infected with DWV_{P0} was significantly lower both in the acquisition phase and the memory retention test compared to the control groups (****, p-value < 0.0001). The two control groups did not show a statistically significant difference (n.s., p - value > 0.05) in learning performance and memory retention when the CS was presented. White columns represent the results of non-manipulated control bees (n=153), grey columns of mock-infected control bees (n=114), and black columns of bees experimentally infected with DWV_{P0} (n=119). (B) In the olfactory conditioning experiments performed with DWV_{P1} infected bees, no statistically significant difference (n.s.) in learning performance and memory retention between the four groups of bees was observed (Two-Way ANOVA followed by Tukey´s multiple comparison test, p-values > 0.05 for all tests). White columns represent the results of non-manipulated control bees (n=149), light grey columns of mock-infected control bees (n=144), dark grey columns of all bees experimentally infected with DWV_{P1} (n=159), while black columns represent the results of those bees from the DWV_{P1} infected group that tested positive for DWV in their heads (n=52).

Fig. 5. Neighbor-Joining phylogeny (Tamura-Nei distance) of previously sequenced DWV variants and the master sequences each of three independent DWV_{P0} and DWV_{P1} isolates. The phylogenetic trees show the alignment of the master sequences of (A) the whole genomes, (B) the partial virus protein VP3 genes and (C) the partial RNA-dependent RNA-polymerase (RdRp) genes from DWV_{P0} (red) and DWV_{P1} (green) isolates as well as from previously sequenced and analyzed DWV-A, -B, and -C variants. The trees were generated by using the Tamura-Nei

genetic distance model and 100 bootstraps replicates and bootstrap values (100, 97, 83) are given at the junctions with diverging DWV-A and DWV-B clades. The scale bar represents estimated phylogenetic divergence. Formica exsecta virus 2 was included as outgroup. The DWV-B clusters are highlighted by a gray cloud.

Competing interests

The authors declare no competing interests.

Author contributions

SG, DE, and EG conceived and designed the experiments; SG and NM performed the experiments; SG and EG analyzed the data; SG and EG wrote the paper; all authors reviewed the manuscript.

Originality-Significance Statement

Deformed wings virus is considered a recent global epidemic in honey bees driven by Varroa mites. Mite vectored DWV infections cause elevated colony losses over winter and are a major threat to the world's honey bees. Differentially virulent variants of DWV (DWV-A, -B) exist but the relationship between DWV virulence and the mite is poorly understood. Our data show that DWV exists as a quasispecies and suggest that the mites act as "reaction tube" for generating mutant clouds which are highly virulent for honey bees. However, we show that these virulent variants evolved back into less virulent variants when passaged in bees. Therefore, controlling the mite in honey bee colonies will solve the problem caused by DWV-B because DWV-B is not a fixed genotype circulating on its own but the result of replicative selection in the

mite. This selection can be reversed when DWV is back in its original host, the bee, due to the quasispecies nature of DWV.