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Deformed wing virus and drone mating flights in the honey bee (*Apis mellifera*): implications for sexual transmission of a major honey bee virus

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Abstract – Deformed wing virus (DWV) represents an ideal model to study the interaction between mode of transmission and virulence in honey bees since it exhibits both horizontal and vertical transmissions. However, it is not yet clear if venereal–vertical transmission represents a regular mode of transmission for this virus in natural honey bee populations. Here, we provide clear evidence for the occurrence of high DWV titres in the endophallus of sexually mature drones collected from drone congregation areas (DCAs). Furthermore, the endophallus DWV titres of drones collected at their maternal hives were no different from drones collected at nearby DCAs, suggesting that high-titre DWV infection of the endophallus does not hinder the ability of drones to reach the mating area. The results are discussed within the context of the dispersal of DWV between colonies and the definition of DWV virulence with respect to the transmission route and the types of tissues infected.

honey bee / DWV / drone congregation areas / vertical transmission

1. INTRODUCTION

The spread and persistence of pathogens in a host population is determined by the dynamic interaction between pathogen transmission and virulence (Lipsitch and Moxon 1997; Chen et al. 2006; Alizon et al. 2009). Moreover, the manner in which a pathogen is transmitted to host individuals is expected to affect its virulence (Galvani 2003), with horizontal transmission routes (between individuals of the same generation) selecting for increased virulence so as to maximise de novo infection of healthy individuals (Bull 1994; Read 1994) and vertical transmission (from parent to offspring) selecting for reduced virulence so as to maximise transmission and minimise the impact of infection on host reproduction (Lipsitch and Moxon 1997; Fries and Camazine 2001; Stewart et al. 2005). However, the relationship between transmission and viru-

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lence is more complicated when the pathogen has multiple transmission routes operating simultaneously (Lipsitch et al. 1996). Such is the case with deformed wing virus (DWV: Iflaviridae) of honey bees (*Apis mellifera*).

DWV is the best characterised of the approximately 18 viruses known to infect honey bees (Ribière et al. 2008; de Miranda and Genersch 2010). It is present in all developmental stages and castes (Yue and Genersch 2005; Chen et al. 2005; Tentcheva et al. 2006) and, at high titres, causes characteristic wing deformities, shortened abdomens, discolouring, social and behavioural abnormalities and a severely reduced lifespan of adult honey bee workers, drones and queens (Bowen-Walker et al. 1999; Yue and Genersch 2005; Iqbal and Mueller 2007; Williams et al. 2009; de Miranda and Genersch 2010). DWV can be transmitted horizontally among adult bees through trophallaxis and from adult bees to larvae through glandular food secretions (Nordström 2000; Chen et al. 2005; Iqbal and Mueller 2007). It can also be vectored between adult bees, and from adults to developing pupae, by parasitic mites (Bowen-Walker et al. 1999; Yue and Genersch 2005, Forsgren et al. 2009; Dainat et al. 2009), representing a different form of horizontal transmission. DWV can also be transmitted venereally, by artificial insemination of queens with DWV contaminated sperm, and subsequently transovarially, from queens to their offspring (Yue et al. 2007; de Miranda and Fries 2008). Although the mating act itself is strictly a horizontal transmission (Chen et al. 2006), its purpose in the context of honey bee reproduction is to establish the conditions for vertical transmission, either directly through the sperm or indirectly through infection of the ovaries (de Miranda et al. 2011), since the drones die in flight after mating and the queens only function in the colony is to produce the subsequent generations. The high DWV titres found naturally in the sperm and the reproductive organs of drones and queens strongly suggest that venereal–vertical transmission also occurs naturally (Fievet et al. 2006; Yue et al. 2006), although this still has to be shown conclusively. Furthermore, the relative contribution of these multiple transmission routes to the epidemiology of DWV is still unclear (de Miranda and Genersch 2010). DWV is therefore an ideal model pathogen for studying the interaction between transmission route and virulence, since it exhibits vertical and horizontal transmission, with both direct (oral) and vector-mediated (*Varroa*) forms of horizontal transmission and both venereal and transovarial components to vertical transmission.

Honey bee mating involves the aggregation of several thousand sexually mature drones from different colonies within a 5-km radius at specific drone congregation areas (DCAs), located at 5–25 m above the ground (Baudry et al. 1998; Jaffé et al. 2009) where they fly for up to 30 min (Koeniger et al. 2005a). Virgin queens visit these DCAs to mate in flight with numerous drones before returning to the hive (Koeniger and Koeniger 2007). The drones die instantly after mating and their semen passes to the queen's spermatheca, where it is retained for fertilising eggs throughout the queen's life (Woyke 1983).

The extent of venereal–vertical transmission of DWV is determined by the ability of DWV-infected drones to inseminate a virgin queen with virus-infected sperm. There is strong selection for drone flight performance to DCAs (Koeniger et al. 2005b; Jaffé and Moritz 2010), as well as for general health and vitality during the 10-day in-hive drone maturation period prior to mating flights. Indeed, most drones infested by mites during the pupal stage are expelled from the colony before reaching sexual maturity (Rinderer et al. 1999). This may be why drones are the least-infected individuals in honey bee colonies, both in the proportion of infected individuals and in terms of normalised DWV titres (Chen et al. 2005), despite the strong preference of *Varroa destructor* mites to reproduce on (and thus infect with DWV) drone pupae (Le Conte et al. 1989; Fries et al. 1994). It is therefore unclear to what extent sexually mature drones harbour DWV and are able to transmit this to virgin queens during mating.

Here, we provide the first clear evidence for a high incidence and considerable titres of DWV in the reproductive organs of sexually mature drones collected from natural DCAs, i.e. drones that could potentially mate with a virgin queen. By genetically tracing the maternal origin of the drones from the DCAs and comparing these with those of nearby hives, we also test if natural DWV infection of the drone endophallus hinders the ability of drones to join a DCA.

2. MATERIALS AND METHODS

2.1. Sample collection, preservation and storage

Samples of honey bee drones were collected at four geographic locations exhibiting different levels of *Varroa* infestation:

1. Ezemvelo Nature Reserve, South Africa (25°42′34′S, 28°59′60′E), hosting a wild *Apis mellifera scutellata* population and lacking any beekeeping activity or *V. destructor* control within a radius of 25 km. DWV quantification and drone genetic analysis were performed on 75 drones captured at a single DCA. *V. destructor* infestation levels in wild honey bee populations from the Cape Region are currently estimated to be 0.06 mites per 100 bees (Allsopp 2006). A few feral colonies were located in the vicinity of the DCA, but they contained no drones at the time.
2. Caher, Ireland (52°21′13′N, 7°57′27′W), hosting an *Apis mellifera mellifera* population subjected to selective breeding and *Varroa* control treatment. DWV quantification and drone genetic analysis were performed on 50 drones captured at a DCA and 50 drones collected from five local hives: ten drones each from hives DA4, DA7, DA11, DA14 and DA15. The colonies were treated in autumn with either Bayvarol® or a combination of Apiguard® and oxalic acid, and had an average *Varroa* infestation rate of 1.9±0.4 mites per 100 bees. The DCA was located 5 km from the hives.
3. Gotland, Sweden (57°04′37′N, 18°12′26′E), hosting an isolated hybrid honey bee population that has survived without *Varroa* control treatment for over 8 years (Fries et al. 2006). DWV quantification and drone genetic analysis were performed on 24 drones captured at a DCA and 50 drones collected from six local hives—23 drones from hive 3B, 13 drones from hive KB, seven drones from hive 27, three drones from hive 4B, three drones from hive 1 and one drone from hive 701. The colonies had an average *Varroa* infestation rate of 15.5±3.4 mites per 100 bees. The DCA was located 150 m from the test hives.
4. Halle, Germany (51°30′20′N, 11°56′58′E), hosting an *Apis mellifera carnica* population subjected to regular *Varroa* control treatment. DWV quantification and drone genetic analysis were performed on 49 drones captured at a DCA and 45 drones collected from five local hives:—ten drones each from hives B, G, R; and eight and seven drones from hives W and Y, respectively. The colonies had an average *Varroa* infestation rate of 19±4.1 mites per 100 bees. The DCA was located 250 m from the test hives.

At each location, drones were captured from the DCAs using a trap baited with synthetic queen mandibular pheromone (E-9-oxo-2-decenoic acid) attached to a weather balloon filled with helium (Williams 1987). The pheromone trap was flown between 13:00 and 18:00 under sunny and non-windy conditions at previously identified DCAs, keeping it about 20 m above ground and taking it down at 10-min intervals to remove the trapped drones. In Caher, Gotland and Halle, random samples of drones were also collected from several hives within the recruitment areas of the DCAs. In Caher and Gotland, these drones were collected from the outer honey frames inside the hives, where the sexually mature drones gather to feed on honey. However, immature drones can also be found here, while only mature drones will be found in the DCAs, potentially biasing the data if there are quantitative DWV differences between mature and immature drones. Therefore, in Halle, newly emerged drones were marked on the thorax with different coloured tags for each hive and returned to their maternal hive for maturation. These marked drones were later re-captured at the DCA and at the Hives, thus ensuring that all drones were mature and of a similar age.

Eversion of the endophallus of each drone was stimulated by gently squeezing the abdomen. Once everted, the whole endophallus and the expelled semen contained in it was cut off at its base with fine scissors, immediately stored in 350 μL Qiagen RLT extraction buffer (including β–mercaptoethanol), transported on ice and finally stored at −20°C until RNA extraction. The remainder of each drone was stored in 95% ethanol for genetic analysis.

2.2. DNA extraction and microsatellite assays

Since drones are produced by arrhenotokous parthenogenesis and thus only carry alleles from their mother, genotyping drones allows for their assignment to a specific mother queen and the subsequent reconstruction of the queen's genotype. The DNA from each drone was extracted using a Chelex protocol (Walsh et al. 1991). Three sets of four, tightly linked microsatellite markers were used to identify individual drone genotypes (Shaibi et al. 2008). The three linkage groups are located on different chromosomes:

Chromosome 3 (LG-3)-loci HB-SEX-01, UN351 and HB-SEX-03 (spanning 11.6 kbp)

Chromosome 13 (LG-13)-loci HB-THE-01, HB-THE-02, HB-THE-03 and HB-THE-04 (spanning 11.2 kbp)

Chromosome 16 (LG-16)-loci HB-16–01, AC006, HB-16–02, HB-16–03 and HB-16–05 (spanning 16.4 kbp).

Microsatellite loci were amplified by multiplex polymerase chain reactions (PCRs) using fluorescence-labelled primers and the Promega PCR Master Mix (see Shaibi et al. 2008 for PCR conditions). Samples containing no DNA were included in all plates as negative controls. The PCR products were resolved in a MegaBACE 1,000 capillary sequencer to determine allele sizes. Genetic Profiler software (Amersham Biosciences) was used to score alleles.

2.3. Queen genotype reconstruction and drone colony assignment

For each geographic location, the drone genotypes were organised by their allelic profile for the microsatellite loci of the three linkage groups. Individuals sharing the same allelic combination at all loci within each linkage group were assigned to a single haplotype. The diploid genotypes of the drone-producing queens were then reconstructed based on the associations of haplotypes found among the three linkage groups (Jaffé et al., 2009). Since this reconstruction is based on 12 highly polymorphic microsatellite loci, in three tight-linkage groups on different chromosomes, the chance that two queens have identical haplotypes is very low, $P=0.0075$ (Jaffé et al. 2009; Boomsma and Ratnieks 1996). The accuracy of the reconstruction depends on the number of drones representing each queen. Drones sampled from hives were deemed to represent the resident queen of that hive, unless their haplotype was inconsistent with the reconstruction, identifying them as drifting drones. Also, queens in hives represented by few drones (i.e. hives #1, #4B and #701 in Gotland) could be distinguished from the queens of other hives through a unique haplotype pattern, even if it was not possible to fully reconstruct both haplotypes. Drones in the DCAs were only matched to the hives if their haplotype matched perfectly that of the reconstructed hive queens, including those represented by few hive drones. Those that could not be positively allocated to hives were allocated to unknown queens outside the study, as described before (Shaibi et al. 2008; Jaffé et al. 2009).

2.4. RNA extraction, reverse transcription and quantitative PCR assays

The normalised DWV titre in the endophallus of individual drones was determined by real-time reverse transcription-quantitative polymerase chain reactions (RT-qPCR), using primers specific for DWV RNA and intron-spanning primers specific for the mRNA of RP49 (Table I). RP49 is a common honey bee reference gene (Lourenço et al. 2008) and is used here to normalise the DWV data for differences between samples in RNA quality and quantity. Primer-pairs F1/B1 and F1153/B1806 have been described previously (Genersch 2005; Forsgren et al. 2009). All other primers were designed with the assistance of the Beacon Designer software (BioRad), in the first instance by virus variability criteria for

Table I. Primers used for the detection and relative quantification of DWV in the endophalli of drones from the four geographic locations.

Location	Target	Primers	Sequence (5′–3′)	Size, bp	qPCR efficiency
Ezemvelo South Africa	DWV	DWV-F8668	TTCATTAAAGCCACCTGGAACATC	136	1.97
		DWV-B8757	TTTCCTCATTAACTGTGTCGTTGA		
		DWV-F1	CCTGCTAATCAACAAGGACCTGG	355	1.94
		DWV-B1	CAGAACCAATGTCTAACGCTAACCC		
		DWV-F1153	ATTAAAAATGGCCTTTAGTTG	694	qualitative RT-PCR
		DWV-B1806	CTTTTCTAATTCAACTTCACC		
	RP49 mRNA	RP49-qF	AAGTTCATTCGTCACCAGAG	205	1.76
		RP49-qB	CTTCCAGTTCCTTGACATTATG		
Caher Ireland	DWV	DWV-F1425	CGTCGGCCTATCAAAG	417	1.70
		DWV-B1806	CTTTTCTAATTCAACTTCACC		
	RP49 mRNA	RP49-qF	AAGTTCATTCGTCACCAGAG	205	1.68
		RP49-qB	CTTCCAGTTCCTTGACATTATG		
Gotland Sweden	DWV	DWV-F1425	CGTCGGCCTATCAAAG	417	1.74
		DWV-B1806	CTTTTCTAATTCAACTTCACC		
	RP49 mRNA	RP49-qF	AAGTTCATTCGTCACCAGAG	205	1.73
		RP49-qB	CTTCCAGTTCCTTGACATTATG		
Halle Germany	DWV	DWV-F8668	TTCATTAAAGCCACCTGGAACATC	136	2.08
		DWV-B8757	TTTCCTCATTAACTGTGTCGTTGA		
	RP49 mRNA	RP49-for	TCGTCACCAGAGTGATCGTT	243	2.09
		RP49-rev	CCATGAGCAATTTCAGCACA		

Primer RP49-qB bridges the junction between exon-2 and exon-3 (*underlined*), with RP49-qF located in exon-1 of the RP49 gene. Primers RP49-for and RP49-rev are located in *exon-1* and *exon-3*, respectively. The sizes of the targeted regions and the efficiencies of the qPCR reactions for each pair of primers are indicated

DWV (de Miranda 2008) and intron-spanning criteria for RP49 mRNA (de Miranda and Fries 2008), secondarily by PCR protocol compatibility (similar T^m, 100–450 bp product) and finally by predicted qPCR performance. The optimal annealing temperatures and primer concentrations were determined empirically with respect to amplification specificity (different DWV strains; RP49 mRNA vs. DNA; absence of non-specific products) through PCR assays and sequencing of representative products.

RNA from the Caher and Gotland drone samples was extracted using the Total RNA Kit II (E.Z.N. A™). The RT-qPCR assays were performed using the Bio-Rad iScript One-Step RT-PCR Kit with SYBR Green as the detecting chemistry, in 20-μL volumes containing 0.1 μg template RNA, 10 μL of 2× SYBR Green RT-PCR Reaction Mix, 0.4 μL of iScript Reverse Transcriptase and 0.2 μM each of the forward and reverse primers for either DWV or RP49 mRNA (Table I). The RT-qPCR cycling profile consisted of 10 min incubation at 50°C for cDNA synthesis, 5 min incubation at 95°C and 40 cycles of 10 s at 95°C for denaturation and 30 s at 58°C for annealing, extension and data collection. To verify the specificity of the PCR products, the amplification was followed by a melting curve analysis by incubating for 60 s at 95°C, 60 s at 55°C and then reading the fluorescence at 0.5°C increments from 55°C to 95°C.

RNA from the Ezemvelo and Halle drone samples was extracted using the RNeasy kit (Qiagen). cDNA was synthesised in 15-μL reactions containing 0.1 μg RNA, 0.5 μg/μL oligo-dT primer (Promega) and 80 units M-MLV H(−) Point Mutant reverse transcriptase (Promega). The qPCR assays were performed using the BioRad iQ SYBR Green qPCR kit, with SYBR-green as the detection chemistry, in 10 μL volumes containing 5 μL of iQ SYBR-green Supermix, 1 μL cDNA and 0.4 μM each of the forward and reverse primers for either DWV or RP49 mRNA (Table I). The qPCR cycling profile consisted of 3 min incubation at 95°C to activate the Taq polymerase, followed by 40 cycles of 15 s at 95°C for denaturation, 30 s at 58°C for annealing and 30 s at 72°C for extension and data collection. Each sample was run in duplicate. To verify the specificity of the PCR products, the amplification was followed by a melting curve analysis by reading the fluorescence at 1.0°C increments from 50°C to 90°C. Each reaction plate included at least one negative (H_2O) control and at least four positive controls for each primer pair, obtained from tenfold serial dilutions of purified PCR products (Caher and Gotland) or pooled cDNA from 20 drones (Ezemvelo, Halle). The RT-qPCR products from the Ezemvelo samples were also resolved on 1% agarose–TBE gels.

2.5. Generation and primary analysis of RT-qPCR data

The data were first screened for the presence of specific target PCR product, as determined by the melting curve analyses. Failed RP49 mRNA reactions and the corresponding DWV reactions were excluded from all analyses. RNA samples were considered DWV-free if the assays did not produce specific DWV products.

The fluorescence threshold for determining the C_q values (cycle for quantification; Bustin et al. 2009) of the reactions was set at 1.0× standard deviation over cycle range after baseline subtraction using the global minimum option by the Opticon Monitor three software (Biorad). The average of the duplicate C_q values was used in subsequent analyses.

The external dilution standards data from all RT-qPCR runs for each location (Ezemvelo, Caher, Gotland or Halle) were combined in a single regression analysis of C_q value onto the log[template] for converting the sample C_q values into estimated absolute copy numbers of each target in each sample. The regression slopes were also used to determine the reaction efficiencies of the different primer pairs at each of the four locations (Table I; Bustin et al. 2009). The DWV titres were normalised to the average amount of RP49 mRNA for all samples, in order to correct for differences between the samples in the quantity and quality of RNA. These data were then adjusted by the various experimental dilution factors to arrive at an estimated DWV copy number (titre) per drone endophallus.

2.6. Statistical analyses

The DWV titres of the individual drones covered a wide range of values, spanning nearly eight orders of magnitude. Logarithmic-scale variation in DWV titre

has been observed previously at all levels of bee and colony organisation, including tissue distribution (Fujiyuki et al. 2006; Fievet et al. 2006), developmental stage (Tentcheva et al. 2006; Chen et al. 2005), worker castes (Fujiyuki et al. 2004; Rortais et al. 2006) and the sexes (Tentcheva et al. 2006; Chen et al. 2005; Fievet et al. 2006) and is a logical consequence of the exponential nature of virus replication and proliferation (Brunetto et al. 2009). This means that DWV titres require a power transformation for parametric quantitative analyses (Box and Cox 1964; Bickle and Doksum 1981) or a conversion to ranks for non-parametric analyses. The data were therefore log-transformed prior to analysis and are presented graphically as mean Log_{10}[DWV]/endophallus±standard error. There were several samples where DWV was not detected, i.e. the DWV amplification curve did not reach the C_q fluorescence threshold before cycle 40 of the PCR, thus returning a zero value for relative quantification. Since it is not possible to take the logarithm of zero values, the common statistical solution for including zero values in log-transformed datasets is to add to each value a minimal constant. We derived this constant using a hypothetical C_q value of 41 for DWV (effectively <1 DWV particle per PCR) and converting this to an estimated DWV titre as described above.

The genetic data were used to partition the drone DWV titre data according to genetic colony of origin in order to evaluate the DWV titre differences between colonies at any one locality, as well as to compare the DWV titres of DCA-caught and hive-collected drones from individual colonies. Groups of bees were compared by one-way and two-way analysis of variance (ANOVA) using the SPSS 15.0 statistical software (Norusis 2006). The data were also analysed by non-parametric methods: the Kruskal–Wallis Test for multiple comparisons and Mann–Whitney *U* tests for pairwise comparisons. All non-parametric results were consistent with the parametric analyses, so only the latter are presented.

3. RESULTS

The distribution of drone endophallus DWV titres in the four DCAs and nearby hives varied considerably (Figure 1), as did the average colony *Varroa* infestation rates in hives (Figure 1). DWV could not be detected by any of the three DWV RT-PCR assays used in the drones captured at our DCA in the Ezemvelo Nature Reserve, a region in South Africa known to be largely free of *Varroa* infestation (Allsopp 2006). By contrast, DWV was present in >95% of the

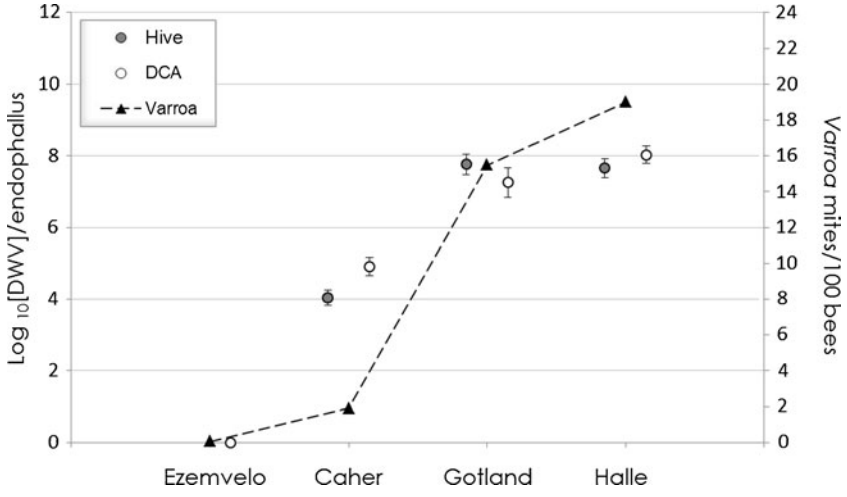
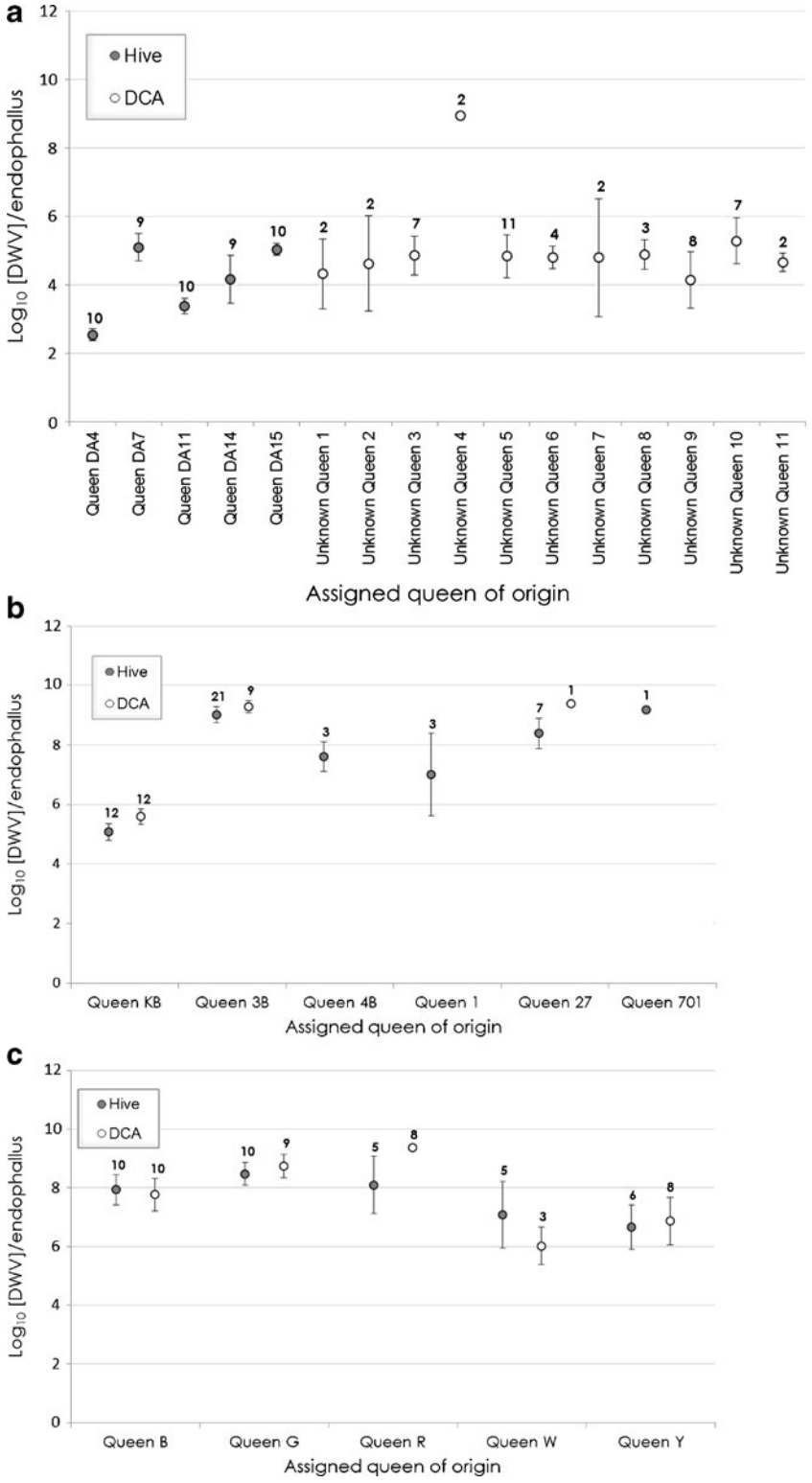


Figure 1. Comparison of DWV titres and *Varroa* infestation levels in Ezemvelo, Caher, Gotland and Halle. DWV titres in the endophallus of drones collected at hives (*black circle*) and DCAs (*white circle*), with the standard error represented by the *error bars*. *Varroa* infestation level (*black triangle*) of the apiaries nearby the DCAs, given as the mean number of mites found in 100 bees.


a
Hive
DCA
Log10 [DWV]/endophallus
Queen DA4
Queen DA7
Queen DA11
Queen DA14
Queen DA15
Unknown Queen 1
Unknown Queen 2
Unknown Queen 3
Unknown Queen 4
Unknown Queen 5
Unknown Queen 6
Unknown Queen 7
Unknown Queen 8
Unknown Queen 9
Unknown Queen 10
Unknown Queen 11
Assigned queen of origin
b
Hive
DCA
Log10 [DWV]/endophallus
Queen KB
Queen 3B
Queen 4B
Queen 1
Queen 27
Queen 701
Assigned queen of origin
c
Hive
DCA
Log10 [DWV]/endophallus
Queen B
Queen G
Queen R
Queen W
Queen Y
Assigned queen of origin

◀ **Figure 2.** Mean drone endophallus DWV titres in Caher (**a**), Gotland (**b**) and Halle (**c**), partitioned according to genetic colony of origin and drone collection site (hive or DCA), with the standard error represented by the *error bars*. The number of drones genetically assigned to each queen/colony is given *above* the *bars*.

drones sampled in the European locations, each with different *Varroa* infestation rates.

Our quantitative analyses therefore concern these three European localities, where we are able to compare DCA-caught and hive-collected drones. Firstly, there was no obvious difference between the endophallus DWV titres of drones captured at DCAs and those collected from hives at any of the three European locations (Figure 1). Secondly, there are significant differences in average DWV titres between the three European locations (one-way ANOVA, $F_{2,262}$= 99.347, P<0.001), which exhibited a positive relationship with the corresponding *Varroa* infestation rates. Thirdly, there was enormous variation in endophallus DWV titres between individual drones, covering several orders of magnitude, as shown by the standard error bars (Figure 1).

To see if these general observations also held true at the level of individual colonies, the genetic origins of the drones collected at the DCAs and in the hives were determined. The drones collected in the Ezemvelo DCA in South Africa came from at least 27 different queens.

In Caher, Ireland, 47 of the 50 drones collected from the five sampled hives (DA4, DA7, DA11, DA14 and DA15) could be genetically assigned to their queen/colony of origin (Figure 2a). The remaining three drones had drifted into the test hives, one from colony DA11 to DA14 and the other two from an unknown colony. The drones in the DCA at Caher came from at least 11 different maternal colonies, but none of those colonies were among the sampled hives, possibly because the DCA was located quite far (5 km) from the sampled hives. The difference in DWV titre between hive-collected and DCA-captured drones was significant (one-way ANOVA, $F_{1,98}$=6.445, P=0.013), with DCA-caught drones having slightly higher DWV titres than hive-collected drones (Figure 2a). Significant variation was also detected in drone DWV titres between the five sampled hives ($F_{4,43}$=8.939, P<0.001), but not among drones of the 11 unknown colonies contributing to the DCA ($F_{10,39}$=1.193, P=0.325).

In contrast to Caher, most drones captured in the DCAs at Gotland and Halle could be genetically assigned to one of the local hives, which is undoubtedly because the sampled hives were much closer to their DCAs than at Caher. In Gotland, Sweden, 47 of the 50 drones collected from the six sampled hives (KB, 3B, 4B, 1, 27 and 701) could be genetically assigned to their queen/colony of origin (Figure 2b). Of the three remaining drones, one had drifted from colony 3B to colony KB and two drones of unknown genetic origin had drifted into colony 3B. The 24 drones captured at the DCA were assigned largely to colony KB (12) and colony 3B (9). A single drone was assigned to colony 27; the origin of one drone was ambiguous and the DNA from one drone failed to amplify.

In Halle, Germany, 36 of the 45 drones collected from the five sampled hives (B, G, R, W and Y) could be genetically assigned to their queen/colony of origin (Figure 2c). The remaining nine drones had drifted into the test hives, all of them from unknown source colonies: five drones into colony R, two drones into colony W and two drones into colony Y. Of the 49 drones collected at the DCA, 38 could be assigned to the sampled hives: ten drones to colony B, nine drones to colony G, eight drones to colony R, three drones to colony W and eight drones to colony Y. The remaining 11 drones were from unknown source colonies with at least nine different queens.

The data for each location (Gotland or Halle) was analysed by two-way ANOVA, involving only those colonies with both DCA- and hive-collected drones. There was no significant difference in DWV titres between drones caught at the DCA and their genetic siblings sampled inside their maternal colony, at both the Gotland

and Halle locations ($F_{1,57}$=1.908, P=0.173 for Gotland; $F_{1,64}$=0.056, P=0.814 for Halle), nor was there any statistical interaction between colony and sample origin (hive or DCA; $F_{2,57}$= 0.222, P=0.802 for Gotland; $F_{4,64}$=0.643, P= 0.634 for Halle). However, as with Caher, there was considerable variation in the drone DWV titres between different colonies, both in Gotland ($F_{2,57}$=83.631, P<0.001; Figure 2b) and in Halle ($F_{4,64}$=4.344, P=0.004; Figure 2c).

4. DISCUSSION

Two main observations derive from these experiments. First, there was no significant difference in mean endophallus DWV titre between drones captured in DCAs and their genetic siblings collected from the same maternal hives. This suggests that DWV infection of drone genitalia has no apparent effect on drone mating flight performance over a very wide range of DWV titres. As a result, the endophalli of the drones found in our European DCAs were frequently infected with high titres of DWV. Secondly, there was enormous variation in the DWV titre found in the endophalli of individual drones from the same colony, with further significant differences between individual colonies within the recruitment area of a particular DCA. There is also highly significant variation in these DWV titres between different geographic regions.

The most important implication of these findings is that sexual DWV transmission is likely to occur naturally in honey bees, in the same way that it occurs by artificial insemination (Yue et al. 2007; de Miranda and Fries 2008) and that DWV is seemingly able to develop to relatively high titres in the drone endophallus (up to $10^8 \sim 10^9$ copies/endophallus) apparently without adverse effects for drone flight performance. This range is similar to previous estimates of DWV titres in the drone reproductive tissues (Fievet et al. 2006). This suggests that, for this tissue, transmission route and at these titres, DWV is a virus of low virulence. A similar conclusion was also drawn from a large-scale analysis of DWV infection of honey bee queens and their ovaries (Gauthier et al. 2011). Comparable normalised DWV titres of whole, DWV-symptomatic adult worker bees from the Gotland colonies are 10^{12}–10^{13} estimated DWV copies/bee (J.R. de Miranda, O. Yañez, R.J. Paxton and I. Fries, unpublished results), i.e. just a few orders of magnitude higher than the titres found in the endophalli of these asymptomatic drones. Although this comparison is distorted by the distinct concentration of DWV in the reproductive organs (Fievet et al. 2006) and the natural susceptibility differences between drones and worker bees (Chen et al. 2005), these endophallus DWV titres are clearly high, albeit with no discernible effect on the host. What still needs to be established is if the endophallic DWV infection affects semen quantity or quality, or if the drone's ability to capture and mate with the queen is lower when in competition with non-infected or less infected drones. A second implication of our results is that sampling of drones in the DCA could be an efficient means to determine the DWV status of contributing colonies, which would be especially useful for wild or feral bee populations. The accuracy of such a strategy depends on factors that affect drone abundance in the DCA which, among others, is influenced by general colony health and distance from the DCA (Jaffé et al. 2010).

It is not clear whether the apparent absence of any effect of high endophallus DWV titres on the drone flight ability supports the theoretical prediction that vertical pathogen transmission favours reduced pathogen virulence, relative to horizontal transmission (Fries and Camazine 2001; Alizon et al. 2009; Stewart et al. 2005). This would require a comparable study of the virulence effects of DWV titres for the principal horizontal DWV transmission routes and the extent to which venereal transmission should be regarded as a strictly horizontal transmission or a pre-vertical transmission. Furthermore, such virulence estimates may well depend on which host fitness parameters are investigated and which tissues are studied. It has been shown that drones are under strong selection for flight performance and wing symmetry during mating flights (Koeniger et al. 2005b; Jaffé and Moritz

2010). However, the endophallus has no direct influence on flight ability. It is used here as an indirect marker for DWV titres elsewhere in the body. It may be able to tolerate DWV titres that would be damaging in those tissues more directly related to orientation and flight ability, such as the brain or wing imaginal disks during pupal development (Fievet et al. 2006). A more direct fitness parameter for the endophallus would be whether the quantity or quality of sperm is affected by endophallus DWV titres, and this hypothesis is currently being investigated.

A second factor that influences the interpretation of our results concerns the selection that occurs during the 2-week drone maturation period, from emergence to mating flights. Drones parasitized by *Varroa* during the pupal phase have progressively lower emergence weight and flight performance than unparasitised drones (Duay et al. 2002; 2003), and most do not survive the maturation period prior to initial mating flights (Rinderer et al. 1999; Duay et al. 2002). This is not entirely unexpected, given the close relationship between *Varroa* infestation and DWV titres in drone pupae (Tentcheva et al. 2006) and the severe wing pathologies associated with pupal DWV transmission by *Varroa* (Bowen-Walker et al. 1999; Yue and Genersch 2005; Gisder et al. 2009). However, it has also been shown that drones from *Varroa*-infested colonies that do survive to maturity have no mating disadvantage when placed in direct competition with drones from uninfested or miticide-treated colonies (Sylvester et al., 1999). The crucial stage for drones therefore seems to be well before mating flights, when *Varroa* infestation reduces the number of drones available for mating, compared with uninfested colonies, but less so, the flight performance of drones that do survive to mate (Rinderer et al. 1999; Sylvester et al. 1999; Duay et al. 2002). A similar interpretation may also apply to our results, i.e. the ability of mature drones to reach a DCA is unaffected by DWV infection of the endophallus, over a large range of titres, and it may be that drones with DWV titres beyond this range (with or without symptoms) disappeared from the population before reaching maturity. This hypothesis is also being investigated.

The second observation concerns the enormous variation in endophallus DWV titres and how this is distributed within and between colonies, and between geographic locations.

DWV could not be detected in our South African DCA by three different RT-PCR assays. The primers for these assays are highly conserved and were designed to avoid non-amplification due to virus variability (Genersch 2005; Forsgren et al. 2009). Although DWV has previously been detected in South Africa, by serology (Allen and Ball 1996; Ellis and Munn 2005), this result is not unusual. African honey bees are much more tolerant to *Varroa* mites than their European counterparts, and the *Varroa* infestation rates of the wild bee populations of the Ezemvelo reserve are very low (Allsopp 2006). *Varroa*-free bee populations in France (Tentcheva et al. 2004; Gauthier et al. 2007) and Sweden (de Miranda and Fries 2008; Yue et al. 2006) also frequently have undetectable, or barely detectable, levels of DWV. African honey bee subspecies are also known to disperse via long distance migratory swarms, abscond more readily and have a faster generation time and smaller colonies than European honey bees (Ratnieks, 1991; Hepburn and Radloff 1998; Schneider et al. 2004). All these factors could contribute to the apparent absence of DWV from our African study population.

Our European study populations were more informative with respect to the variability of DWV titres. Despite the large variation between individuals of the same colony, significant differences could still be observed between colonies in the same location, and more variation still between different geographic locations. At the geographic level, these average DWV titres are roughly linked to the average *Varroa* infestation rates of the colonies in the area. However, within each geographic location, at the colony level, there is much less of a relationship between endophallus DWV titre and colony *Varroa* infestation rates. There are of course other factors (e.g. local *A. mellifera* genotype, DWV strain, environment) that may

contribute to the observed geographic differences in drone endophallus DWV titres. However, it is certainly plausible that *Varroa* infestation could indirectly affect venereal–vertical transmission by influencing the amount of DWV available for transmission, given the primary role of *Varroa* in generating and maintaining high DWV titres in honey bee populations (Bowen-Walker et al. 1999, Nordström 2003, Yue and Genersch 2005). This will depend on whether the overall DWV titre affects the specific DWV titre in the endophallus or semen (Fievet et al. 2006) and by the DWV titre threshold for venereal transmission. If this threshold is low, then the important parameter for venereal–vertical transmission and DWV epidemiology is more the proportion of DWV infected drones in the DCA, rather than the DWV titres in these drones. If this threshold is high, then the titres in the drones also become important and by inference, so does the *Varroa* infestation status of the drone donor colonies. These different perspectives of the importance of virus titre on the probability of transmission highlight the effect antiviral or anti-*Varroa* treatments can have on the epidemiology and evolution of a virus (Brunetto et al. 2009; Lee et al. 2008). The high endophallus DWV infection rate itself suggests that venereal transmission is a major transmission route for DWV (Fievet et al. 2006; Yue et al. 2006; 2007; de Miranda and Fries 2008), which may in part explain the wide distribution of DWV in Europe. The high endophallus DWV infection titres in DCA-captured drones suggest that there is also a quantitative component to venereal transmission efficiency. Effective *Varroa* control may therefore indirectly help minimise the risk of venereal DWV transmission as well as reduce the risk of lethal DWV titre accumulation in honey bee populations.

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Virus des ailes déformées et vols d'accouplement des mâles chez l'abeille (*A. mellifera*): implications dans la transmission sexuelle d'un important virus de l'abeille

Abeille / virus des ailes déformées / place de rassemblement des mâles / transmission verticale

Deformed wing virus und Paarungsflüge von Drohnen der Honigbiene (*A. mellifera*): Folgen für die sexuelle Transmission eines bedeutenden Honigbienen Virus

Honigbiene / DWV / Drohnensammelplatz / vertikale Transmission

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