Vertical-transmission routes for deformed wing virus of honeybees (Apis mellifera)

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Deformed wing virus (DWV) is a viral pathogen of the European honeybee (Apis mellifera), associated with clinical symptoms and colony collapse when transmitted by the ectoparasitic mite Varroa destructor. In the absence of V. destructor, DWV infection does not result in visible symptoms, suggesting that mite-independent transmission results in covert infections. True covert infections are a known infection strategy for insect viruses, resulting in long-term persistence of the virus in the population. They are characterized by the absence of disease symptoms in the presence of the virus and by vertical transmission of the virus. To demonstrate vertical transmission and, hence, true covert infections for DWV, a detailed study was performed on the vertical-transmission routes of DWV. In total, 192 unfertilized eggs originating from eight virgin queens, and the same number of fertilized eggs from the same queens after artificial insemination with DWV-negative (three queens) or DWV-positive (five queens) semen, were analysed individually. The F0 queens and drones and F1 drones and workers were also analysed for viral RNA. By in situ hybridization, viral sequences were detected in the ovary of an F0 queen that had laid DWV-positive unfertilized eggs and was inseminated with DWV-positive semen. In conclusion, vertical transmission of DWV from queens and drones to drone and worker offspring through unfertilized and fertilized eggs, respectively, was demonstrated. Viral sequences in fertilized eggs can originate from the queen, as well as from drones via DWV-positive semen.

INTRODUCTION

In insect virology, the descriptive terms overt and covert are used widely to categorize the infections caused by insect viruses. Typically, overt infections are those in which the virus-infected host develops obvious disease symptoms, and covert infections are defined as conditions in which the virus is present within the host in the absence of clear disease symptoms. Additional characteristics of covert infections, deduced from work on baculoviruses, are (i) that the viruses remain fully competent and can re-emerge to cause overt infections (Burden et al., 2003) and (ii) that the viruses persist beyond the current life stage and can be transmitted vertically to a subsequent generation (Burden et al., 2002; Kukan, 1999). This definition of a covert infection gives a clear distinction from an inapparent infection, which is also characterized by the absence of disease symptoms. True inapparent viral infections are defined as short-term infections with high levels of virus production, albeit without visible symptoms, and are transmitted solely horizontally (Dimmock & Primrose, 1987).

Deformed wing virus (DWV) is a positive-stranded RNA virus (Lanzi et al., 2006) that is pathogenic for both honeybees and bumblebees (Genersch et al., 2006). DWV can be detected in all life stages of the honeybee in the absence of visible disease symptoms (Chen et al., 2005a; Tentcheva et al., 2006; Yue & Genersch, 2005). Transmission of DWV by Varroa destructor is often associated with clinical symptoms (crippled wings, bloated and shortened abdomen and discoloration) (Ball & Allen, 1988; Bowen-Walker et al., 1999; Martin, 2001; Martin et al., 1998; Tentcheva et al., 2006; Yue & Genersch, 2005), suggesting that, even in the absence of disease symptoms, infecting viruses remain fully competent and can result in clinically apparent infections when vectored by V. destructor.

Whilst presence of the virus in the absence of disease symptoms and occasional overt outbreaks fit with the definition of a covert infection outlined above, the possibility that the asymptomatic DWV infection is an inapparent one, rather than a genuine covert infection, cannot be ruled out. To distinguish between a covert and an inapparent infection, the mode of transmission is crucial (Dimmock & Primrose, 1987). Inapparent infections are transmitted horizontally, whereas covert infections can also be transmitted vertically from parent to offspring. It has been suggested that differences in the mode of transmission influence the virulence of a pathogen (Fries & Camazine, 2001; Lipsitch et al., 1996). Horizontal transmission allows the development of more virulent forms of the pathogen with high negative impact on the fitness of the host.
Vertical transmission, however, relies on host survival and reproduction and, therefore, less virulent forms will be favoured, allowing for long-term persistence of the virus within the host population (Burden et al., 2003; Oldstone, 2006).

Recent studies on the localization of DWV in honeybee queens and drones revealed that the reproductive organs of both were strongly positive for viral sequences (Chen et al., 2006b; Fievet et al., 2006), suggesting the possibility of vertical-transmission routes. In addition, DWV sequences were recently demonstrated in sperm (Yue et al., 2006), further supporting the hypothesis that DWV may be transmitted vertically through drones. Concerning the detection of DWV RNA in eggs, conflicting results are reported (Chen et al., 2005a, b, 2006a; Tentcheva et al., 2006), preventing researchers from finally answering the question of vertical DWV transmission through the queens’ eggs.

Here, we present a detailed analysis of the presumed vertical-transmission routes of DWV to answer the question of whether DWV can cause true covert infections. By using RT-PCR and in situ hybridization, we analysed viral RNA in reproductive organs of both queens and drones, in sperm before using it for artificial insemination (AI), individually in unfertilized and fertilized eggs, in larval food and in offspring. Our results strongly support the hypothesis that DWV is transmitted vertically through queens and drones and, therefore, that DWV can cause genuine covert infections. The impact of true covert DWV infections on the maintenance of the virus in the population will be discussed.

**METHODS**

**Sample collection and mating scheme.** Eight healthy-looking, virgin queens were selected randomly from different colonies and put into eight queenless experimental mini-colonies free of *V. destructor* (Table 1). Only in colony 65 were three DWV-positive mites observed at the end of the experiments. Unfertilized eggs laid by these virgin queens were analysed for viral sequences (24 eggs each). Some unfertilized eggs were allowed to develop into F1 drones, which were then analysed for viral RNA. Semen for AI of the virgin queens was collected from sexually mature honeybee drones showing no overt signs of DWV infection. These F0 drones originated from four different, apparently healthy colonies (Table 2). Drones were stimulated to ejaculate by pressing on the thorax until the cream-coloured semen and a white mucus plug were released on the end of the penis. Under a dissecting microscope (×10), the semen was collected without mucus into a syringe, designed by Schley (1982). As only a small quantity, <1 μl of semen can be collected from each drone, the semen of five drones per colony was pooled to obtain a volume of around 4 μl. Half of the volume was used for the detection of viral RNA; the remaining volume was used for AI. Subsequent to the collection of the F0 drones’ semen, these drones were analysed for DWV RNA. The eight virgin queens (after analysing the viral status of their unfertilized eggs) were inseminated artificially with either DWV-negative or DWV-positive sperm to obtain the following combinations (Table 1): (i) DWV-negative unfertilized eggs/DVW-negative sperm (*n* = 2), (ii) DWV-negative unfertilized eggs/DVW-positive sperm (*n* = 2).
sperm \( (n=3) \), (iii) DWV-positive unfertilized eggs/DJV-negative sperm \( (n=1) \), and (iv) DWV-positive unfertilized eggs/DJV-positive sperm \( (n=1) \). One queen that had produced a small proportion of DWV-positive unfertilized eggs (three of 24) was also inseminated with DWV-positive sperm. Subsequently, fertilized eggs (24 eggs per colony), as well as F1 workers, were analysed for viral sequences. At the end of the experiment, the F0 queens were analysed for DWV RNA by using RT-PCR and in situ hybridization. Larval food was collected from uncapped cells of larvae not older than 2 days. Eggs, bees and larval food were frozen at \(-70^\circ C\) immediately after sampling and stored at \(-70^\circ C\) until analysis.

**Tissue dissection.** After sperm collection and at the end of the experiment, the F0 drones and queens were sacrificed for tissue dissection and analysis of viral RNA. Some of the F1 drones were also sacrificed for tissue dissection. The bee heads were cut off and the eviscerated body was fixed with insect pins on the wax top of a dissecting dish. The abdomen was opened with scissors; the tissues were removed carefully from the body with forceps and separated into testis, vesicular glands and seminal vesicles (drones) or gut, spermatheka and ovaries (queens) under a dissecting microscope (\( \times 10 \)). Contaminating haemolymph was washed off by rinsing the tissues carefully with 1 \( \times \) PBS and nuclease-free water. One ovary was used for RNA extraction and subsequent RT-PCR analysis; the other ovary was embedded in a polymerizing resin (Technovit 8100; Heraeus Kulzer) according to the manufacturer’s protocol and subjected to in situ hybridization. All other tissues were used for RNA extraction and RT-PCR analysis only.

**RNA extraction.** Total RNA extraction from eggs, sperm, adult bees (body parts and tissue of F0 queens, F0 drones, F1 drones and F1 workers) and larval food was performed as described previously (Yue & Genersch, 2005; Yue et al., 2006), using standard methods following the manufacturer’s protocols (RNeasy kit for eggs, sperm and bees; Viral RNA kit for larval food; both from Qiagen). The air-dried RNA pellets were resuspended in elution buffer (Qiagen) and stored at \(-70^\circ C\).

**One-step RT-PCR for the detection of DWV RNA.** One-step RT-PCR was performed according to standard protocols (One-Step RT-PCR kit; Qiagen) and as described previously (Genersch, 2005). The following temperature scheme was used: 30 min at 50 \( ^\circ C \), 15 min at 95 \( ^\circ C \), followed by 35 cycles of 1 min at 94 \( ^\circ C \), 1 min at 54.3 \( ^\circ C \) and 1 min at 72 \( ^\circ C \), including a final elongation step for 10 min at 72 \( ^\circ C \). The following primer pairs, covering different regions of the viral genome, were used (see Supplementary Table S1, available in JGV Online): F0/B0 (designed for this study), F1/B1, F0/B16 and F15/B23 (Genersch, 2005). PCR products (8 \( \mu \)l per reaction) were analysed on a 1.0 % agarose gel. The DNA bands were stained with ethidium bromide and visualized by UV light. Specificity of the amplicons was further verified by sequencing (Medigenomix, Germany) of random samples.

**In situ hybridization.** 5’-Digoxigenin (DIG)-labelled oligonucleotides (TIB MOLBIOL) were used as probes for the detection of viral positives and negative-stranded RNA. Six oligonucleotides hybridizing to different three regions of the DWV genome were used (positions according to GenBank accession no. NC_004830: 4780–4810 [DIG-TAACTGAGACACGTGAGAAGACATTTGGCT (DWV4810-minus) and DIG-AGCAATGTCTCTACAGTGTCAGTTA (DWV4780plus)], 6580–6610 [DIG-TAGAGCTGGTGATGATGATTCCGTGGTGCC (DWV6610-minus) and DIG-GGGCAACGTGAGATTTACCTTTACAGGCTCTA (DWV6580plus)] and 9240–9270 [DIG-TCCGTATTGAGAATCCGTGAAATTCG (DWV9270-minus) and DIG-CTGAAATTTGCTCAATTCAGTTCTCGCAATTAACGC (DWV9240plus)], as well as a nonsense oligonucleotide [DIG-GGCTAGGTGAGAGTTAGC (nonsense)]. Oligonucleotides DWV4810minus, DWV6610minus and DWV9270minus represent antisense probes; oligonucleotides DWV4780plus, DWV6580plus and DWV9240plus are sense probes. For hybridization, tissues were fixed in 4 % paraformaldehyde in 1 \( \times \) PBS at 4 \( ^\circ C \) for 24 h and embedded in a polymerizing resin (Technovit 8100; Heraeus Kulzer) at 4 \( ^\circ C \) according to the manufacturer’s protocol. Polymerized resin blocks were stored at 4 \( ^\circ C \) until the preparation of semi-thin sections (4 \( \mu \)m), which were mounted on poly-l-lysine-coated slides and stored at 4 \( ^\circ C \) until use. The in situ hybridization protocol was adapted from Heiles et al. (1988) and Mitta et al. (2000). Briefly, semi-thin sections were incubated for 20 min at room temperature in 0.2 M HCl, washed twice for 5 min each at 37 \( ^\circ C \) in 2 \( \times \) SSC (standard saline citrate) and then incubated for 15 min at 37 \( ^\circ C \) in 1 \( \mu \)g proteinase K ml \(^{-1}\). Subsequently, the sections were washed three times for 5 min each at 37 \( ^\circ C \) in 1 \( \times \) PBS, incubated for 10 min at room temperature in 0.1 M glycine, 0.2 M Tris/HCl (pH 7.5), and washed twice for 5 min each at 37 \( ^\circ C \) in 2 \( \times \) SSC, 5 mM EDTA. A post-fixation step was performed for 20 min at room temperature in 4 % paraformaldehyde in 1 \( \times \) PBS, followed by a final washing step for 5 min at room temperature with 1 \( \times \) PBS. DIG-labelled oligonucleotides (mixture of DWV4780plus, DWV6580plus and DWV9240plus, 20 ng each, or DWV4810minus, DWV6610minus and DWV9270minus alone) were diluted in 50 \( \mu \)l hybridization buffer containing 6 \( \times \) SSC, 45 % formamide, 10 % dextran sulfate, 0.5 % SDS, 5 \( \times \) Denhardt’s solution, 0.2 \( \mu \)g salmon sperm DNA ml \(^{-1}\). Hybridization was performed overnight at 37 \( ^\circ C \) in a humid chamber. The sections were then washed twice in 6 \( \times \) SSC, 45 % formamide for 15 min each at 42 \( ^\circ C \), once in 6 \( \times \) SSC for 5 min at 42 \( ^\circ C \), twice in 2 \( \times \) SSC for 10 min each at 50 \( ^\circ C \) and twice in 0.2 \( \times \) SSC for 15 min each at 50 \( ^\circ C \). Detection of DIG-labelled, hybridized oligonucleotides was performed by using alkaline phosphatase-conjugated anti-DIG antibodies according to the manufacturer’s protocol (Roche). Bound antibodies were visualized by incubation (overnight, darkness, room temperature) in 0.1 M Tris/HCl (pH 9.5), 50 mM MgCl\(_2\), 0.1 M NaCl, 375 \( \mu \)g nitro blue tetrazolium (NBT) ml \(^{-1}\) and 188 \( \mu \)g 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) ml \(^{-1}\). The chromagen reaction was stopped by rinsing the slides in 0.1 M Tris/HCl (pH 7.5), 0.1 M NaCl, and then coverslips were mounted on the slides with AquapolyMount (Polysciences Europe).

**RESULTS**

**Detection of DWV sequences in unfertilized eggs and sperm**

To evaluate the incidence of maternally originating DWV sequences in eggs, unfertilized eggs laid by eight asymptomatic, virgin queens selected randomly from different, healthy-appearing colonies were analysed for the presence of DWV RNA (Table 1; Fig. 1a). Five of eight queens produced only DWV-negative eggs (24 of 24), one queen laid DWV-negative (21 of 24) as well as DWV-positive (three of 24) eggs, and two queens laid 100 % DWV-positive eggs (24 of 24). Analysis of sperm for DWV sequences revealed that two of the four randomly selected colonies produced drones with DWV-positive sperm and the other two produced drones with DWV-negative sperm (Table 2), supporting earlier results (Yue et al., 2006). Further analysis of the virus-positive samples with a panel of PCR primers covering four evenly spaced segments of the viral genome (Supplementary Table S1, available in http://vir.sgmjournals.org}
JGV Online) was successful, thus excluding the possibility that the positive signals in sperm and unfertilized eggs represent defective interfering (DI)-like RNAs (Fig. 1b).

Detection of DWV sequences in fertilized eggs

To evaluate the impact of virus transmission via semen, fertilized eggs laid after AI with DWV-negative or DWV-positive semen were analysed for the presence of DWV sequences (Table 1; Fig. 1a). AI with DWV-positive sperm resulted in 100 % DWV-positive fertilized eggs. One control queen with DWV-negative unfertilized eggs and inseminated artificially with DWV-negative sperm laid 100 % DWV-negative eggs. The other control queen showed a small proportion of DWV-positive eggs (three of 24), suggesting a low background of DWV-positive unfertilized eggs not detected in the first batch of analysed eggs. Again, analysis of DWV-positive fertilized eggs with a panel of PCR primers (Supplementary Table S1, available in JGV Online) revealed the presence of the entire viral genome rather than the presence of DI-like RNAs (Fig. 1b).

Detection of DWV sequences in the F1 generation

None of the drones or workers of the F1 generation showed any visible symptoms of disease or DWV infection (crippled wings, discoloration and bloated abdomen). Detailed analysis of two drones of the F1 generation from each colony revealed the presence of DWV sequences in thorax and/or abdomen or, when analysed in detail, in testis, seminal vesicles and vesicular glands (Table 1). This was true even for drones originating from queens with 100 % DWV-negative unfertilized eggs. No DWV sequences could be detected in total RNA isolated from head, supporting earlier reports that asymptomatic bees rarely test positive for DWV sequences in head (Yue & Genersch, 2005). Detailed analysis of three F1 workers from each colony showed no strong correlation between the detection of DWV sequences in fertilized eggs and in workers. Although no DWV sequences could be detected in fertilized eggs in mini-colony 66, only one F1 worker was DWV-negative; in two F1 workers, weak signals for DWV could be detected in RNA isolated from thorax (w1) or abdomen (w3). In contrast, in mini-colony 67, all eggs tested positive for DWV but, in two of three workers, no DWV sequences could be detected. All other workers analysed were DWV-positive. Surprisingly, we detected a weak signal for DWV in total RNA isolated from head in four F1 workers. Three belonged to mini-colony 28 (Fig. 2a) and originated from insemination of a queen laying DWV-positive unfertilized eggs with DWV-positive sperm (Table 1). The presence of the entire viral genome in DWV-positive F1 bees could be substantiated by further analysing DWV-positive bees with a panel of PCR primers representing different regions of the viral genome (Fig. 2b). It is noteworthy that, in all colonies, larval food tested positive for DWV sequences (Table 1).

Detection of DWV sequences in the F0 generation

Although none of the queens or drones of the F0 generation showed any pathological signs of disease or DWV infection, they were all DWV-positive, as revealed by qualitative RT-PCR analysis of different body parts and tissues. F0 drones...
were positive for DWV RNA in testis, seminal vesicles and vesicular glands, regardless of whether they had produced DWV-negative or -positive sperm (Table 2). All queens tested positive for the entire viral genome in gut, spermatheca and ovaries (Table 3; Fig. 3a, b). Surprisingly, the ovaries of the queen heading mini-colony 66 were DWV-positive, although this queen laid 100% DWV-negative fertilized eggs and produced DWV-negative F1 workers (Table 1). Similarly, the spermatheca was DWV-positive even for the queens of mini-colonies 66 and 48, which had laid DWV-negative unfertilized eggs and were inseminated with DWV-negative sperm, resulting in 100 and 88% DWV-negative fertilized eggs, respectively (Table 1). No DWV RNA could be detected in head (Table 3).

Table 3. Detection of DWV sequences in head, gut, spermatheca and ovaries of the F0 queens, shown with respect to the viral status of unfertilized eggs, sperm used for AI and fertilized eggs

<table>
<thead>
<tr>
<th>Mini-colony</th>
<th>Viral status of:</th>
<th>DWV sequences in h/g/sp/o</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unfertilized eggs/sperm</td>
<td>Fertilized eggs</td>
</tr>
<tr>
<td>66</td>
<td>−/−</td>
<td>−/+/++/++</td>
</tr>
<tr>
<td>48</td>
<td>−/−</td>
<td>−/++/++/++</td>
</tr>
<tr>
<td>65</td>
<td>−/+</td>
<td>−/++/++/++</td>
</tr>
<tr>
<td>68</td>
<td>−/+</td>
<td>−/++/++/++</td>
</tr>
<tr>
<td>69</td>
<td>−/+</td>
<td>−/++/++/++</td>
</tr>
<tr>
<td>67</td>
<td>±/+</td>
<td>−/++/++/++</td>
</tr>
<tr>
<td>49</td>
<td>+/−</td>
<td>−/++/++/++</td>
</tr>
<tr>
<td>28</td>
<td>+/+</td>
<td>−/++/++/++</td>
</tr>
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</table>

Abbreviation: h/g/sp/o, head/gut/spermatheka/ovaries. −, Negative; +, positive; ±, some eggs were DWV-positive and others were DWV-negative. See Fig. 1 for details.

In situ hybridization analysis of DWV RNA in ovaries

The puzzling results concerning DWV detection in ovaries and eggs prompted us to analyse the ovaries of the queens heading mini-colonies 66 and 49 via in situ hybridization. Strong, DWV-specific signals could be observed in the ovary of the queen heading mini-colony 49 (Fig. 4), which had produced 100% DWV-positive unfertilized eggs and had been inseminated with DWV-positive sperm (Table 1; Fig. 1). Positive-stranded viral RNA was detected in the fully developed oocytes, as well as in the surrounding tissue of the terminal part of the ovary. No viral negative-stranded RNA could be detected, although it was present according to strand-specific RT-PCR (data not shown). Localization of viral sequences in the ovary of the queen heading mini-colony 66 (DWV-negative unfertilized eggs, inseminated with DWV-negative sperm, DWV-negative fertilized eggs; Table 1) was unsuccessful, despite the presence of DWV RNA in the ovary of this queen when analysed by RT-PCR (Table 3; Fig. 3).

DISCUSSION

DWV is widespread all over the world (Berenyi et al., 2006; Chantawannakul et al., 2006; Chen et al., 2004; Martin et al., 1998; Tentcheva et al., 2004) and outbreaks of clinical DWV infection have been reported to be associated with the collapse of honeybee colonies due to infestation with the ectoparasitic mite V. destructor (Bailey & Ball, 1991; Ball & Allen, 1988; Martin, 2001; Martin et al., 1998; Nordström et al., 1999). Vectorial transmission of DWV through V. destructor is well documented (Bailey & Ball, 1991; Bowen-Walker et al., 1999; Chen et al., 2005a; Shen et al., 2005) and recent studies suggested that V. destructor might serve as a biological rather than a mechanical vector (Yue & Genersch, 2005). As DWV was also demonstrated in bees that had never had contact with V. destructor (Yue et al., 2006; Chantawannakul et al., 2006; Chen et al., 2004; Martin et al., 1998; Tentcheva et al., 2004) and outbreaks of clinical DWV infection have been reported to be associated with the collapse of honeybee colonies due to infestation with the ectoparasitic mite V. destructor (Bailey & Ball, 1991; Ball & Allen, 1988; Martin, 2001; Martin et al., 1998; Nordström et al., 1999). Vectorial transmission of DWV through V. destructor is well documented (Bailey & Ball, 1991; Bowen-Walker et al., 1999; Chen et al., 2005a; Shen et al., 2005) and recent studies suggested that V. destructor might serve as a biological rather than a mechanical vector (Yue & Genersch, 2005). As DWV was also demonstrated in bees that had never had contact with V. destructor (Yue & Genersch, 2005).
Here, we provide evidence that DWV can be transmitted vertically through the maternal as well as through the paternal route. Although the reproductive organs of all F0 queens tested positive for DWV RNA by RT-PCR, the majority (six of eight) of the analysed queens laid exclusively (n=5) or predominantly (n=1) DWV-negative unfertilized eggs. However, the unfertilized eggs of two of eight analysed queens contained DWV RNA, indicating the possibility of a transovarial route for the vertical transmission of DWV. Analysis of the F1 drones revealed that all drones, including those hatching from DWV-negative eggs, were positive for DWV, emphasizing the impact of the horizontal route through feeding of DWV-containing larval food on the viral status of the F1 generation.

Recently, we demonstrated the presence of DWV RNA in semen collected from healthy drones (Yue et al., 2006). Here, we present further evidence for the vertical transmission of DWV through semen. AI of queens laying 100% DWV-negative unfertilized eggs with DWV-positive semen resulted in 100% DWV-positive fertilized eggs, demonstrating venereal transmission of an insect virus for the first time and indicating that DWV transmission via semen is a very efficient route. All F1 workers originating from these eggs were DWV-positive. As the F1 workers that developed from DWV-negative eggs were also DWV-positive, the influence of the horizontal-transmission route through feeding of DWV-containing larval food is demonstrated further. Unfortunately, our experimental design did not allow us to exclude the influence of larval food, which was positive for DWV RNA in all colonies. Further studies involving laboratory-reared F1 drones and workers are necessary to analyse separately the true impact of the vertical routes on the viral status of the offspring.

Recently, the existence of DI-like RNAs was demonstrated for Israeli acute paralysis virus (IAPV), a honeybee-pathogenic dicistrovirus (Maori et al., 2007). As our RT-PCR primer pairs and in situ hybridization probes cover the entire DWV genome from the 5’ end, coding for the structural proteins, to the 3’ end, coding for the non-structural proteins (Lanzi et al., 2006), we can rule out the possibility that our results originate solely from such DI-like RNAs. Likewise, we can rule out the possibility that they are due to the detection of transcribed viral sequences integrated into the host genome, as only a small, 428 bp fragment of the structural-protein cistron of IAPV was found to be integrated into the host genome (Maori et al., 2007), whereas we can demonstrate the presence of the entire DWV genome, as well as viral replication.
organs tested positive for DWV RNA in RT-PCR. We believe that our positive in situ hybridization result is due to the fact that the analysed queen laid DWV-positive eggs, as we also failed to detect DWV sequences in the ovaries of the analysed queen laying DWV-negative eggs, although these ovaries were positive for DWV according to RT-PCR analysis. Therefore, the detection of DWV in ovaries via in situ hybridization might correlate with the viral status of the unfertilized eggs. In situ hybridization analysis of ovaries for DWV will probably give negative results for queens laying DWV-negative eggs and positive results for queens laying DWV-positive eggs, because only then is enough viral RNA present to be detected by in situ hybridization. Further studies to elucidate this transovarial-transmission route for DWV are necessary.

In contrast to an earlier report, where we did not find DWV sequences in total RNA extracted from the heads of healthy workers (Yue & Genersch, 2005), in the current study, four bees showed weak signals for DWV sequences in head RNA. Attempts to localize DWV via in situ hybridization of head sections of healthy bees failed. Therefore, we can only speculate that, for example, hypopharyngeal glands might be infected. Persistent infection of the hypopharyngeal glands of some otherwise-healthy worker bees would be a plausible explanation for the DWV-positive larval food and, thus, could be the source of horizontal transmission of DWV within an infected colony.

All F0 bees and almost all (30 of 33) F1 bees were DWV-positive, but none showed any visible symptoms of disease, suggesting that, in the absence of V. destructor, even the combination of horizontal- and vertical-transmission routes within the colony does not have a high negative impact on the fitness and fecundity of the infected bees. We therefore conclude that, in the absence of V. destructor, DWV causes true covert infections, with DWV being present in the absence of disease symptoms and being transmitted vertically from parent (queen, drones) to offspring (workers, drones, queens). Vertical transmission of pathogens selects for the development of less virulent forms, as the pathogen needs a relatively fit host that is able to reproduce at least once to be passed on the next host generation (Burden et al., 2003; Fries & Camazine, 2001; Oldstone, 2006). Hence, our findings that DWV transmitted vertically within the colony in the absence of V. destructor had little, if any, impact on the fitness of the individual infected honeybee are not surprising, as this is a prerequisite for the vertical transmission of DWV between colonies. An infected colony, even when harbouring predominantly such covertly infected animals, will develop normally and eventually swarm, transmitting the virus vertically to the next colony generation, allowing long-term persistence of DWV in the honeybee population. So far, clinical outbreaks of DWV infections followed by colony collapse have been associated with infestation of V. destructor (Ball & Allen, 1988; Bowen-Walker et al., 1999; Martin, 2001; Martin et al., 1998; Tentcheva et al., 2006; Yue & Genersch, 2005), suggesting that DWV has developed a very well-balanced co-existence with honeybees and, therefore, needs a strong trigger, such as immunosuppression by V. destructor (Yang & Cox-Foster, 2005) or V. destructor as biological vector (Yue & Genersch, 2005), to re-emerge as an overt infection lethal to colonies. This principal ability to re-emerge as an overt infection demonstrates the full competence of the infecting virus and is the third hallmark of genuine covert infections. We therefore propose to classify DWV as a covert-infecting virus. Further studies will identify the target tissues and cells that allow the virus to persist, and unravel the mechanisms of viral persistence.

![Fig. 4. Detection of DWV by in situ hybridization in one ovary of the queen heading colony 49. Consecutive ovary sections were challenged with DIG-labelled antisense (a, d, e, f), sense (b) and nonsense (c) probes. Signals were visualized after chromagen reaction by light microscopy. The terminal part of the ovaries, containing nearly fully developed oocytes, is shown. Positive signals were obtained with a mixture of the antisense probes DWV4810minus, DWV6610minus and DWV9270minus (a), as well as with DWV4810minus (d), DWV6610minus (e) or DWV9270minus (f) alone.](http://vir.sgmjournals.org)
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