Horizontal transmission of deformed wing virus: pathological consequences in adult bees (Apis mellifera) depend on the transmission route

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Recent reports on a steady decline of honeybee colonies in several parts of the world caused great concern. There is a consensus that pathogens are among the key players in this alarming demise of the most important commercial pollinator. One of the pathogens heavily implicated in colony losses is deformed wing virus (DWV). Overt DWV infections manifested as deformed-wing syndrome started to become a threat to honeybees only in the wake of the ectoparasitic mite Varroa destructor, which horizontally transmits DWV. However, a direct causal link between the virus and the symptom ‘wing deformity’ has not been established yet. To evaluate the impact of different horizontal transmission routes, and especially the role of the mite in the development of overt DWV infections, we performed laboratory infection assays with pupae and adult bees. We could demonstrate that pupae injected with DWV dose-dependently developed overt infections characterized by deformed wings in adult bees, suggesting that DWV, if transmitted to pupae by the mite, is the causative agent of the deformed-wing syndrome. The OID50 (overt infection dosage) was approximately 2500 genome equivalents. Injecting more than $1 \times 10^7$ DWV genome equivalents into adult bees also resulted in overt infections while the same viral dosage fed to adult bees only resulted in covert infections. Therefore, both infection of adult bees through DWV-transmitting phoretic mites and infection of nurse bees through their cannibalizing DWV-infected pupae might represent possible horizontal transmission routes of DWV.

INTRODUCTION

Managed honeybees are indispensable for profitable and sustainable agriculture and for many non-agricultural ecosystems because of the extraordinary pollination service they provide. Hence, reports on a steady decline in honeybee populations in Europe, USA and Japan in the recent past (for a review see vanEngelsdorp & Meixner, 2010) caused great concern and several studies were initiated to identify the underlying cause. From these studies it became more and more evident that the key players in most of the ‘inexplicable’ colony losses are pathogens (Ratnieks & Carreck, 2010). Especially, the ectoparasitic mite Varroa destructor in concert with certain pathogenic bee viruses played a major role in the observed colony collapses (Cox-Foster et al., 2007; Genersch et al., 2010; Guzmán-Novoa et al., 2010; Highfield et al., 2009; Siede et al., 2008; vanEngelsdorp, 2008; vanEngelsdorp et al., 2009).

One of the viruses heavily implicated in colony losses is Deformed wing virus (DWV), a plus-stranded RNA virus belonging to the genus Iflaviridae (Lanzi et al., 2006). DWV normally causes covert infections in honeybees (de Miranda & Fries, 2008; Hails et al., 2008; Yü et al., 2007). Overt DWV infections characterized by the occurrence of visible disease symptoms (deformed wings, bloated and shortened abdomen, and miscolouring) are associated with V. destructor infestation (Ball & Allen, 1988; Bowen-Walker et al., 1999; Martin, 2001; Martin et al., 1998; Santillán-Galicia et al., 2010; Tentcheva et al., 2006; Yue & Genersch, 2005). However, the exact role of the mite in the pathogenesis of DWV is still not fully understood and a direct causal link between the virus and the symptom ‘wing deformity’ has not been established yet (de Miranda & Genersch, 2010).

Recently, it has been shown that DWV infections can be vertically transmitted through drones and queens and that this transmission route results in covert infections (presence of the virus in the absence of visible symptoms) (de Miranda & Genersch, 2010; de Miranda & Fries, 2008; Yue et al., 2007), which follows the general assumption that vertical transmission selects for less virulent forms of a pathogen (Ewald, 1983; Ewald & De Leo, 2002; Fries & Camazine, 2001). These vertically infected bees also lacked another hallmark of overt DWV infection, the detection of DWV RNA in bees’ heads (Yue & Genersch, 2005; Yue et al., 2007). The clinical importance of this characteristic was demonstrated recently. When bee colonies were analysed for the presence of DWV and the diagnosis was restricted to the detection of DWV in total RNA extracted from bees’ heads, colony losses were significantly related to
DWV infection (Genersch et al., 2010). The clinical importance of DWV infection in bees’ heads was also substantiated by a study that demonstrated that injecting DWV into forager bees resulted in learning deficits (Iqbal & Mueller, 2007). This study suggested the possibilities, (i) that phoretic mites are able to transmit DWV horizontally to adult bees when feeding on the bees’ haemolymph (Santillán-Galicia et al., 2010) and (ii) that transmission leads to an infection, presumably of the brain and the nervous system (Shah et al., 2009), characterized by subtle clinical symptoms like learning deficits. Such learning deficits can affect the performance of individual bees and can add to the damage inflicted upon honeybee colonies by mites.

There is evidence that DWV can also be horizontally transmitted to larvae via larval food containing DWV without causing any overt disease symptoms (Gisder et al., 2009; Yue & Genersch, 2005). Detection of DWV in the midgut content (Fievé et al., 2006) and bee faeces (Chen et al., 2006) suggests the additional possibility of a faecal–oral route of transmission between adult bees. Oral transmission of DWV might also occur through trophalaxis or when DWV-infected pupae are cannibalized by nurse bees in order to remove these pupae as part of the social immune response.

The different horizontal routes of transmission are poorly studied by controlled experiments and the pathological consequences of these transmission routes for individual bees and colonies have not been elucidated yet. Therefore, in the present study we performed injection bioassays as well as feeding experiments using purified DWV and both pupae and adult bees to evaluate the outcome of infections transmitted via different horizontal transmission routes. Based on the results obtained, we discuss the role of the mite in the development of symptomatic DWV infections.

**RESULTS**

**Injection bioassays with pupae to mimic vectorial DWV infection during pupal development**

To provide experimental proof that injecting DWV into pupae (i.e. mimicking their infection through V. destructor) can cause clinical DWV infections we performed injection bioassays with purified DWV and white-eyed pupae. Injecting increasing dosages of DWV \((8 \times 10^1–1 \times 10^7\) DWV equivalents per bee) resulted in an increasing proportion of bees showing symptoms of an overt DWV infection (malformed wings, shortened and bloated abdomen, Fig. 1a, and positive detection of DWV in bees’ heads, Fig. 1b). By plotting the proportion of bees with positively detected DWV in their heads (mean ± SD) against the DWV dosage used for injection we obtained a dose–response curve [linear regression, \(f(x) = 15.77 \log(x) - 3.607\), \(r^2 = 0.9418\), \(P = 0.0295\); Fig. 1c]. As few as 80 DWV equivalents were sufficient to induce an overt infection in approximately 30 % of the injected individuals. The ‘OID50’, i.e. the dose sufficient to cause an overt infection in 50 % of the injected animals, was approximately 2500 DWV equivalents. Following injection of \(1 \times 10^7\) DWV equivalents or more into pupae, 100 % of the injected individuals developed an overt infection. No DWV-induced pupal mortality could be observed in our experiments, supporting the notion that DWV is a rather benign virus that allows infected pupae to develop until emergence (de Miranda & Genersch, 2010).

**Injection bioassays with adult bees to mimic vectorial DWV infection through phoretic mites**

To mimic horizontal DWV transmission through phoretic mites we performed injection bioassays with naïve, DWV-free, adult bees. Analysis of the bees 3 days post-infection revealed that injecting between \(8 \times 10^1\) and \(8 \times 10^4\) DWV equivalents did not result in the establishment of any detectable DWV infection. Neither in total RNA extracted from head nor in the thorax or abdomen could any DWV sequences be detected (Fig. 2a). Injecting \(1 \times 10^7\) DWV equivalents, the dosage which caused an overt infection in 100 % of the injected pupae, resulted in weak signals in thorax (15.89 ± 22.88 %, mean ± SD) and abdomen (62.57 ± 8.65 %, mean ± SD). No overt infection (detection of DWV signals in head RNA) could be demonstrated (Fig. 2a). However, after injection of \(1 \times 10^8\) DWV equivalents, strong viral signals were detected in all bees, indicating a successful DWV infection. In 100 ± 0 % (mean ± SD) of the bees, DWV could be detected in the abdomens although signal intensity varied (Fig. 2a). In 88 ± 11 % (mean ± SD) of the bees DWV could also be detected in their heads, indicating an overt infection (Genersch et al., 2010; Yue & Genersch, 2005). Control bees injected with PBS were always negative for DWV (Fig. 2a).

**Exposure bioassays with adult bees to mimic oral DWV transmission**

To determine the impact of oral transmission of DWV, DWV-free adult bees were orally infected with a dose of \(1 \times 10^6\) DWV equivalents, the same dose that was sufficient to infect 100 % of the injected adult bees. In none of the exposed bees could any DWV sequences be found in the head. Also DWV sequences could not be detected in the thorax. However, 87.5 ± 17.7 % (mean ± SD) of the bees tested positive for DWV in total RNA extracted from abdomen (Fig. 2b). To rule out the possibility that these signals were because of ingested virus circulating in the gut lumen, we performed DWV-specific fluorescence in situ hybridization (FISH) analysis of gut sections (Fig. 2c). DWV-specific signals could be detected in the cytoplasm of midgut epithelial cells of infected adult bees suggesting that the oral transmission route leads to infection of the digestive tract, which is among the primary tissues orally administered viruses have access to.
Fig. 1. DWV-injection bioassays with white-eyed pupae. White-eyed pupae were injected with $8 \times 10^1$, $8 \times 10^2$, $8 \times 10^4$ and $1 \times 10^7$ DWV genome equivalents. Hatching bees were analysed for obvious disease symptoms (a) and the presence of DWV sequences in total RNA extracted from bees’ heads as a molecular marker for an overt DWV-infection (b). A dose–response curve for DWV was established based on the results from the molecular detection of overt DWV infection (c; linear regression, $f(x)=15.77\log(x)-3.607; r^2=0.9418; P=0.0295$). Representative results obtained after injection of PBS or $1 \times 10^7$ DWV genome equivalents are shown in (a) and (b).

Fig. 2. Infection assays with adult bees via injection of DWV (a) or orally administering DWV (b and c). (a) Adult bees were injected with $8 \times 10^1$, $8 \times 10^2$, $8 \times 10^4$, $1 \times 10^7$ and $1 \times 10^8$ DWV genome equivalents or injected with PBS as a control and analysed for DWV in head (h), thorax (t) and abdomen (a) 3 days post-infection. Representative results are shown. (b) Adult bees were individually fed a single dose of $1 \times 10^8$ DWV genome equivalents and analysed for DWV in head, thorax and abdomen (labelled h, t and a, respectively) 3 days post-infection. Representative results are shown. (c) Midguts of infected bees were analysed by using DWV-specific FISH to confirm true infection and rule out mere acquisition of DWV following oral infection. DWV-specific signals appear as yellow dots in the red-stained cytoplasm of infected midgut cells. A representative section of the midgut is shown.
**DISCUSSION**

In the absence of *V. destructor*, DWV is considered a virus with low virulence that manages to establish vertical transmission routes through both drones and queens (de Miranda & Fries, 2008; Yue et al., 2007). Such vertically transmitted infections are mainly if not exclusively covert, which might present a mechanism for long-term persistence in the honeybee population as has also been described for other insect viruses (Bonsall et al., 2005; Burden et al., 2003; de Miranda & Genersch, 2010; Hails et al., 2008). Only in association with the ectoparasitic mite *V. destructor* have overt outbreaks of DWV infection, characterized by deformed wings as the most prominent symptom, been recorded (Ball, 1989; Ball & Allen, 1988; Bowen-Walker et al., 1999; Martin, 2001). However, a direct causal link between the virus and the symptom ‘wing deformity’ had not been established.

The present study demonstrated that injection of DWV isolated from overtly infected, deformed bees into pupae resulted in an overt infection characterized by deformed wings and DWV detection in the heads of emerging infected bees. Thus our results prove for the first time a direct causal relationship between DWV and the symptoms ‘wing deformity’ and ‘viral infection in the bees’ heads’, i.e. prove Koch’s postulates for DWV and the deformed-wing syndrome. Because our experimental design simulated DWV transmission to pupae by *V. destructor* our data also add to the understanding of the role of the mite in the development of overt DWV infection.

We recently showed that horizontal transmission of DWV to pupae through *V. destructor* is necessary, although not sufficient, for an overt infection in the emerging bee since in any given colony the majority of the vectorially infected pupae still emerge as asymptomatic adult bees (Gisder et al., 2009; Yue & Genersch, 2005). Our recently published data also indicate that *V. destructor* needs to act as biological vector, i.e. replication of DWV in the parasitizing mite prior to transmission is an imperative prerequisite for the deformed-wing syndrome to develop (Gisder et al., 2009; Yue & Genersch, 2005). Whether this replication just provides a high-enough viral titre in the mite or is associated with especially virulent forms of DWV still remains elusive. The latter possibility could be true if the mite represents a genetic bottleneck allowing only certain viral variants to accumulate in the vector.

In the present study, laboratory assays revealed that as few as approximately 2500 genome equivalents were sufficient to induce the deformed-wing syndrome in 50% of the injected pupae. However, we recently demonstrated that mites supporting DWV replication and associated with the development of the deformed-wing syndrome contained between $2 \times 10^{10}$ and $1 \times 10^{12}$ genome equivalents while mites not associated with wing deformity in parasitized pupae and not harbouring replicating DWV contained between $5 \times 10^4$ and $1 \times 10^8$ DWV genome equivalents (Gisder et al., 2009).

How do these figures fit with each other? Theoretically, the majority of the mites, irrespective of whether or not they contain replicating DWV, should have high enough viral titres to allow a sufficient number of DWV particles ($\sim 2500$) to enter the pupal haemolymph and cause overt DWV infections. Practically, according to our previous results, only mites containing replicating virus, and thereby viral titres of more than $2 \times 10^{10}$ genome equivalents, were able to induce overt infections. There are three possible explanations for this discrepancy. Firstly, it is not at all clear how viruses, which have no motility of their own, can enter the haemolymph of the pupa against the flow created while the mite is sucking haemolymph. If the mite is starting its blood meal by injecting a small volume of immune suppressing saliva, as suggested recently by Yang & Cox-Foster (2005), then it might very well be possible that the viral titre in the mite must exceed $1 \times 10^8$ DWV genome equivalents to ensure that this tiny aliquot of saliva entering the pupa contains 2500 DWV particles. This would leave only mites supporting viral replication as possible sources for overt infections. Secondly, if up to $1 \times 10^8$ DWV genome equivalents in the mite are borderline sufficient to allow 2500 particles to enter the pupa, the frequency of such an event might be so low that these bee–mite combinations have been overlooked in our studies. Thirdly, it is not the viral titre in the mite that determines the outcome of an infection but rather the viral variant enriched in the mite, i.e. a variant with a reduced host and tissue specificity, which is able to replicate in mite tissue and which is highly virulent to bees. Although it is tempting to speculate that DWV variants of differing virulence exist, so far we have not been able to prove this. Unfortunately, our attempts to isolate enough DWV from covertly infected bees or from mites collected from emerging healthy-looking bees to perform injection assays with these, presumably less virulent, variants failed. Therefore, it is still unclear whether or not ‘covert’ (low virulence, no replication in mites) and ‘overt’ (high virulence, replication in mites) variants are circulating in the bee and mite populations.

Recently, Iqbal and Müller reported infection experiments with adult bees using extracts from DWV-infected bees (Iqbal & Mueller, 2007). Injecting these crude extracts into adult bees resulted in detectable infection of the bees accompanied by impairments in sucrose responsiveness and associative olfactory learning, suggesting that brain structures involved in these two tasks were affected by the treatment. This study suggested that phoretic mites might also serve as a DWV vector by horizontally transmitting DWV to adult bees and possibly leading to overt infections (DWV detected in head) accompanied by subtle neurological symptoms. Unfortunately, the authors injected crude extracts of normal-winged bees and did not determine the amount of DWV or viral nucleic acid in their extracts, making it impossible to compare their results with ours. According to our results, $1 \times 10^8$ DWV genome equivalents had to be injected into adult bees to
reproducibly establish detectable DWV infections. At this dosage, overt infections characterized by detection of DWV in bees’ heads could be demonstrated in 88 ± 11 % of the injected bees. However, considering the DWV titre present in mites (Gisder et al., 2009) only mites containing replicating DWV (viral titre >2 × 10^{10}) will have a small chance of vectorially transmitting such a high dosage of DWV while feeding on adult bees. Therefore, while it is not impossible that some phoretic mites horizontally transmit DWV, and even cause overt infection in these bees, it is rather unlikely that under natural conditions the frequency of this event will be high enough for a negative effect on colony performance due to learning deficits.

The above-mentioned study also demonstrated that after offering adult bees sucrose contaminated with crude extracts containing DWV no infection could be established in the bees (Iqbal & Mueller, 2007), suggesting that the oral transmission route is rather ineffective. In contrast, our results showed that by feeding a single dose of 1 × 10^8 DWV genome equivalents to adult bees, all exposed bees developed an infection that was restricted to abdominal organs involving an infection of the midgut epithelium. Ingested viruses remain ‘external’ as long as they stay in the lumen of the digestive tract. To successfully establish an infection ingested viruses first need to breach the epithelial barrier lining the digestive tract. Accordingly, we could detect DWV in the cytoplasm of gut epithelial cells suggesting that the gut epithelium provides a means of entry for DWV. This is in agreement with other studies, which either showed high viral titres in the abdomens of infected bees (Chen et al., 2006; Iqbal & Mueller, 2007; Yue & Genersch, 2005) or directly identified the digestive tract as one of the predominantly infected tissues (Fievet et al., 2006). Further experiments are needed to analyse the spread of the virus from the midgut to other tissues.

There are studies showing qualitatively that DWV is present in the midgut content (Fievet et al., 2006) and in faeces (Chen et al., 2006), suggesting a faecal–oral route of transmission, but there is no quantitative data allowing the estimation of the absolute viral titre. The same is true for absolute DWV titres in different developmental stages of the bee. There is only one study showing relative quantification of DWV levels in larvae, pupae and adult bees (deformed and healthy looking). In this study it was demonstrated that pupae had the highest level of DWV, even higher than for deformed bees (Chen et al., 2005). Because of this lack of absolute data on DWV levels in faeces and bees, we can only speculate on the effectiveness of the oral transmission route under natural conditions.

Bees normally defecate outside the hive limiting the contact of adult bees with faeces mostly to circumstances where nestmates are already affected by disease conditions involving dysentery like nosemosis (Fries, 1993, 2010) and chronic bee-paralysis virus infection (Ribiere, 2006). Therefore, the faecal–oral transmission of DWV might play a role in such diseased colonies and DWV infections may then add to the damage already inflicted. However, the faecal–oral route of DWV transmission should not play a major role in otherwise healthy colonies.

Bees remove and cannibalize diseased larvae and pupae as part of the social immune response (Evans & Spivak, 2010). Colonies infested by V. destructor produce a considerable number of pupae damaged by both the mites and DWV transmitted by the mite. These pupae, which contain high levels of DWV (Chen et al., 2005), are removed and cannibalized in the nurse bees’ hygienic efforts to combat the parasitic mite syndrome. Therefore, cannibalism of mite/DWV-damaged pupae should represent an efficient route for horizontal transmission of DWV to adult bees. This might become more and more important in the wake of DWV spreading within the mite population and might lead to an increase in fatal DWV-infections vectorially transmitted to pupae.

The impact of a DWV infection of the digestive tract on the health status and performance of an adult bee is unclear; however, it is rather unlikely that this will have a negative impact on the entire colony. However, if the infection is able to spread from the initial infection site (gut tissue) to other tissues including brain, the establishment of an overt infection accompanied by learning deficits could be possible. Foragers displaying the learning deficits described (Iqbal & Mueller, 2007) will negatively impact upon colony performance and add to the damage already present in mite-infested colonies. In this context it is noteworthy that colonies that harboured detectable numbers of healthy-looking bees that tested positive for DWV in total RNA extracted from head had a significantly lower chance of surviving the winter season than colonies without such overtly infected adult bees (Genersch et al., 2010).

METHODS

**DWV detection via RT-PCR analysis.** Qualitative RT-PCR analysis of eggs, white-eyed pupae, adult bees and body parts of adult bees for the presence of DWV was performed essentially as already described. Total RNA was extracted using an RNeasy kit (Qiagen) following the manufacturer’s protocols (Yue & Genersch, 2005). Eluted RNA (50 μl) was stored at −70 °C for subsequent analysis. One-step RT-PCR was performed according to standard protocols (OneStep RT-PCR kit; Qiagen) as previously described (Yue & Genersch, 2005), using the primer pair F1/B1 (F1, 5′-CCTGCTAATCAACAAGGAC-CCTGG-3′; B1, 5′-CAGAAACATCTAAGCTTAACC-3′) resulting in a DWV-specific amplicon of 355 bp (Genersch, 2005).

Quantitative real-time RT-PCR analysis (qRT-PCR) allowing the absolute quantification of DWV genome equivalents was performed essentially as previously described (Gisder et al., 2009), by using a QuantiTect reverse transcription kit (Qiagen) for the reverse transcription reaction, and a QuantiTect SYBR green PCR kit (Qiagen) and a Chromo4 real-time PCR thermal cycler (Bio-Rad) for the PCR amplification, which were performed according to the manufacturer’s instructions.

**FISH analysis.** Midguts from experimentally infected bees (n=5) were carefully prepared and fixed in 4 % Histofix (Roth) overnight at 4 °C on a shaker. Fixed midguts were incubated in 6.8 % sucrose in
Preparation and evaluation of DWV suspensions. For virus extraction, deformed bees were collected from heavily mite-infested colonies. For each preparation twenty bees were each crushed in 5 ml potassium phosphate buffer (0.01 M, pH 7). Subsequently, the homogenate obtained was filtered through a nylon cell strainer (Falcon) with 100 µm mesh diameter, followed by centrifugation for 15 min at 15,000 g to separate the virus-containing supernatant from the chitin debris of the bees. The supernatant was immediately centrifuged through a 30 % sucrose cushion at 100,000 g for 6 h at 4 °C. Following centrifugation, the sucrose solution was carefully removed and the pelleted viruses were dissolved in 500 µl potassium phosphate buffer (0.01 M, pH 7). For further purification, the virus suspension was centrifuged for 24 h at 132,000 g at 4 °C through a caesium chloride gradient with 1.37 g cm\(^{-2}\) initial density. Virus-containing fractions were determined by measurement of A\(_{280}\) and electron microscopy (data not shown). The presence of DWV and the absence of Israeli acute paralysis virus (IAPV), sacbrood virus (SBV), acute bee paralysis virus (ABPV) and chronic bee paralysis virus (CBPV) was confirmed by RT-PCR (Fig. 3a). Quantification of DWV equivalents using qRT-PCR was performed as previously described (Gisder et al., 2009). DWV suspensions were stored at 4 °C in the dark until use.

**V. destructor-free bee material for injection bioassays with pupae.** To obtain suitable pupae for infection experiments, in early summer several colonies from the institute’s apiary were analysed for their mite infestation level. Colonies with low mite-infestation levels were treated against *V. destructor* with formic acid, a chemical acaricide, killing *V. destructor*. Efficiency of the treatment was controlled both during the application of the formic acid (bottom board control) and 14 days after the treatment. The latter was performed by randomly checking brood cells for the presence of parasitizing mites and by calculating the number of mites that died naturally and fell down onto the bottom board over a period of 5 days. In addition, the colonies were checked for vertical transmission of DWV by analysing unfertilized and fertilized eggs for DWV by using RT-PCR. Furthermore, the colonies were checked for signs of clinical DWV infection [deformed appendages in newly emerging bees and/or the presence of DWV in total RNA extracted from bees’ heads (Genersch et al., 2010; Yue & Genersch, 2005)]. One colony that could be considered free of *V. destructor*, that did not show any signs of clinical DWV-infection and was headed by a queen that was not vertically transmitting DWV (see above), was kept in the institute’s bee yard but was separated from the other colonies to minimize reinvasion by *V. destructor*. White-eyed pupae were carefully collected exclusively from brood cells not containing any parasitizing mites and directly used for injection bioassays.

**DWV-free bee material for infection assays (injection and exposure bioassays) with adult bees.** DWV-infection assays with adult bees are not expected to produce any obvious disease symptoms (deformed wings). Evaluation of such assays will therefore rely on molecular detection of the virus in bee tissue, which will only produce meaningful results if the experiments are performed with naive bees. Hence, the availability of naive, DWV-free bees is an imperative prerequisite for such studies. Because under natural conditions it is hardly possible to find a DWV-free bee, we had to establish a DWV-free colony.

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Fig. 3. Analysis of the virus and bee material used for infection assays. (a) DWV isolated from deformed-winged bees was tested for the absence of contaminating viruses like Israeli acute paralysis virus (IAPV), sacbrood virus (SBV), acute bee paralysis virus (ABPV) and chronic bee paralysis virus (CBPV) by using RT-PCR. (b) Eggs, white-eyed pupae and adult bees (h, head; t, thorax; a, abdomen) from a colony that was considered free of *V. destructor* and DWV were tested for the absence of DWV by using RT-PCR. All material analysed did not contain detectable levels of DWV and representative results are shown.
V. destructor-free colonies (see above) were analysed for their DWV-status (see above). A colony that did not show any signs of clinical DWV-infection (see above) and was headed by a queen that was not vertically transmitting DWV (see above) was put into quarantine, i.e. placed in a flight room equipped with time-controlled daylight lamps and kept at a constant temperature of 25 °C. Every day, fresh pollen and water, and every other day, fresh invert sugar solution, were offered. The colony was regularly checked for the absence of V. destructor. After 3 months, when the initial bee generation had been completely replaced by a new bee generation, eggs, white-eyed pupae and adult bees (12 individuals of each) were analysed for their viral status and proved to be free of DWV (Fig. 3b) and other contaminating viruses (IAPV, SBV, ABPV and CBPV; not shown). Every four weeks the colony was checked again for its viral status by repeating this analysis. The bees from this DWV-free colony were then used for infection assays with adult bees (see below). Unfortunately, the availability of this mite- and DWV-free bee material was limited (approx. 300 bees over time) so that only a limited number of experiments and replicates were possible.

Injection bioassays with pupae. Brood combs from the selected V. destructor-free colony (see above) were checked for the presence of brood cells containing engorged larvae. These cells, expected to be caged soon after, were marked and approximately 1 day after cell capping white-eyed pupae were carefully extracted from these cells with forceps, transferred into cellulose-lined cavities of 24-well microtitre plates, and kept in an incubator (35 °C, 60–70 % humidity). Cells were carefully checked again for the absence of V. destructor. Pupae that showed handling injuries or melanization after 12 h were excluded from the experiments. Pupae were infected via injection assay by using various dosages of DWV (8 × 10², 8 × 10³, 8 × 10⁴ or 1 × 10⁵ DWV equivalents) suspended in 2 µl PBS (pH 7.0). DWV suspensions were injected ventrally through the intersegmental membrane between the third and fourth abdominal segment (the typical feeding place of V. destructor) using a microlitre hollow needle (Unimied). Control pupae were mock infected with 2 µl PBS (pH 7.0) or not manipulated at all. All pupae were kept in an incubator (35 °C, 60–70 % humidity) until the end of metamorphosis (fully developed adult bees). Pupae fatally injured by coarse piercing or handling became noticeable within <12 h post-infection (p.i.) by melanization and death. These pupae were removed from the experiments. The experiments were repeated several times (n = 4–5) resulting in totals of 48 (n = 5), 45 (n = 4), 45 (n = 5), 45 (n = 5), 38 (n = 4) and 49 (n = 5) pupae, which were non-manipulated, PBS-injected or injected with 8 × 10², 8 × 10³, 8 × 10⁴, 1 × 10⁵ DWV equivalents, respectively. Adult bees were evaluated for obvious disease symptoms (deformed wings) and subsequently stored at −70 °C for further molecular analysis. Bees suffering from overt DWV infection are characterized by deformed wings and DWV being detected in all body parts, especially in total RNA extracted from head (Yue & Genersch, 2005). The latter characteristic was recently shown to be of clinical importance (Genersch et al., 2010). We therefore chose ‘detection of DWV in total head RNA’ as an additional molecular marker for overt DWV infection in our assays. Bees were decapitated for analysis; total RNA was extracted from each head separately and analysed for the presence of DWV. The proportion of overtly infected bees is given as a percentage mean ± SD.

Observed mortality in the experimental groups was low although it varied substantially and was not because of DWV infection, as can be deduced from the fact that mortalities in the non-manipulated group, the PBS-injected group and, for example, the group injected with 1 × 10⁴ DWV equivalents were 6.6 ± 8.7 %, 12.1 ± 11.5 %, and 5.6 ± 7.9 % (mean ± SD), respectively.

Injection bioassays with adult bees. Adult bees from the DWV-free flight-room colony were collected from the hive entrance and immobilized on ice. Bees were infected with various dosages of DWV (8 × 10¹, 8 × 10², 8 × 10³, 1 × 10⁴ or 1 × 10⁵ DWV equivalents) suspended in 2 µl PBS and injected into the thorax haemolymph of each bee. Control bees were mock infected with 2 µl PBS (pH 7.0). Subsequently, the experimental groups were kept in small cages in an incubator (dark, 34 °C, 60–70 % humidity) and provided ad libitum with sucrose solution (0.88 M) and water. After 3 days, surviving bees were sacrificed by freezing at −70 °C. Bees were stored at −70 °C until analysis. For analysis, total RNA was extracted from head, thorax and abdomen, was extracted separately from each bee and was analysed for the presence of DWV. The experiments were repeated three times (n = 3) and each experimental group comprised between 14 and 18 animals depending on the availability of suitable bees. Observed mortality varied considerably but was not due to DWV infection as can be deduced from the fact that mortalities in the PBS-injected group and, for example, the group injected with 1 × 10⁴ DWV equivalents was 34.9 ± 1.3 % and 22.3 ± 1.2 % (mean ± SD), respectively.

Exposure bioassays (oral infection) with adult bees. Adult bees from the DWV-free flight-room colony were collected from the hive entrance, immobilized on ice and fixed in metal tubes for feeding. Two hours after collection, the bees were orally infected with DWV by feeding them individually with a dosage of 1 × 10³ DWV particles in 4 µl of 0.88 M sucrose solution. Control bees were fed the same volume of 0.88 M sucrose only. After being fed the DWV particles, the bees were kept at 34 °C and 60–70 % humidity in the dark (incubator), and provided ad libitum with sucrose solution (0.88 M) and water (modified after Iqbal & Mueller, 2007). We performed two independent experiments, each with two experimental groups and one control group (nine adults). In the first replicate each experimental group comprised eight adults, in the second replicate one experimental group comprised eight and the other experimental group nine adults. After three days, bees were sacrificed by freezing at −70 °C and stored at −70 °C until analysis. For analysis, total RNA was extracted from head, thorax and abdomen, was extracted separately from each bee and was analysed for the presence of DWV. No mortality was observed during these experiments.

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