

The Transmission of Deformed Wing Virus between Honeybees (*Apis mellifera* L.) by the Ectoparasitic Mite *Varroa jacobsoni* Oud

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INTRODUCTION

Under field conditions, *Varroa jacobsoni* were shown to be highly effective vectors of deformed wing virus (DWV) between bees. Adult female mites obtained from honeybee pupae naturally infected with DWV contained virus titers many times in excess of those found in their hosts and, beyond that, which might be expected from a concentration effect. It is therefore possible that DWV may be capable of replicating within *V. jacobsoni*. Bees which tested positive for DWV exhibited characteristic morphological deformity and/or they died during pupation. Asymptomatic bees had much lower virus titers than those which were deformed or had died during pupation. It is therefore suggested that for DWV to cause pathology it must be present in pupae above a certain concentration. The amount of DWV vectored by *V. jacobsoni* will depend on the mites' level of infection, which will in turn depend on whether they had fed previously on dead or deformed bees and also on the rate of replication of the virus within the mites. Consequently, developing bees infested with large numbers of mites could suffer a high incidence of deformity if the mites are heavily infected or harbor an especially virulent strain of virus. A positive relationship was found between increasing numbers of mites on individual bees and the incidence of morphological deformity and death. This probably reflected the large number of viral particles transmitted by the mites, which resulted in many multiply infested bees dying before emergence. These results demonstrate the importance of the role of viruses when considering the pathology of *V. jacobsoni* and that much of the pathology previously associated with the effects of mite feeding could be attributed directly to secondary pathogens vectored by *V. jacobsoni*. © 1999 Academic Press

Key Words: *Varroa jacobsoni*; honeybees; *Apis mellifera*; deformed wing virus; vector; bee deformity; bee death; disease transmission.

Varroa jacobsoni Oud. is associated with the appearance of deformed bees in colonies of *Apis mellifera* and colony death normally occurs within 3–5 years of initial infestation (Korpela *et al.*, 1992). Bee deformity includes malformed appendages (crumpled/vestigial wings), shortened abdomens, reduced weight at emergence (DeJong *et al.*, 1982; Schatton-Gadelmayer and Engels, 1988), possibly a reduction in life span (Kovac and Crailsheim, 1988), and eventually colony effects such as patchy/irregular brood and dwindling bee numbers (Shimanuki *et al.*, 1994).

Several theories have been proposed to explain these effects. *V. jacobsoni* is known to feed on the hemolymph of developing larvae and has been shown to deplete total hemolymph volume and hemolymph protein titers (Gliński and Jarosz, 1984; Weinberg and Madel, 1985). Visible bee deformity could therefore be attributed to both reduced hydrostatic pressure during development and to a reduction in protein availability during development (Daly *et al.*, 1988). Consequently, with increased numbers of mites on individual bees there would be a greater probability of emerging bees exhibiting deformity (DeJong *et al.*, 1982). Unfortunately, bees with very few or no mites can be found exhibiting the same deformity as highly parasitized bees, and highly parasitized bees exhibiting no deformity have also been recorded (Marcangeli *et al.*, 1992; Bowen-Walker, unpublished observations). This casts serious doubt on the ability of mite numbers alone to explain bee deformity and colony collapse.

Another theory is that bee deformity is related to microbial septicemia, caused by microorganisms transmitted by *V. jacobsoni* (Shabanov, 1984; Gliński and Jarosz, 1992; Liu, 1996). Again, other evidence has not been supportive (Koch and Ritter, 1989, 1991) or is in direct opposition (Alippi *et al.*, 1995) to the hypothesis.

Under experimental conditions, *V. jacobsoni* transmits certain viruses (e.g., Acute Paralysis Virus and Cloudy Wing Virus) (Batuev, 1979; Ball and Allen,

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1988), raising the possibility that these might be involved in causing bee deformity. One of these viruses, the Japanese strain of Egypt Bee Virus, now called Deformed Wing Virus (DWV), causes deformity, reduction in emergence size, and mortality of infected honeybees (Ball, 1989; Bailey and Ball, 1991); these symptoms are commonly associated with mite feeding behavior. Furthermore, because female mites feed regularly and move between both bee brood and adult bees (Bowen-Walker and Gunn, in press) they have the potential to act as vectors of pathogenic organisms.

The purpose of this investigation was to provide evidence that under natural field conditions, mites (1) acquire and carry viruses and (2) act as vectors of these viruses and that (3) the pathology associated with *V. jacobsoni* is linked to the viruses they carry and not necessarily their feeding activities.

MATERIALS AND METHODS

All experiments were conducted in May and June 1997 in Devon (UK). Six untreated *A. mellifera* colonies were used: three source colonies and three target colonies. Source colonies were severely infested with *V. jacobsoni* (daily mite drop, 30+ mites) and many bees (both emerging and older) exhibited noticeable morphological deformity, such as crumpled/vestigial wings, and some bees were reduced in size. The target colonies had undetectable varroa populations (no mite drop over 5 days) and the bees exhibited no morphological deformity. All colonies were within the same apiary. The target colonies were recent introductions to the apiary—hence their low mite populations.

Transmission of Deformed Wing Virus by V. jacobsoni

Collection of mites from the source colony. Frames of emerging brood were removed from the source colonies and female mites were collected from both deformed and nondeformed emerging worker bees. The mites were kept overnight on damp tissue at 33°C in a Gallenkamp incubator before introduction into the target bee cells the next day.

Introduction of mites into the target colony brood. The queens of the target colonies were marked, caged onto drawn worker comb, and released following oviposition. Brood development was monitored daily. On the day of capping between one and six mites from the source colonies were introduced into the recently sealed cells. Inoculation was accomplished by making a small incision in the capping using a pin, inserting a mite which had mounted a needle (flamed immediately before picking up each mite) into the incision, and then melting a small piece of beeswax from the same frame over the incision. Control cells were prepared in the same way but no mites were introduced into the cells.

Each cell was identified by being marked on an overlying sheet of acetate pinned to the frame.

The frame was then inserted into a Gallenkamp incubator maintained at 33°C and 55–60% relative humidity (monitored by two hair hygrometers) until the bees were due to emerge. Frames were not returned to the hives, owing to the possibility of the bees rejecting the manipulated cells (S. Martin, personal observation). Developing bees into whose cells the mites were introduced were termed “target hosts”; the original emerging bees from which the mites were collected were termed “source hosts.”

Emerging target bees, classified as being deformed or nondeformed, were collected at emergence together with their infesting mites and frozen (–20°C) for later viral analysis. This procedure was repeated three times, the mites being drawn from the same three source colonies, but each frame of target brood came from a different hive.

Identification of Deformed Wing Virus in V. jacobsoni and Target Hosts

Due to the nature of the morphological deformity [deformed wings, reduction in overall size, and mortality of brood (Ball, 1989)] mites were tested for DWV, as other known viruses in the United Kingdom (e.g., Slow Paralysis Virus and Cloudy Wing Virus) do not show similar symptoms (Bailey and Ball, 1991). The mites and target bees were analyzed by the indirect ELISA technique of Allen *et al.* (1986) as modified by B. Ball (pers. comm.) for the identification of DWV. All analyses were conducted at IARC Rothamstead, Harpenden.

Whole bees and whole mites were homogenized in 1 and 0.5 ml, respectively, of extraction buffer, pH 7.4 [per 100 ml: 2 g polyvinylpyrrolidone (44000 Av mol wt), 500 µl 10% Tween 20, 0.2 g ovalbumen, 10 ml stock phosphate-buffered saline (stock-PBS) (1.4 M NaCl, 0.01 M KH₂PO₄, 0.08 M Na₂HPO₄ · 12 H₂O, 0.02 M KCl)] using a Pro 2000 electric homogenizer. Carbon tetrachloride (100 µl) was then added to each homogenate to remove fatty material and the samples were centrifuged at 8000 rpm for 10 min in a 5417C Eppendorf centrifuge. Aliquots of the supernatants were then diluted with extraction buffer (1:40 for bees, 1:20 for mites) and assayed for the presence of DWV as detailed below.

Cliniplate polystyrene microtiter plates (Labsystems) were coated with 200 µl of DWV-specific F(ab')₂ fragments (1:4000) in 0.0125 M sodium carbonate buffer (pH 9.6) and incubated at 30°C for 4 h. The plates were then rinsed with 10% stock-PBS containing 0.05% Tween 20 (Tween-PBS) in a Skatron A/S Microwash III. Diluted bee or mite supernatant (200 µl) was then added to each well and the microtiter plates were incubated at 4°C overnight. The plates were then rinsed with Tween-PBS, after which 200 µl of DWV-

specific IgG in extraction buffer ($4 \mu\text{g ml}^{-1}$) was added to each well. The plates were then incubated at 30°C for 3 h, after which they were rinsed with Tween-PBS and $200 \mu\text{l}$ of protein A–peroxidase conjugate in extraction buffer ($25 \mu\text{g ml}^{-1}$) was added to each well. The plates were then incubated at 30°C for 3 h after which they were rinsed with Tween-PBS and $200 \mu\text{l}$ of a peroxidase substrate (per 20 ml: 2 ml sodium acetate–citric acid buffer, $200 \mu\text{l}$ 3,3',5,5'-tetramethylbenzidine in dimethyl sulfoxide, $20 \mu\text{l}$ hydrogen peroxide) was added to each well. The plates were then incubated in the dark at 20°C for 20 min, after which the reaction was stopped by the addition of $50 \mu\text{l}$ of 3 M H_2SO_4 . The optical density of each well at 450 and 690 nm was recorded against a reagent blank using a Titertek Multiskan MCC/340 MKII microtiter plate reader equipped with Titresoft II software.

The antiserum we used is not known to cross-react with other bee viruses (B. Ball pers. comm.). Bees were analyzed for DWV individually, but all the mites within the same cell were pooled for analysis. DWV research is presently in its infancy and no standard curve for absorbency against virus particle concentration exists (B. Ball pers. comms).

RESULTS

Bee Deformity in Source and Target Colonies

Mites (462) in three trials were introduced into a total of 179 recently capped worker cells. Of these, 97 bees (54%) survived to emergence. By contrast, all the bees in randomly opened, nonexperimental cells on the frames ($n = 674$) survived. All of the control bees emerged ($n = 30$) and were nondeformed, even though one bee had a natural (nonexperimental) parasitosis of 4 mites. With an increasing number of introduced mites, fewer nondeformed bees emerged, more bees died, and more bees emerged with morphological deformity (Fig. 1). Source host deformity was associated with target host deformity; thus, the target was more likely to be deformed if the source host was deformed

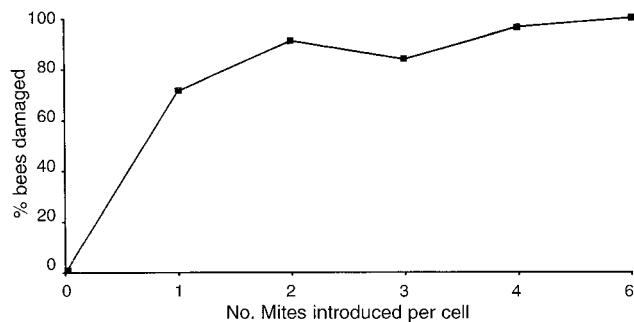


FIG. 1. The relationship between the number of *Varroa jacobsoni* introduced into developing *Apis mellifera* cells and the percentage of bees which emerged deformed or died.

TABLE 1

The Percentage Deformity Recorded in Emerging Worker *Apis mellifera* Parasitized by *Varroa jacobsoni* When the Mites Had Previously Infested Deformed and Nondeformed Honeybees

	Original <i>A. mellifera</i> host deformed?	
	Yes	No
Second <i>A. mellifera</i> host deformed?		
Yes	82% $n = 23$	43% $n = 13$
No	18% $n = 5$	57% $n = 17$

and less likely to be deformed if the source host was not deformed ($\chi_1^2 = 4.42$, $P < 0.05$) (Table 1).

Identification of Deformed Wing Virus in V. jacobsoni and Target Hosts

High DWV ELISA optical densities were recorded in all deformed and dead bees and in all the mites that were infesting dead and deformed bees (Fig. 2). Low or undetectable DWV levels were recorded in uninfested and infested but nondeformed bees (Fig. 2). Interestingly, those mites recovered from nondeformed bees exhibited a wide range of optical densities, which were essentially platykurtic (Sokal and Rohlf, 1995) in their distribution ($g_{2n=17} = -2.05$). There were highly significant associations (i) between a bee being infected with DWV and emerging deformed ($\chi_1^2 = 38.2$, $P < 0.001$) (Table 2), (ii) between bee deformity and the presence of DWV in their parasitising mites ($\chi_1^2 = 22.6$, $P < 0.001$) (Table 3), and (iii) between a bee being infected with DWV and its parasitising mites being infected with DWV ($\chi_1^2 = 13.45$, $P < 0.001$).

The mean weight of adult female *V. jacobsoni* was $0.34 \pm 0.01 \text{ mg}$ ($n = 5$), while that of an emerging worker honeybee was $116.4 \pm 0.61 \text{ mg}$ ($n = 165$) (Bowen-Walker, unpublished). Using these data, and the mean DWV ELISA optical densities (measured in optical density units) for five singly infested bees (2.623 ± 0.221 OD units) and their associated mites (2.891 ± 0.115 OD units), it was possible to compare, albeit crudely, the relative amounts of virus present (estimated as optical density mg^{-1} tissue live weight) in the mites and in the bees. As an a priori our calculation assumed that the standard curve for the DWV ELISA would be of the typical sigmoid shape (Allen *et al.*, 1986; Clark and Barbara, 1987) and that the measurements fell on the linear portion of the curve. The results suggested that the mites contained virus particles that led to an average absorbency of $85.03 \text{ OD units mg}^{-1}$ live weight and that the bees contained virus particles that lead to an absorbency of $0.904 \text{ OD units mg}^{-1}$ live weight.

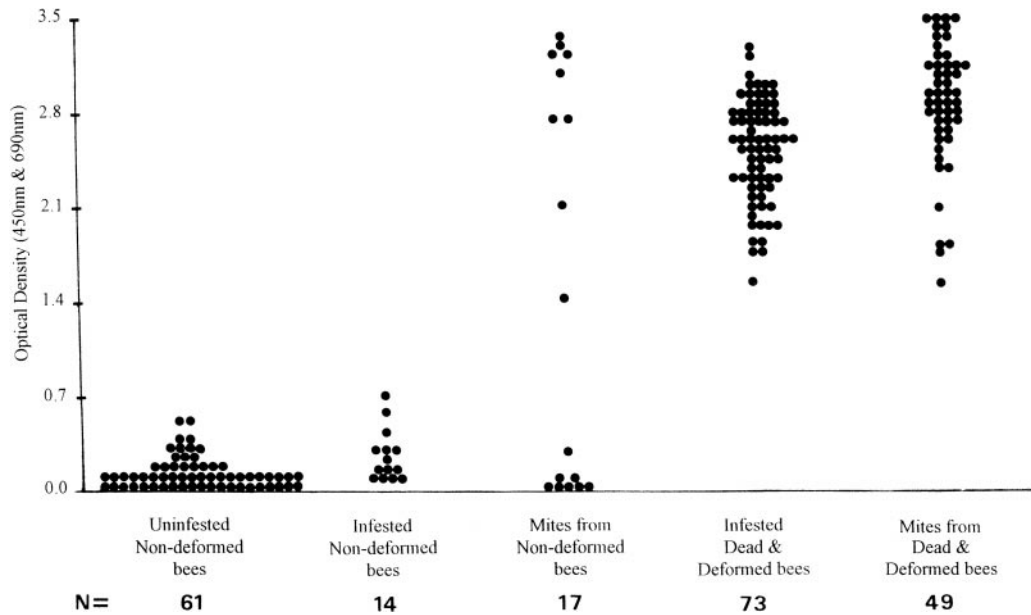


FIG. 2. Deformed Wing Virus ELISA optical densities measured at 450 and 690 nm of (i) uninfested nondeformed bees, (ii) infested deformed bees, (iii) mites removed from deformed bees, (iv) deformed and dead bees, and (v) mites removed from deformed and dead bees.

DISCUSSION

There are two principal mechanisms whereby *V. jacobsoni* could spread viruses within and between bee colonies. First, the mites feeding activities could potentiate inapparent virus infections already present in the bees, i.e., until activated by the mites the viruses do not cause noticeable pathology and their levels are low or undetectable. Second, the mites could act as vectors of viruses by directly transmitting them between bees, for example, in their saliva or in regurgitated gut contents.

Our results prove that *V. jacobsoni* can act as a highly

effective vector of DWV. The evidence for this is, first, that the mites are capable of acquiring DWV from infected bees—all the mites feeding on deformed bees themselves tested positive for the virus. Second, we found that a bee was more likely to die or emerge deformed if the mite feeding on it had previously fed on a deformed bee. This association would be baseless if the mites were not capable of transmitting DWV between hosts. If *V. jacobsoni* acted solely as a potentiator of DWV then the introduction of mites into the target bee cells would have resulted in little more than 13% deformity among the emerging bees. That is, the level

TABLE 2

The Relationship between Deformed Wing Virus ELISA Optical Densities at 450 and 690 nm in *A. mellifera* and Their Deformity at Emergence

	Levels of DWV in emerging <i>A. mellifera</i>		
	DWV absent	DWV likely	DWV present
<i>A. mellifera</i> deformed?			
Yes	0%	1%	99%
	<i>n</i> = 0	<i>n</i> = 1	<i>n</i> = 72
No	0%	57%	43%
	<i>n</i> = 0	<i>n</i> = 8	<i>n</i> = 6
Uninfested nondamaged bees	44%	43%	8%
	<i>n</i> = 27	<i>n</i> = 26	<i>n</i> = 8

Note. DWV ELISA optical densities were placed into the following groups according to Kemeny (1991): (i) DWV absent, below 1.5 × mean plate background (mpb); (ii) DWV likely, 1.5 × mpb to 0.2 OD units above mpb; and (iii) DWV present, higher than 0.2 OD units above mpb.

TABLE 3

The Relationship between Deformed Wing Virus ELISA Optical Densities at 450 and 690 nm in *V. jacobsoni* Removed from Emerging *A. mellifera* Which Were Deformed or Non-deformed

	Levels of DWV in mites removed from emerging <i>Apis mellifera</i>		
	DWV absent	DWV likely	DWV present
<i>A. mellifera</i> deformed?			
Yes	0%	0%	100%
	<i>n</i> = 0	<i>n</i> = 0	<i>n</i> = 49
No	35%	6%	59%
	<i>n</i> = 6	<i>n</i> = 1	<i>n</i> = 10

Note. DWV ELISA optical densities were placed into the following groups according to Kemeny (1991): (i) DWV absent, below 1.5 × mean plate background (mpb); (ii) DWV likely, 1.5 × mpb to 0.2 OD units above mpb; (iii) DWV present, higher than 0.2 OD units above mpb.

of deformity would be similar to the natural incidence of virus among the bees in the colony (13%).

The existence of a low frequency of DWV within the colony indicates that its transmission can also be independent of *V. jacobsoni*. This would explain the occasional outbreaks of DWV in the UK before the arrival of *V. jacobsoni* in 1992 (B. Ball pers. comm.). The absence of bee deformity among naturally infected bees could result from either the virus normally being inhibited by the bee immune system or because, for some reason, the virus might be replicating slowly.

The spread of *V. jacobsoni* into a new area is often associated with an increased prevalence of viral diseases, including DWV, which were previously not reported or rare (Ball, 1993). However, there is usually a lag period of about a year between the arrival of *V. jacobsoni* and reports of colony damage (Martin, 1997). This indicates that *V. jacobsoni* infestation does not immediately or inevitably result in honeybee pathology. The outcome of infestation will depend on numerous factors such as (i) the number of mites in the colony, (ii) the presence and level of natural viral infection among the bees, (iii) the prevalence of virus in the mite population, (iv) the pathogenicity of the strain of virus, (v) the susceptibility of the bees (and possibly also the mites) to the virus, and (vi) the amount/caste of brood present in the colony, which in turn will influence the mite population dynamics.

In our experiments uninfested nondeformed bees had similar DWV ELISA optical densities. Again, this shows that *V. jacobsoni* infestation does not always result in bee deformity. However, dead and deformed bees invariably expressed optical densities considerably in excess of those of the nondeformed bees. This suggests that it is the level of DWV present in the bees which determines whether they are deformed at emergence rather than just the presence or the absence of virus.

Mites collected from dead and deformed bees exhibited optical densities similar to those of the bees they were infesting. Furthermore, our calculations indicated that weight for weight the mites actually contained much higher levels of virus than the bees. This could arise from two possible mechanisms. First, the virus particles may somehow be concentrated within the mites. However, this would require an exceptionally efficient and hitherto unknown process to account for such high optical densities in the mites. Alternatively, once the mites have acquired DWV from the bees the virus may be replicating within them as well. Such replication has not previously been described in other viruses transmitted by *V. jacobsoni* but considering the close affinity between the mites and the bees, which extends to the mites absorbing and utilizing unmodified bee proteins (Tewarson and Engels, 1982), it would appear to be at least a plausible explanation, which warrants further investigation. The ability of viruses to

replicate successfully within several unrelated hosts is well-documented, e.g., rabies virus (class V, Rhabdoviridae) and yellow fever virus (class IV, Flaviviridae) (Dimmock and Primrose, 1994).

Additional circumstantial evidence of DWV replication within *V. jacobsoni* is our finding of extremely high optical densities in some of the mites collected from nondeformed bees. These bees had low virus levels and it is unlikely that their mites could have obtained their levels by concentration effects alone.

The relationship among honeybees, DWV, and *V. jacobsoni* is obviously complex but it is possible to postulate a progression of events on the arrival of the mites in a colony. Having infested a colony, *V. jacobsoni* will either acquire DWV from naturally infected (but asymptomatic) bees or bring it with them from their previous colony. The viruses may then replicate within the mites and, ultimately, high virus levels of DWV will be transmitted to the brood. This may help to explain the positive correlation we found between increasing numbers of mites infesting developing bees and an increasing percentage of deformed emerging bees, which has also been reported by DeJong *et al.* (1982) and Marcangeli *et al.* (1992). With increasing mites on developing bees, there would be an increased probability that a developing bee would receive enough DWV from either a single mite with a high virus titer (e.g., one that had previously fed on a dead or deformed bee) or from the combined contributions of several mites with low virus titers. Where two or more mother mites enter a cell, only one needs to be carrying virus to cross-infect the other(s) and the offspring by infecting the common food source, i.e., the developing bee. Ultimately, there would be a depletion in the bee population, both adult and brood, and eventually the colony may collapse.

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