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ORIGINAL RESEARCH ARTICLE



The role of deformed wing virus in the initial collapse of varroa infested honey bee colonies in the UK

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Summary

The mite *Varroa destructor* has been associated with the collapse of millions of *Apis mellifera* honey bee colonies world-wide. During the past decade, a large body of research has revealed various interactions between varroa, the honey bee and various viral pathogens. One pathogen in particular, deformed wing virus (DWV), has emerged as the key pathogen involved in colony collapse. As varroa has permanently changed the viral landscape in which honey bees exist, we present a large body of data on the effects of DWV during the initial phase of varroa infestation in the UK during 1998. This provides baseline data for further comparative studies. We carried out DWV transmission studies, and observed the effects of DWV on bee longevity. As the ELISA technique used in these studies had a detection limit of $\sim 10^7$ viral particles per bee, only high viral (overt) titres were detected. During the initial phase of varroa establishment, DWV was detected in 0.6% of non-infested sealed brood, but in 89% of sealed brood invaded by varroa. Once DWV was introduced into the bee's haemolymph via mite feeding on either pupae or adults, an overt virus infection was rapidly produced in 3-4 days. In sealed brood the presence of varroa was fatal for 21% of the brood, caused wing deformity in some emerging adults and significantly reduced longevity as an adult. However, adult bees that became infected after they had emerged, did not develop wing deformity nor show any reduced longevity, but acted as reservoirs of DWV infection.

El papel de los virus de las alas deformadas en el colapso inicial de las colonias de abejas infestadas por varroa en el Reino Unido

Resumen

El ácaro *Varroa destructor* se ha asociado con el colapso de millones de colonias de abejas *Apis mellifera* en todo el mundo. Durante la última década, un amplio volumen de investigaciones ha puesto de manifiesto diversas interacciones entre varroa, la abeja de la miel y diversos patógenos virales. Un patógeno en particular, el virus de las alas deformadas (DWV), se ha convertido en el agente patógeno clave implicado en el colapso de colonias. Como varroa ha cambiado para siempre el paisaje viral en el que existen las abejas, se presenta aquí una gran cantidad de datos sobre los efectos del DWV durante la fase inicial de la infestación de varroa en el Reino Unido durante 1998. Esto proporciona datos de referencia para futuros estudios comparativos. Hemos llevado a cabo estudios de transmisión del DWV, y observamos los efectos del DWV en la longevidad de la abeja. Como la técnica de ELISA usada en estos estudios tenía un límite de detección de $\sim 10^7$ partículas virales por abeja, solamente se detectaron concentraciones virales altas (manifestadas). Durante la fase inicial del establecimiento de varroa, el DWV se detectó en el 0,6% de la cría operculada no infestada, y en el 89% de la cría operculada invadida por varroa. Una vez que el DWV se introdujo en la hemolinfa de la abeja a través de la alimentación del ácaro tanto en pupas como en adultos, la infección manifestada por el virus se produjo rápidamente en 3-4 días. En la cría operculada la presencia de varroa fue fatal para el 21% de la cría, causó deformidades del ala en algunos adultos emergentes y redujo significativamente la longevidad en los adultos. Sin embargo, las abejas adultas que se infectaron después de haber salido, no desarrollaron deformidad del ala ni mostraron ninguna reducción de la longevidad, pero pueden actuar como reservorios de la infección DWV.

Keywords: *Varroa destructor*, *Apis mellifera*, deformed wing virus, DWV, colony collapse, honey bee, interactions

Introduction

In all temperate regions where the ectoparasitic mite *Varroa destructor* has become established, it has been closely associated with widespread losses of both managed and feral honey bee (*A. mellifera*) colonies (De Miranda and Genersch, 2010; Neumann and Carreck, 2010) and still remains the number one pest faced by beekeepers. Because of the ability of the mite to transmit viruses, a previously obscure virus, deformed wing virus (DWV) has become the most prevalent honey bee virus after the appearance of varroa in all countries where the mite has become established, thus changing the viral landscape (Carreck *et al.*, 1999; Martin *et al.*, 2012; Schroeder and Martin, 2012). By their very nature, viral pathogens continue to evolve as various selection pressures change (De Miranda and Genersch, 2010; Genersch and Aubert, 2010). This may explain why there was an initial strong correlation between the survival of varroa infested colonies and the presence of a small group of viral pathogens such as slow bee paralysis virus (SBPV) and DWV (Martin *et al.*, 1998; Nordström, 2003; Carreck *et al.*, 2005; Carreck *et al.*, 2010). Recent studies have, however, shown that colonies continue to collapse with high DWV titres despite mite control measures which have ensured very low mite loads, both in the UK (Highfield *et al.*, 2009) and the USA (Cox-Foster *et al.*, 2007).

Furthermore, DWV may now respond differently to varroa control treatments (Locke *et al.*, 2012; Martin *et al.*, 2010). These differences could be due to the continual evolution of the cloud of DWV variants that has been accelerated by the initial spread of the varroa mite (Martin *et al.*, 2012; Schroeder and Martin, 2012).

Varroa was first found in the UK in 1992 (Bew, 1993), and in this paper we present previously unpublished key baseline data collected in 1998 during the early phase of infestation in the UK, representing an early stage in the evolution between DWV, varroa and the honey bee host. This will allow comparisons of the death rates, transmission rates and effects of DWV on bee longevity found in this study with data generated from new epidemiological studies.

Materials and methods

DWV was detected in individual bees and mites using an indirect enzyme linked immunosorbent assay (ELISA) (Allen *et al.*, 1986; Nordström, 2000), except that after homogenization, the bees were diluted 1/40 to reduce any cross-reactivity with bee protein. Due to the occasional carryover of DWV during extraction, the raw data was adjusted by halving any low value (<60 times mean plate background) when it was preceded by a high reading (>100 times mean plate background). It is not possible to accurately quantify the amount of DWV in a sample due to its instability during the purification procedure, so we compared the relative amounts of DWV in an individual bee or mite by performing a dilution series of, 1/40 to 1/100,000 for a bee,

and 1/1 to 1/1,000 for a mite. Since the dilution end point for DWV is 10^{-5} and the detection limit is around 10^7 , an estimate of 10^{11} particles ($\approx 1\mu\text{g}$) of DWV in an overtly infected bee was made. This is consistent with the number of particles found in bee larvae killed by sacbrood virus (Bailey and Ball, 1991) and those found in overtly infected honey bees calculated using DWV standards (Highfield *et al.*, 2009; Martin *et al.*, 2012). Therefore, the detection limit of the ELISA can be considered to separate a covert (undetectable) and overt (detectable) infection and therefore may be very useful in large scale epidemiological studies such as this where hundreds or thousands of samples need to be analysed (De Miranda *et al.*, 2013), although it will miss carriers with lower infection titres i.e. those between 10^5 and 10^7 .

To investigate transmission efficiency between January and October 1998, naturally infested worker sealed brood cells with their associated varroa mites were collected from 14 colonies monthly and analysed for DWV. The development stage of the sealed brood and mites were recorded. In addition, adult bees which had mites attached in the feeding position between the third and fourth abdominal tergites (Bowen-Walker *et al.*, 1997) were collected between September and December and both analysed for DWV.

To estimate brood mortality, naturally infested combs containing brood of a uniform age were removed in July 1998 from five bee colonies immediately after the brood cells had been sealed and placed into an incubator maintained at 35°C and 60-70% RH, thus preventing worker honey bees from removing any dead brood. After 10 days, the combs were removed from the incubator and 200-300 cells from each comb were randomly chosen and opened. The number of live or dead bee pupae and the degree of mite infestation were recorded. A high proportion of dead pupae were then analysed individually for the presence of DWV along with up to a maximum of 30 live pupae from each group.

The effect of DWV on honey bee longevity was studied in two varroa free queen-right honey bee colonies held in separate outdoor bee proof flight enclosures (4 x 3 x 8 m) that were supplied with pollen, water and syrup and erected on a concrete floor. To investigate seasonal differences, the experiments were carried out in summer (1 June 1998 to 20 July 1998) autumn (17 August 1998 to 4 October 1998) and winter (21 October 1998 to 13 April 1999) using two new varroa free queen-right colonies each time. Into each of these colonies, two groups of newly emerged marked bees and their mites were introduced. These were obtained by the coordinated confinement of the queen to a single comb, in five naturally infested colonies, since the time of emergence of a large number of adult bees could then be predicted. These bees were individually collected upon emergence and marked with different coloured paint according to whether or not their cells had been infested by varroa. The two groups of adult bees (infested or un-infested as pupae), were divided equally and introduced into the two caged colonies. Due to the large adult bee sample size required for the winter experiment, it was not possible to check

Table 1. Association of sealed worker brood mortality in five colonies naturally infested by *Varroa destructor*.

Hive #	% infestation of sealed brood	Uninfested [DWV+ / n sampled]		Infested [DWV+ / n sampled]	
		Alive n = 437	Dead n = 13	Alive n = 617	Dead n = 164
22	40	194 [1/30]	3 [1/2]	86 [10/30]	46 [23/24]
6	45	140 [0/30]	4 [2/4]	103 [17/31]	7 [6/6]
2	58	37 [1/5]	5	45 [9/9]	13 [7/8]
3	86	46 [0/23]	0	238 [28/30]	44 [30/30]
16	90	20 [0/15]	1	145 [30/30]	54 [21/22]
Averages	64%	[2%]	[50%]	[72%]	[97%]

whether each individual bee was infested or not, so bees emerging from severely infested colonies were used and it was assumed that the majority of the introduced bees were from infested cells. Therefore, no survivorship data for adults infected with overt DWV was collected during the winter. As all bees that emerged from non-infested cells were assumed to be DWV free (Table 1), so any bees from the non-infested group later found to have overt DWV titres were assumed to have been fed on by a phoretic mite that were introduced into the varroa-free experimental colonies attached to the group of infested bees. All dead bees were collected daily (or weekly during the winter) from the concrete floor of the cage, sorted into groups of marked infested, and marked un-infested bees. Samples from each group were tested individually for DWV. Comparison of survivorship curves were performed used the Log-rank (Mantel-Cox) test in GraphPad Prism. We lost one of the autumn caged colonies due to vandalism so only one dataset exists for that season. All our experimental colonies that were either naturally or artificially infested with varroa subsequently

collapsed if no mite control measures were taken (Carreck *et al.*, 2010; Martin *et al.*, 2010).

Results

The incidence of overt (detectable) DWV infections in un-infested sealed worker brood in the 14 colonies studied was very low, with only six positive cases (0.6%) from the 987 cells analysed. It is possible that some, if not all, of these six cases were false positives due to the practical difficulty of detecting the presence of the mite within a cell with complete accuracy.

An experiment to study the transmission efficiency of DWV between varroa and its host was conducted using 373 mite-honey bee pupa pairs (Fig. 1A) and 43 mite-adult honey bee pairs (Fig. 1B). Both figures show a continual cluster of points along the y-axis, indicating that the amount of DWV detected in the mites was highly variable. In the

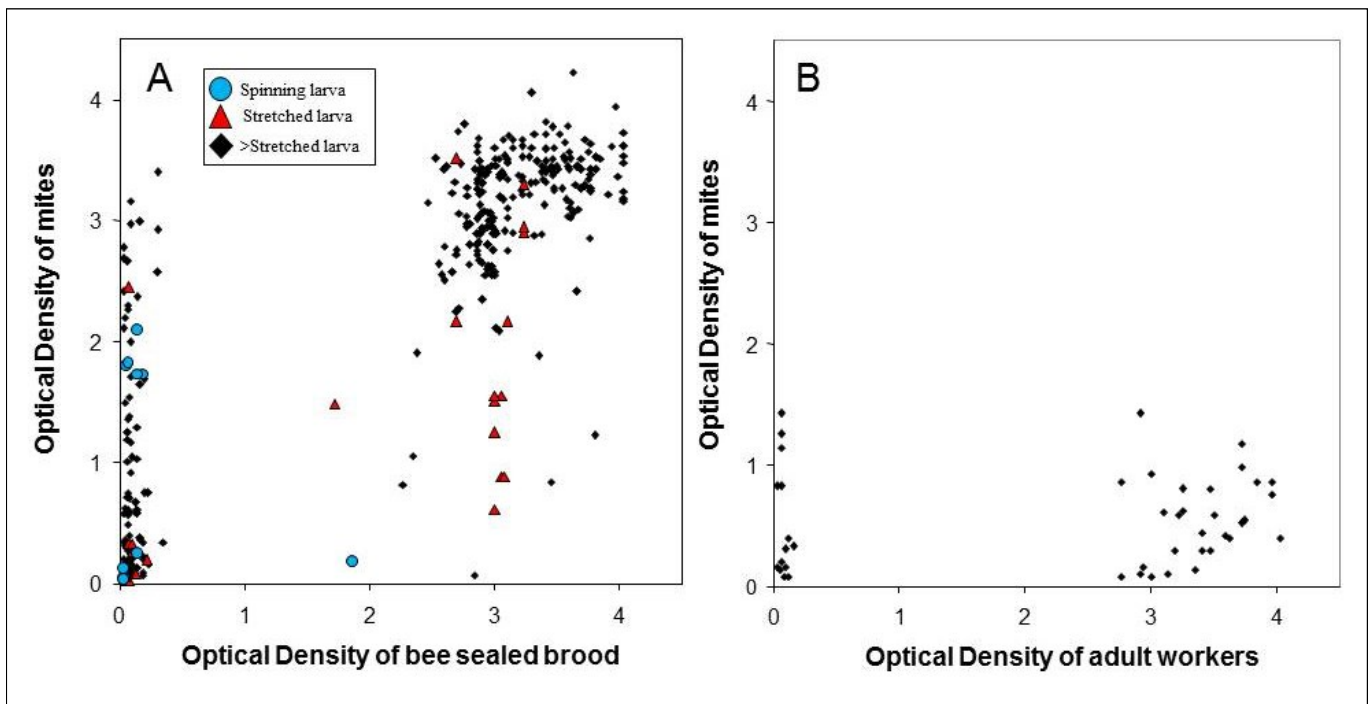


Fig. 1. Relationship between overt DWV (A) in 373 sealed larvae or pupae and (B) 43 adult bees and their associated mites. The different ages of sealed brood are indicated by a circle (spinning larva), triangle (stretched larva) and diamonds (pupa in A and adults in B). The higher the optical density up to the saturation value of 4, the greater the quantity of DWV, values less than 0.5 represent no detectable DWV.

sealed brood, between 10-20% of mites carrying DWV either did not transmit it to their host, or the virus failed to become established as an overt infection. The upper estimate assumes that all mites were carrying DWV whilst the lower estimate considers only those mites carrying large amounts of DWV (optical density >2). In the case of the adult workers, no mites were carrying large amounts of DWV, so if all mites are considered, 33% did not transmit the virus to the host or it failed to become an overt infection within the host.

No DWV was detected in eight of the nine sealed brood aged between 0-30 h after the cells were sealed (spinning larva stage) despite five of the associated mites having large amounts (optical density around 2) of virus present (Fig. 1A). However, during the next 30 to 85 h (stretched larva stage) there was a sudden increase from no virus being detectable to >10⁷ particles per bee being present in the sealed brood. Thereafter, the amounts of detectable virus remained at the upper limit (optical density >3) of detection (Fig. 1A). In the case of the adult bees, DWV was again detectable at the upper limit of detection, but only small amounts were detectable in the associated mites (Fig. 1B).

Brood mortality was measured in 781 infested and 450 un-infested sealed brood cells from five honey bee colonies. DWV was the only virus found (SBPV and APV were absent) and it was detected in the 94% of dead brood and 41% of live sealed brood tested (n = 96 and 233 respectively) (Table 1). DWV was detected in only six (6%) of the 109 un-infested brood but 82% of the 220 infested brood tested positive for DWV (Table 1). Therefore, the majority (79%) of the sealed brood infected with DWV were still alive after 10 days, indicating that varroa infestation was fatal to the brood in 21% of infested cells (Table 1), although high titres of DWV were associated with 97% of the dead infested sealed-brood (Table 1).

Over the course of the three seasonal longevity trials, 862 un-infested and 2911 infested newly-emerged adult bees were individually marked. Of these, 2735 (72%) were recovered and 1199 (44%) were analysed for DWV. Survival curves of dead marked bees in which only DWV was detected were constructed for each experiment, and the results compared against published data (Free and Spencer-Booth, 1959; Fukuda and Sakagami, 1966) for bees emerging in mite free colonies at a similar time of the year (Fig. 2). The curves show that bees infected with DWV during their pupal development had their longevity significantly reduced relative to either control bees or bees infected with DWV after emergence in the summer ($X^2 = 51.4$, $df = 1$, $p < 0.001$), autumn ($X^2 = 31.4$, $df = 1$, $p < 0.001$) and winter ($X^2 = 16.8$, $df = 1$, $p < 0.001$). The decrease in longevity appeared to be proportional to the normal lifespan of bees at that time of year (Fig. 2). Bees with wing deformity all died within 48 hours of emergence. Bees emerging from un-infested cells that then subsequently acquired DWV from the mites had a longevity not significantly different than non-infested control bees in the summer ($X^2 = 5.2$, $df = 1$, $p = 0.08$) and autumn ($X^2 = 1.2$, $df = 2$, $p = 0.5$) (Fig. 2).

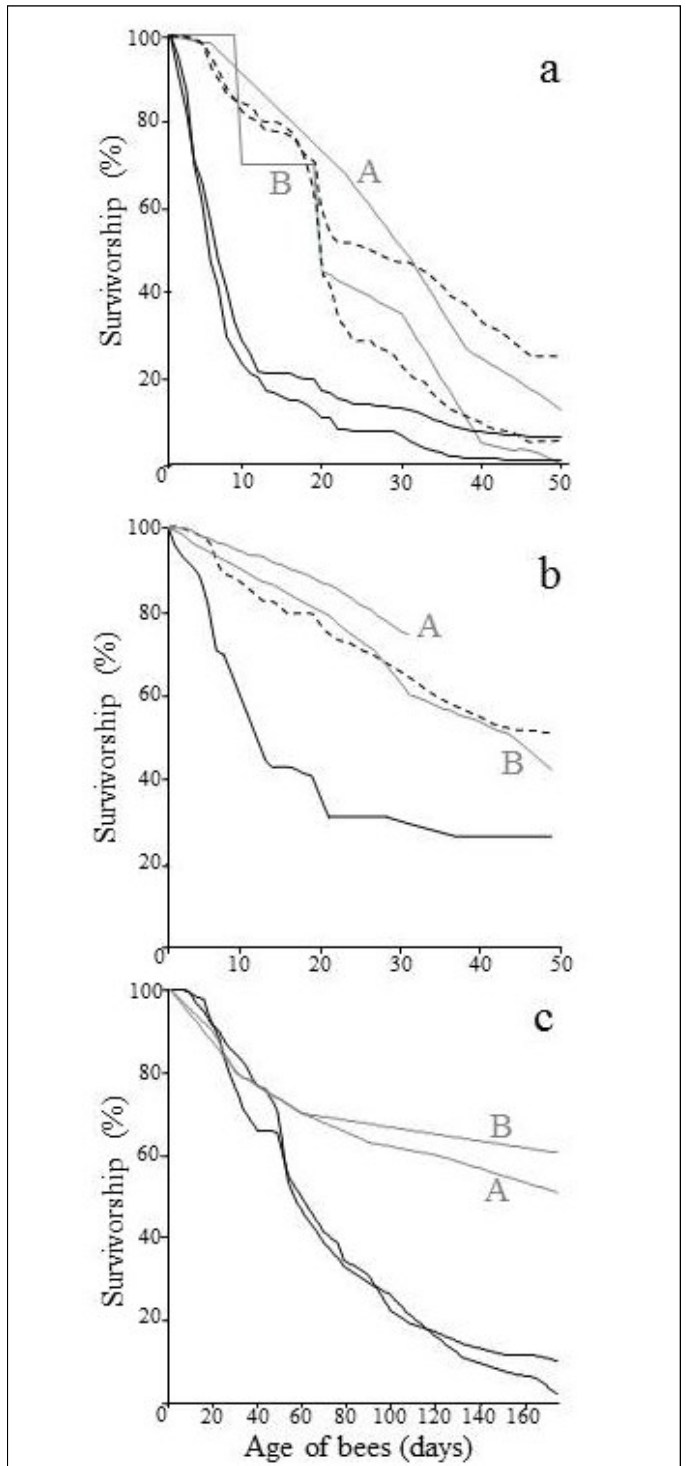


Fig. 2. Survivorship curves of bees from the two replicate experiments conducted in (a) summer, (b) one experiment in autumn, (c) winter that were infected with overt DWV as pupae (solid black line), or as adults (black dashed line) since these individuals were un-infested at emergence and so assumed to be DWV free (Table 1), so could only have become infected with DWV from being fed on by phoretic mites that were introduced into the caged colonies along with the newly emerged infested bees. No marked non-infested adults were introduced into the winter experiment (see methods). Survivorship data for healthy bees (grey solid lines) from mite free colonies derived from Free and Spencer-Booth (1959) (A) and Fukuda and Sakagami (1966) (B).

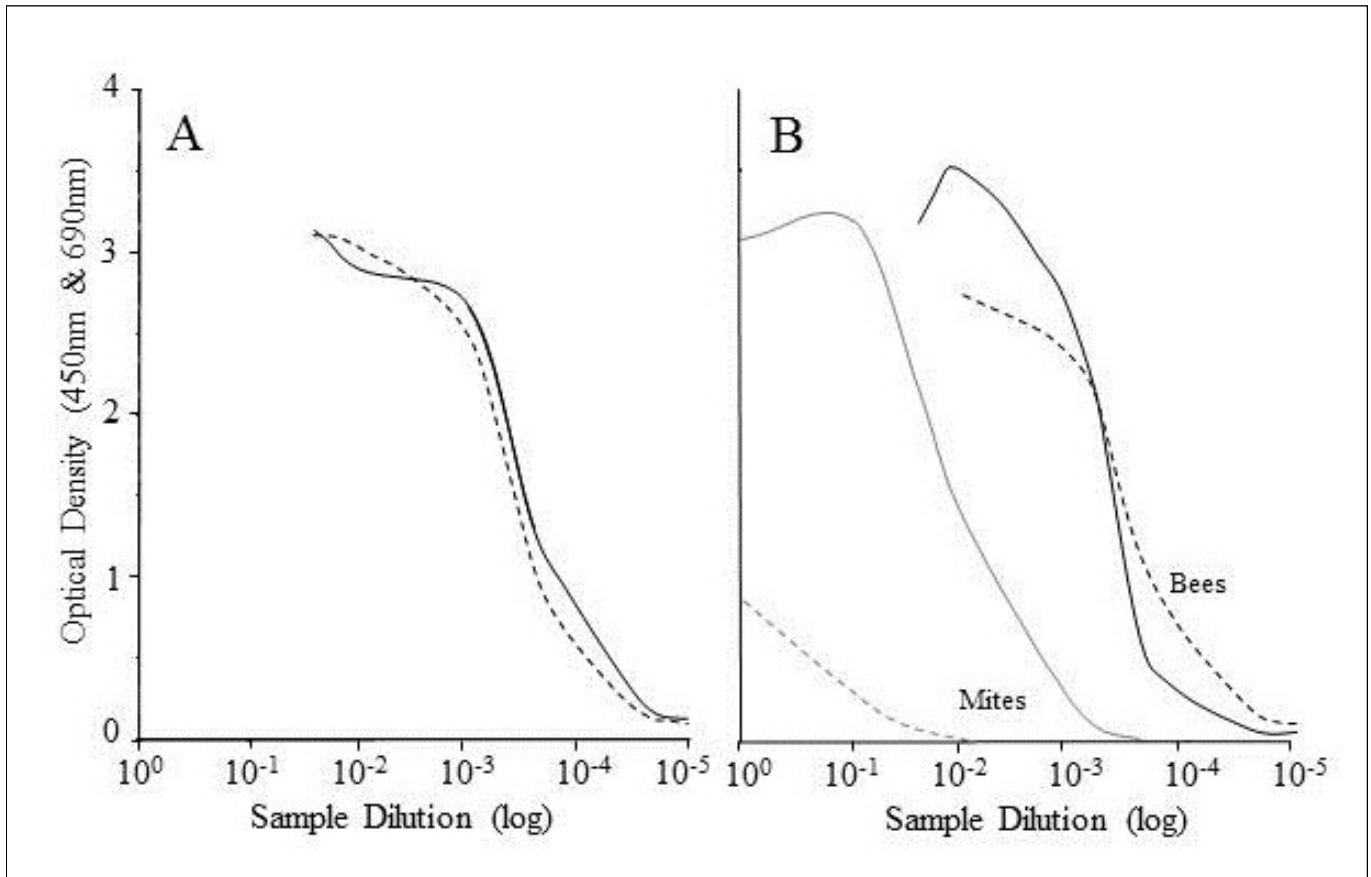


Fig. 3. Dilution curves indicating the mean relative amounts of DWV in (A) 11 living (solid line) and 16 dead bees (dashed line), along with the dilution curves for (B) ten *Varroa* mites that just invaded brood cells (grey dashed line), ten mites emerging from infected brood cells (grey line), and adult honey bees that became infected with DWV during pupal ($n = 12$, dashed line) or the adult stage ($n = 10$, black line).

Despite these different pathologies, the relative amount of DWV in dead or living adult worker bees had almost identical dilution curves (Fig. 3A). Furthermore, DWV levels were similar whether the honey bee became infected during the pupal or adult stage (Fig. 3B). Mites entering brood cells contained very low amounts of DWV relative to mites emerging from sealed brood cells that contained an overtly DWV infected pupae (Fig. 3B), which is supported by the data from the transmission studies (Fig. 1).

Discussion

During this study which represents the early stages of varroa and DWV establishment in the honey bee community, the transmission of overt DWV infections between adult bees to brood appeared to be negligible, since we detected only a very small proportion (0.6%) of un-infested brood that were overtly infected with DWV, whereas 89% of sealed brood infested with varroa tested positive for DWV. In contrast, Nordström (2000) found that DWV may be transmitted by nurse bees to larvae in severely mite-infested colonies. Mites are very effective vectors of DWV (Bowen-Walker *et al.*, 1999; Chen *et al.*, 2006) since 89% (this study) and 71%-76% (Nordström, 2003) of varroa

infested sealed brood had overt DWV infections. *Varroa* mites start feeding on the developing bee brood within six hours of the cell being sealed, and feeding episodes occur regularly thereafter (Donzé and Guerin, 1994). Therefore, varroa can potentially infect the brood with DWV within hours of the cell being sealed. Thirty hours later, DWV becomes detectable in the developing bee and reaches the saturation point of the ELISA method i.e. $>10^{11}$ particles per bee, after just 85 h, indicating a rapid multiplication of the pathogen within the host. This high viral titre causes fatality in around 21% of infested developing brood, since 97% of dead brood had high DWV titres, which helps explain the spotty brood patterns typically associated with heavy varroa infestations. Akrananukul and Burgett (1975) found that varroa killed between 6-35% of the infested sealed brood, which is similar to the 21% of dead infested sealed-brood found in this study (Table 1). Of the brood that emerges, up to 66% of individuals can have wing deformity in severely infected colonies (Nordström, 1999), which died within 48 h (this study; Yang and Cox-Foster, 2007). The development of crippled wings in the honey bee depends not only on DWV transmission by varroa, but also on viral replication within the mite (Gisder *et al.*, 2009). Although DWV replication within the mites is not consistent with our finding that DWV titres are significantly higher in mites leaving sealed brood cells relative to phoretic mites invading

cells (Fig. 3). As crippled wings normally occur in only a small proportion of infested honey bees, DWV replication within mites may be a rare event, and serial feeding on non-infested bees can reduce DWV titre (Brenda Ball unpublished data), this may help explain our findings. The remaining infested pupae that emerge successfully and appear normal suffer a reduced longevity (Fig. 2) as was also found by Yang and Cox-Foster (2007) and could explain the results of earlier studies (e.g. De Jong and De Jong, 1983; Schneider and Drescher, 1987; Kovac and Crailsheim, 1988) that attributed the reduced honey bee survival directly to the varroa mite. The mechanism by which DWV effects longevity is currently unknown, but virus replication often shuts down the normal protein production of an infected cell. This may explain the reduced protein content of haemolymph observed in infested bees (Glinski and Jarosz, 1984; Schatton-Gadelmayer and Engels, 1988), and this could affect longevity. However, longevity of adult honey bees is unaffected when bees are infected during the adult stage (Fig. 2; Martin *et al.*, 2003). This is despite DWV titres reaching similar levels in bees infected as pupae or adults (Fig. 3). Therefore, DWV may be affecting the developmental pathway from larvae to adult, hence reducing lifespan only if infection occurs at the pupal stage. Further studies into tissue tropisms and bee-viral interactions are needed to confirm this idea. Although, this phenomenon explains why queens from mite infested colonies on the verge of collapse, which tested positive for DWV (unpublished data) were still alive and laying eggs. Mites rarely, if ever, invade queen cells and survive to feed on the developing queen pupae, all queens must therefore have become infected as adults, thus any DWV will not affect their longevity. Although adults infected with DWV after emergence may not suffer reduced longevity, they become key DWV reservoirs that allow the pathogen to persist within the honey bee population, even after the mites have been removed (Locke *et al.*, 2012; Martin *et al.*, 2010).

It has been suggested that the feeding activities of varroa may activate covert DWV infections already present in the honey bees (Yang and Cox-Foster, 2005), rather than actively transmit the pathogen. Although it is almost impossible to prove a negative, using the most sensitive molecular methods such as q-PCR, DWV was not detected in large numbers of honey bee colonies in areas never exposed to varroa (Martin *et al.*, 2012), but more importantly, the commonly observed time lag of two to three years between the arrival of varroa and start of colony collapse has now been explained by time needed for virulent clouds of DWV strains to evolve to the new mite-honey bee transmission route (Martin *et al.*, 2012). Such a time lag would not exist if mite feeding activated an already present covert DWV infection.

The variable effects of DWV on the bees' longevity due to time of infection and season helps to explain why colonies infected with DWV do not suddenly collapse (Martin, 2001; Sumpter and Martin, 2004). DWV is now known to consist of a naturally large cloud of variants which has been significantly reduced by the new varroa-bee transmission route (Martin *et al.*, 2012). The evolution of this cloud of

variants that affects factors such as virulence, growth and transmission rate, will be the focus of many new studies. Since varroa continues to be the most serious world-wide pest of honey bees, the data provided by this study during the initial phase will be useful in seeing whether such changes are occurring.

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