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RESEARCH ARTICLE

Evidence for damage-dependent hygienic behaviour towards *Varroa destructor*-parasitised brood in the western honey bee, *Apis mellifera*

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SUMMARY

The ectoparasitic mite *Varroa destructor* and honey bee pathogenic viruses have been implicated in the recent demise of honey bee colonies. Several studies have shown that the combination of *V. destructor* and deformed wing virus (DWV) poses an especially serious threat to honey bee health. Mites transmitting virulent forms of DWV may cause fatal DWV infections in the developing bee, while pupae parasitised by mites not inducing or activating overt DWV infections may develop normally. Adult bees respond to brood diseases by removing affected brood. This hygienic behaviour is an essential part of the bees' immune response repertoire and is also shown towards mite-parasitised brood. However, it is still unclear whether the bees react towards the mite in the brood cell or rather towards the damage done to the brood. We hypothesised that the extent of mite-associated damage rather than the mere presence of parasitising mites triggers hygienic behaviour. Hygienic behaviour assays performed with mites differing in their potential to transmit overt DWV infections revealed that brood parasitised by 'virulent' mites (i.e. mites with a high potential to induce fatal DWV infections in parasitised pupae) were removed significantly more often than brood parasitised by 'less virulent' mites (i.e. mites with a very low potential to induce overt DWV infections) or non-parasitised brood. Chemical analyses of brood odour profiles suggested that the bees recognise severely affected brood by olfactory cues. Our results suggest that bees show selective, damage-dependent hygienic behaviour, which may be an economic way for colonies to cope with mite infestation.

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Key words: *Apis mellifera*, *Varroa destructor*, deformed wing virus, biological vector, hygienic behaviour, brood odour.

INTRODUCTION

The Western honey bee, *Apis mellifera*, is among the most important productive livestock because of its indispensable role as commercial pollinator of many agricultural crops and fruit. In addition, honey bees contribute to the pollination of wild flowers, thereby contributing to the maintenance of natural ecosystems and biodiversity. Increasing honey bee colony losses both over winter and during the season have been reported, particularly from European countries, Japan and North America (Potts et al., 2010; Ratnieks and Carreck, 2010; vanEngelsdorp and Meixner, 2010). In the USA, the term colony collapse disorder (CCD) has been coined to describe the various symptoms associated with some of the observed unexplainable colony losses: the disappearance of most of the worker population while the queen is still alive, accompanied by a few nurse bees taking care of the remaining brood and provisioned by ample food stores (Cox-Foster et al., 2007; vanEngelsdorp et al., 2009). Although in Europe cases of CCD *sensu stricto* have not been reported so far, a steady increase in colony losses mainly over winter is causing as much concern as CCD does in the USA. Honey bee decline as part of a more general pollinator decline is becoming a global problem and is of great concern for agriculturists, apiculturists and scientists alike. From the many studies trying to unravel the causative factors of these enigmatic honey bee colony losses a picture is emerging where pests and pathogens play a major role (Ratnieks and Carreck, 2010), although additional factors like climate change, bee-keeping practice, habitat fragmentation, and pesticide use in agriculture as well as acaricide use in apiculture may add to the problem.

Among the pests and pathogens responsible for colony losses in Europe, the ectoparasitic mite *Varroa destructor*, which causes a devastating disease called varroosis, seems to be the key player. However, certain viruses have also been identified as being involved in unusual colony losses, like members of the ABPV/KBV/IAPV (acute bee paralysis virus/Kashmir bee virus/Israeli acute paralysis virus) cluster of related viruses and the deformed wing virus (DWV) (Genersch et al., 2010; Highfield et al., 2009; Potts et al., 2010; Ratnieks and Carreck, 2010). Remarkably, all these viruses are vectorially transmitted by *V. destructor* (Ball, 1983; Ball, 1989; Bowen-Walker et al., 1999; Chen et al., 2004; Di Pisco et al., 2011; Gisder et al., 2009; Yue and Genersch, 2005) and that at least ABPV and DWV only became virulent after *V. destructor* became established in the populations of *A. mellifera* in the late 1980s (Genersch and Aubert, 2010). In the absence of *V. destructor*, both viruses cause covert infections. However, they can be lethal once transmitted to pupae by parasitising mites (de Miranda et al., 2010; de Miranda and Genersch, 2010; Genersch and Aubert, 2010). The visible symptoms caused by DWV vectorially transmitted to pupae (shortened and bloated abdomen and deformed wings in the

emerging bee) are a hallmark of varroosis and were even mistaken for being induced by *V. destructor* alone in the beginning of varroosis research (Boecking and Genersch, 2008; de Miranda and Genersch, 2010).

Varroa destructor is a specialised parasite of *Apis* species (Anderson and Trueman, 2002). Originating in South-East Asia – where it is a parasite of *A. cerana* – the species made a host switch to *A. mellifera* in the last century and has spread around the world since then (Rosenkranz et al., 2010). The mites feed on the haemolymph of adult bees during the phoretic stage. Female mites enter brood cells with fifth instar bee larvae shortly before cell capping and lay several eggs. Mating takes place among siblings within the capped brood cells. From pupation to emergence of the bee, the mother mite and her offspring regularly feed on the haemolymph of the host pupa. Adult female mites disperse from one colony to another when transported on the bodies of drifting, robbing or swarming worker bees (Rosenkranz et al., 2010). When sucking haemolymph, the mites may transmit viruses including DWV (for references see above).

DWV is a positive-stranded RNA virus belonging to the Iflaviridae (Lanzi et al., 2006). It usually causes covert infections in honey bees (de Miranda and Genersch, 2010) and is transmitted vertically through queens and drones as well as horizontally through larval food (de Miranda and Fries, 2008; Yue et al., 2007). Overt DWV infections with clinical symptoms such as deformed wings, bloated and shortened abdomen, discolouration, and a dramatically shorter life-span or even death during pupal development only occur in association with *V. destructor* infestation. One line of evidence suggests that parasitisation of pupae by *V. destructor* influences the immune system of the pupae, which might result in the activation of pre-existing covert viral infections to overt, fatal infections (Gregory et al., 2005; Navajas et al., 2008; Yang and Cox-Foster, 2005; Yang and Cox-Foster, 2007). In contrast, recent studies have provided indirect (Bowen-Walker et al., 1999) and experimental evidence (Gisder et al., 2009; Möckel et al., 2011; Yue and Genersch, 2005) that the mite needs to act as a biological vector (i.e. with DWV replication in the mite prior to transmission) for an overt infection to develop (horizontal vectorial transmission). While pupae parasitised by mites harbouring no or only non-replicating DWV seemed to develop normally and emerge as healthy-looking adults, pupae parasitised by mites harbouring replicating virus may develop an overt DWV infection and emerge as non-viable, deformed adults. According to these recent studies, the damage done to the individual bee as well as to the colony depends on (i) the *V. destructor* infestation level of the colony, (ii) the proportion of DWV-carrying mites, and (iii) the proportion of mites harbouring replicating DWV (i.e. acting as biological vector of DWV) among these DWV-carrying mites.

Regardless, it is the combination of mite parasitisation and viral infection that poses a serious, if not the most serious, threat to honey bee health and survival. Considering the nearly global distribution of *V. destructor* (Rosenkranz et al., 2010) and DWV (de Miranda and Genersch, 2010), and the fact that despite increasing colony losses the majority of bee colonies are still able to survive and thrive, the question arises as to how honey bees manage to cope with this problem and, more importantly, how the colonies manage to contain and mitigate both mite parasitisation and fatal viral infections. One possible mechanism at the colony level is the so-called hygienic behaviour of honey bees. Hygienic behaviour involves the detection and removal of diseased brood in various developmental stages by adult worker bees tending to the brood (Cremer et al., 2007; Wilson-Rich et al., 2009), thereby preventing or reducing the reproduction and spread of brood pathogens and parasites in the hive. In view of the low number of immune effector genes in the honey bee genome, hygienic behaviour is considered a particularly important 'social immunity' mechanism against brood diseases (Evans et al., 2006; Wilson-Rich et al., 2009). Accordingly, it has been suggested that this behaviour may play a crucial role in the population dynamics of *V. destructor* and mite-vectored viral pathogens (Sumpter and Martin, 2004), and many breeding programmes have been undertaken to enforce the hygienic behaviour of honey bee colonies in order to develop bee lines with a high capacity to recognise and clean out parasitised and/or diseased brood (Büchler et al., 2010; Rinderer et al., 2010). While it has been well established for decades that bees show hygienic behaviour towards mite-parasitised brood (Ibrahim and Spivak, 2006; Peng et al., 1987; Spivak, 1996), it still remains elusive whether (i) the bees recognise the mite in the brood cell and react by a general removal of mite-infested brood or whether (ii) they recognise the damage done to the brood and only remove diseased and moribund pupae.

In order to experimentally answer this question, we took advantage of our recently established *V. destructor*–DWV–bee model where we had convincingly demonstrated that replication of DWV in mites prior to its transmission into pupae is necessary and sufficient for the induction of an overt infection in pupae, which either die in the pupal stage or develop into non-viable bees with deformed wings. Therefore, the extent of damage done to the pupae depends on (i) whether the parasitising mites transmit DWV to pupae and (ii) whether DWV replication takes place in the mites prior to transmission (Gisder et al., 2009; Möckel et al., 2011; Yue and Genersch, 2005). Using this model, we examined the hygienic behaviour of bees towards pupae experimentally infested with mites originating from populations with either a very high or a very low proportion of DWV replication, i.e. with mites differing in their ability to act as a vector for fatal DWV infections and to considerably damage the developing bees. It was found that bees preferentially recognise and remove pupae infested by mites transmitting fatal DWV infections. Mite-free pupae and pupae parasitised by nearly DWV-free mites were allowed to develop equally well undisturbed. Analysis of the odour profiles of the pupae supported the observed differences in removal behaviour and gave a first hint of its sensory basis. We propose that such a selective hygienic behaviour may be beneficial for the colony because (i) it removes the virulent DWV–*V. destructor* combination from the colony and (ii) it limits the removal of parasitised pupae to those that are really adversely affected by mite parasitism, thereby following the guide line to remove 'as much brood as necessary and as little brood as possible'.

MATERIALS AND METHODS

Mite and bee material

In May and June 2010, we screened mites from *A. mellifera carnica* Pollmann 1879 colonies at the institute's apiary in Hohen Neuendorf and at a remote apiary for DWV. These colonies had been excluded from the regular treatment against *V. destructor* in the previous autumn in order to maintain large mite populations for experiments. Mites were collected by dusting bees in powdered sugar. The two colonies with the lowest and highest proportion of mites carrying replicating DWV (henceforth DWVL and DWVH, respectively) were reduced in size (to eight brood frames and three frames with food) and subsequently kept in sealed glasshouses to keep their mite populations isolated. Colonies were provided with water *ad libitum* and honey and pollen as needed. The proportions of mites with DWV and DWV replication reported below apply to samples collected on the day when colonies were confined in glasshouses. All experiments

were carried out within 22 days following colony confinement. For experiments, we collected mites from freshly capped brood cells with fifth instar bee larvae to ascertain that they were in the proper physiological state for reproduction (Rosenkranz et al., 2010).

For the hygienic behaviour assay we selected 10 honey bee colonies (*A. m. carnica*) with good hygienic behaviour against *V. destructor* [as determined earlier in standard hygienic behaviour tests (Büchler et al., 2010)] from the institute's stock. Honey bee brood combs (*A. m. carnica*) for this experiment were obtained from eight colonies at a quarantine apiary, which were treated regularly against *V. destructor* with formic acid to minimise brood infestation levels. For the chemical analyses we also used brood combs from the eight colonies at the quarantine apiary.

RNA extraction and one-step RT-PCR for the detection of DWV RNA

Total RNA from single mites was extracted as described previously (Yue and Genersch, 2005) using an RNeasy Kit (Qiagen, Hilden, Germany). Eluted RNA (25 µl) was stored at –70°C for further analysis. One-step RT-PCR was performed according to standard protocols with a One-step RT-PCR kit (Qiagen) and as described previously (Genersch, 2005). The following temperature regime was used for DWV one-step RT-PCR: 30 min at 50°C, 15 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 54.3°C and 1 min at 72°C, including a final elongation step for 10 min at 72°C. The primers F1 and B1 (Genersch, 2005) are given in supplementary material Table S1. PCR products (5 µl per reaction) were analysed using a 1% agarose gel, stained with ethidium bromide and visualised by UV light.

Tagged RT-PCR for the specific detection of replicative forms of DWV RNA

Strand-specific detection of DWV RNA in mites was performed with the modified one-step RT-PCR protocol and with tagged F15/B23 primers as recently published (Gisder et al., 2009; Yue and Genersch, 2005) (supplementary material Table S1). PCR products (5 µl per reaction) were analysed using a 1% agarose gel, stained with ethidium bromide and visualised by UV light.

One-step RT-PCR for the detection of SBV, ABPV, CBPV, IAPV and KBV

To verify the absence of other bee viruses [Sacbrood virus (SBV), ABPV, chronic bee paralysis virus (CBPV), IAPV, KBV), extracted total RNA from single mites (see above) was pooled for the DWVL and DWVH colonies and standard one-step RT-PCR was performed according to the manufacturer's protocols (One-step RT-PCR Kit, Qiagen) using specific primers (supplementary material Table S1). The following temperature regime was used: 30 min at 50°C, 15 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at the appropriate annealing temperature (supplementary material Table S1) and 1 min at 72°C, including a final elongation step for 10 min at 72°C. PCR products (5 µl per reaction) were analysed using a 1% agarose gel, stained with ethidium bromide and visualised by UV light.

Hygienic behaviour assay and determination of brood damage

Mites from the two mite source colonies were introduced into cells containing recently capped fifth instar bee larvae following established methods (Ibrahim and Spivak, 2006). A small opening in the edge of each cell's wax capping was made using a sharp razor blade, mites were introduced through the opening with a fine paintbrush, and then the cell was carefully resealed. On each experimental comb we treated 30 groups of three adjacent cells each for a total of 90 cells. In each cell triplet, one cell received a mite from the DWVL colony, in another we introduced a mite from the DWVH colony, and the third was opened and resealed without introducing a mite, to serve as a control for manipulation. The locations of all treated cells were marked on a transparent plastic sheet. These combs were introduced into the experimental colonies. After 7 days we removed the comb from the colony and recorded the number of removed pupae. Additionally, we inspected 12 cells in each category (fewer if many pupae had been removed) for mite presence, mite reproduction (i.e. presence or absence of mite offspring in any developmental stage) and symptoms of overt DWV infection in the brood.

Chemical analysis of volatile substances emanating from brood

We collected the volatiles released from healthy non-parasitised brood, brood parasitised by mites from the DWVH colony and brood parasitised by mites from the DWVL colony in order to determine whether these brood types differ in their chemical profiles. Brood combs from the quarantine apiary were prepared in the same way as above and kept in an incubator (34.5±1°C; 70–80% relative humidity). Four days later, 10 cells in each category were opened; the pupae were removed and checked for any obvious damage, mite presence and mite reproduction. The 10 pupae from each category were then placed together in a flask (50 ml) kept in an incubator (34.5±1°C). Their headspace volatiles were collected by open-loop stripping. The flasks were connected *via* Teflon tubes to air pumps that generated a constant airflow of 100 ml min^{-1}. Incoming air was purified by passing it through activated charcoal. The volatiles were adsorbed on a Twister desorption unit (TDU) liner filled with 60 mg Tenax TA (Gerstel, Mühlheim a. d. Ruhr, Germany) for 1 h. Headspace samples were analysed by a coupled gas chromatography–mass spectrometry system (7890 GC-5975C MSD; Agilent Technologies, Waldbronn, Germany) equipped with a TDU and a programmable temperature vaporisation inlet (CIS-4; Gerstel). Samples were thermodesorbed in the TDU at 250°C for 12 min and cryofocused in the CIS-4 at –150°C. After thermodesorption, the CIS-4 was immediately heated to 300°C at 12°C s^{-1}. A fused silica column (DB-5MS, 30 m × 0.25 mm i.d. × 0.25 µm; J&W Scientific, Folsom, CA, USA) was used for separation with a constant helium flow of 1 ml min^{-1}. The oven was programmed from 40°C (4 min isotherm) to 300°C (10 min isotherm) at a rate of 10°C min^{-1}. Electron impact ionisation was 70 eV. Compounds were identified by comparing mass spectra and linear retention indices with those of authentic standards and literature data if available. Otherwise, structural assignment was based on comparison with Wiley mass spectral library data (Wiley Registry™, www.sisweb.com/software/ms/wiley.htm). For each compound, the peak area relative to the total peak area was calculated. Peaks with areas <0.1% in all samples were excluded from further analyses. The amounts of all remaining compounds were considerably higher than the lower limit of quantification and could thus be reliably quantified.

Statistics

To test for differences in removal rates, we compared the number of cleared brood cells between the three categories described above with a repeated measures ANOVA, with Scheffé test for *post hoc* comparisons. The proportions of reproducing mites and the proportions of brood with obvious damage were compared with a two-tailed paired *t*-test. Total volatile profiles of the differently

treated brood types were analysed using principal component analysis (PCA). To reduce the 'closure' effect, data were transformed according to the formula (Aitchison, 1986):

$$z_{ip} = \log[A_{ip} / g(A_p)] , \quad (1)$$

where A_{ip} is the area of peak i for sample p, $g(A_p)$ is the geometric mean of all peaks for sample p, and z_{ip} is the transformed area of peak i for sample p. As the logarithm is not defined for zero values, the constant 0.0001 was added to each relative peak area prior to the transformation. Differences in PCA factor scores between different treatments were evaluated by means of a repeated measures ANOVA with Scheffé test comparisons. Statistical analyses were carried out using PASW Statistics Version 18.0.0 (http://pasw.en.malavida.com/).

RESULTS

To obtain mites differing in their ability to transmit and induce overt DWV infections, we analysed several mite populations for both the presence and replication of DWV. We identified two honey bee colonies infested by mite populations with highly divergent characteristics, DWVH and DWVL (Fig. 1A). In all mites of the DWVH colony (72 of 72) and in 20.8% of the DWVL colony (15 of 72), DWV viral RNA could be detected. Among the DWV-positive mites, replication occurred in 100% of the former group (15 of 15 examined) and in 53.3% of the latter group (eight of the 15 individuals with DWV). This two-step analysis with a sample size of 72 thus allowed us to estimate the true percentage of mites with DWV replication in the two populations at a predetermined precision of 10% with 95% confidence as 100% and 11%, respectively. No viruses other than DWV were detected in the mites of the two populations (Fig. 1B). As expected from recent studies (Boecking and Genersch, 2008; Gisder et al., 2009; Möckel et al., 2011; Yue and Genersch, 2005), the highly divergent proportions of DWV replication were mirrored in the clinical symptoms observable in the two colonies. In the DWVL colony, 116 of 2660 worker bees (4.4%) that died within the first 2 weeks after the colony was confined in the greenhouse had deformed wings and other characteristic signs of overt DWV infection, whereas 966 of 5808 dead worker bees (16.6%) and 63 of 255 dead drones (24.7%) in the DWVH colony showed such symptoms. The proportion of worker bees with symptoms of an overt DWV infection was significantly higher in the second group (χ^2=288.6, d.f.=1, P<0.001). We also performed an incubator experiment (data not shown), which showed the detrimental effects of mite parasitisation similarly to hive experiments.

To answer the question of whether the bees respond to the mite in the brood cell or to the damage done to the brood, we performed hygienic behaviour assays using colonies with known good hygienic behaviour, mites from the DWVH and DWVL populations, and brood from colonies with a low initial level of mite infestation in the brood combs (mites were found in only 1.7% and 1.4% of the control cells in the hygienic behaviour assays and in the chemical analyses, respectively). We found dramatic differences in brood removal between the three cell types (repeated measures ANOVA, $F_{2,18}$=99.9, P<0.0001, Fig. 2). Brood parasitised by DWVH mites was removed at much higher rates (54.33±13.91%) than brood parasitised by DWVL mites (9.67±5.67%) and control brood (2.0±1.63%) (Scheffé test, P<0.001 in both comparisons). There was no significant difference in the removal rates between the last two categories (Scheffé test, P>0.1). To evaluate the efficiency of the bees in detecting and removing damaged pupae, we inspected the brood combs after the experiments. All brood in the examined, non-cleared cells looked healthy, indicating that the bees had been able to remove all conspicuously affected pupae.

The striking differences in removal rates prompted us to examine the volatile compounds emanating from the three brood types in search of olfactory cues eliciting the observed removal behaviour. In these experiments (performed as described in Materials and methods), the

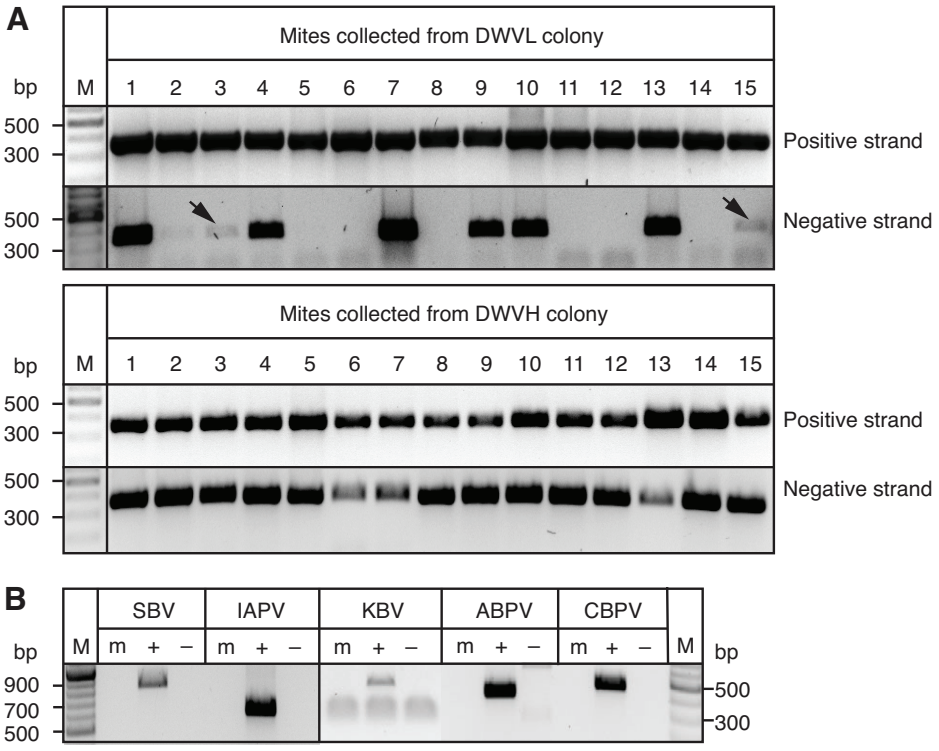

Fig. 1. RT-PCR analysis of honey bee viruses in mites. (A) Mites collected from the DWVL (low proportion of mites with deformed wing virus replication) and DWVH (high proportion of mites with deformed wing virus replication) colonies were individually analysed for the presence of positive- and negative-strand DWV RNA using tagged RT-PCR. For the DWVL colony, all 15 DWV-positive mites from the 72 collected mites are shown. Six mites revealed strong bands and two mites rather weak bands (nos 3 and 15, marked by arrows) for the DWV negative strand indicative of viral replication. For mites collected from the DWVH colony, representative results are shown. (B) Pooled samples of mites collected from the DWVL and DWVH colonies were analysed for the presence of sacbrood virus (SBV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), acute bee paralysis virus (ABPV) and chronic bee paralysis virus (CBPV) and tested negative for all analysed viruses. Representative results for the DWVL colony are shown. M, marker; +, positive control; –, negative control; m, mite.

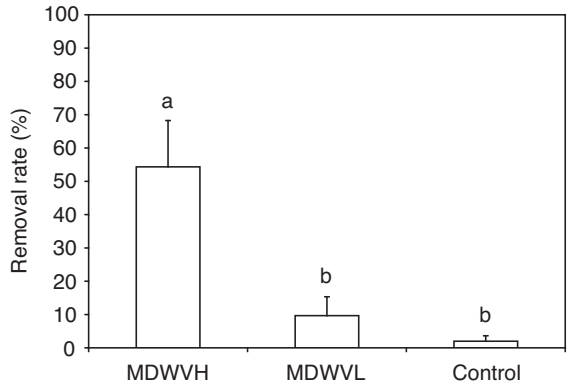

Fig. 2. Hygienic behaviour aimed at mite-parasitised brood in relation to viral replication in the mite in *A. m. carnica* colonies (*N*=10). Mean (+s.d.) cumulative proportions of removed brood over a period of 7 days for three different cell types: MDWVH, pupae parasitised by mites from DWVH colony; MDWVL, pupae parasitised by mites from DWVL colony; Control, pupae not parasitised by mites. Here, 100% is equivalent to 30 pupae. The differences in proportions of brood after 7 days between groups as detected by a Scheffé test are indicated by different letters.

health status of the pupae was evaluated visually at the point of collection, i.e. 4 days after experimental infestation or manipulation of the cells. All pupae parasitised by DWVL mites and the control pupae looked healthy. By contrast, on average 17.14% of the pupae parasitised by DWVH mites were already malformed or discoloured, with one pupa even showing obvious signs of decay despite the rather early phase of infestation. This difference in brood damage was significant (paired *t*-test, t_6=5.46, P=0.002). Altogether, 39 compounds were identified in the headspace of these pupae (supplementary material Table S2). The chemical profiles of all three brood types were dominated by *E*-β-ocimene (supplementary material Table S2). Among the samples of pupae parasitised by DWVH mites, there were several profiles with high proportions of compounds that are either rare (e.g. 2- and 3-methylbutanoic acid) or occur only in single samples (e.g. acetoin and 2,3-butanediol) (supplementary material Table S2).

Based on the results from the behavioural assays, we hypothesised that the volatile blend of brood parasitised by DWVH mites may be distinct from those of the two other groups combined. This hypothesis was supported by significant differences in factor scores for PC1 between brood parasitised by DWVH mites and both other groups (repeated measures ANOVA, $F_{2,10}$=5.70, P=0.022; Scheffé test, P<0.027 for both comparisons; Fig. 3A). There were no differences in PC1 factor scores between brood parasitised by DWVL mites and control brood (Scheffé test; P>0.05). The variables with the highest positive factor loadings on PC1 were mainly monoterpene hydrocarbons, while methyl-branched acids showed the highest negative loadings (Fig. 3B). Factor scores for PC2 were not significantly different between the three groups (repeated measures ANOVA; $F_{2,10}$=0.72; P>0.05). High positive or negative factor scores for PC2 indicate the presence of rare or unique compounds that had high positive or negative factor loadings on PC2.

As earlier studies reported an influence of mite reproduction on the removal rate of parasitised brood (e.g. Harbo and Harris, 2005), we tested whether the differences in removal rates and in chemical profiles were related to differences in reproduction between the two mite populations. In the cells not cleared in the hygienic behaviour experiments the reproduction rates of mites from the two populations did not differ significantly (Table 1). This does not rule out the possibility that the reproduction rate of the DWVH and DWVL mites in the cells that had been cleared by adult bees (and which we, therefore, could not evaluate) was different. However, this is rather unlikely because the reproduction rates of DWVL and DWVH mites used in the experiments performed for the chemical analyses also did not differ (Table 1).

DISCUSSION

Honey bees are attacked by numerous parasites and pathogens during any life stage, some of them inducing fatal infections in individual bees and even causing entire colonies to collapse. Defences against these pathogenic threats are mounted at the individual as well as at the group level, the latter referred to as ‘social immunity’. Hygienic behaviour, i.e. the recognition and removal of diseased or otherwise affected brood by adult bees, is an important part of the bees’ social immune response aimed at fending off brood pathogens and parasites. The hygienic behaviour has extensively been studied for American Foulbrood, caused by the bacterium *Paenibacillus larvae*, and for chalkbrood, caused by the fungus *Ascosphaera apis* (Gilliam et al., 1988; Gilliam et al., 1983; Park, 1937; Rothenbuhler, 1964; Spivak and Reuter, 2001; Woodrow and Holst, 1942). In these two cases it was quite obvious that the cues eliciting the hygienic

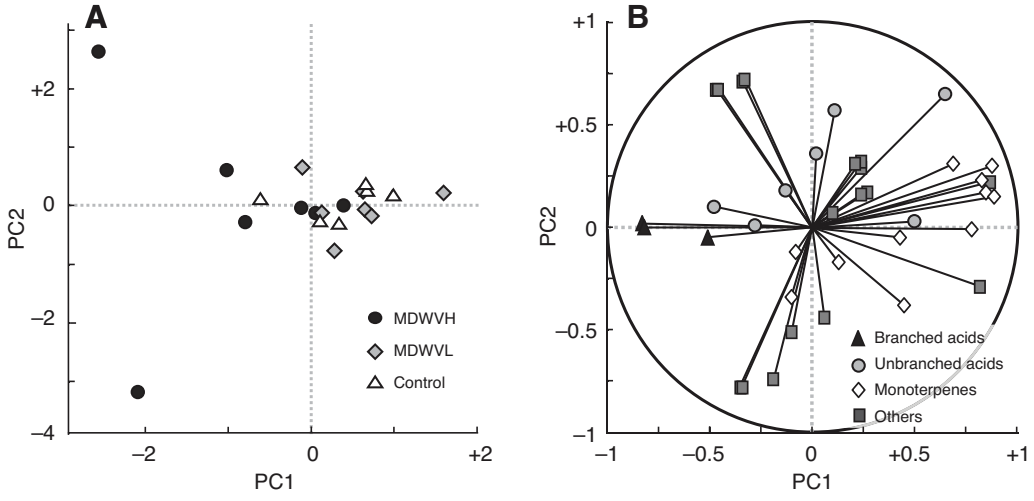

Fig. 3. Score plot (A) and loading plot (B) from a principal component analysis (PCA) based on relative amounts of volatiles collected from the headspace of pupae parasitised by mites from DWVH colonies (MDWVH; *N*=7), pupae parasitised by mites from DWVL colonies (MDWVL; *N*=7), and pupae not parasitised by mites (Control; *N*=6).

Table 1. Proportion of reproducing mites (means ± s.d., *N*) taken from brood used for the hygienic behaviour assays and chemical analyses

Brood type	Hygienic behaviour assays	Chemical analyses
MDWVH	59.3±13.2% (10)	57.1±12.8% (7)
MDWVL	61.7±16.7% (10)	52.9±15.8% (7)
Paired *t*-test*	t=0.49, P=0.64	t=1, P=0.36

MDWVH and MDWVL is the brood parasitised by mites from populations with high or low proportions of individuals with deformed wing virus (DWV) replication (see Results).

*Pairwise *t*-tests comparing the reproduction rates of parasitising mites from the two populations.

behaviour originate from the diseased larvae (Gramacho and Spivak, 2003; Masterman et al., 2001) and it was even possible to identify the volatile compounds of chalkbrood-infected larvae that induce hygienic behaviour (Swanson et al., 2009).

Despite decades of research on the hygienic behaviour towards mite-infested brood (Boecking and Drescher, 1991; Boecking and Drescher, 1992; Boecking et al., 1992; Moretto et al., 2006; Peng et al., 1987; Spivak, 1996), the situation has been and still is much more difficult with *V. destructor* because here two possible triggers are present in each infested brood cell: the ectoparasitic mite and the affected brood. It was suggested that reproduction of the parasitising mother mite in the capped cell influences the probability of detection and subsequent removal of infested brood (Harbo and Harris, 2005) but results of more recent studies do not support this notion (Harris et al., 2010; Ibrahim et al., 2007). Moreover, studies on the source and nature of the cues used by adult worker bees to detect cells with parasitised brood yielded contradictory results (Aumeier and Rosenkranz, 2001; Martin et al., 2001; Nazzi et al., 2004). In summary, it is still controversially discussed (1) whether the bees recognise and remove mite-infested brood in general or only such brood conspicuously affected by mite parasitism (e.g. from mite-vectored overt DWV infection), and hence (2) whether the cues eliciting this hygienic behaviour originate from the mites or the parasitised/infected brood and (3) whether these cues are olfactory or contact chemosensory.

Our hygienic assays performed with two mite populations exhibiting predictably different levels of virulence help to answer all three questions. Brood parasitised by ‘virulent’ mites from the DWVH colony (i.e. mites able to transmit a fatal DWV infection) was removed at a much higher rate than non-parasitised brood and brood parasitised by ‘less virulent’ mites from the DWVL colony (i.e. mites with little potential to transmit a fatal DWV infection). No significant differences between the removal rates of these last two categories were observed. These results clearly indicate that the trigger for hygienic behaviour in our assays was the damage done to the pupae rather than the presence of the mite *per se*. Importantly, mites from the two source populations did not differ in reproduction rate, and mite infestation prior to experimental manipulation was very low. Hence, we can safely conclude that these factors did not bias the experimental results. The results of the chemical analyses were in line with those of the behavioural experiments and show that the chemical profiles of pupae parasitised by ‘virulent’ mites from the DWVH colony differed from those of healthy pupae as well as from those of pupae parasitised by ‘less virulent’ mites from the DWVL colony, with the profiles of the last two groups being indistinguishable. This indicates that the cues eliciting the hygienic behaviour towards mite-infested pupae originated from the parasitised/infected brood and not from the mites. It also suggests that olfactory cues played a role in triggering the hygienic behaviour, although additional contact-chemosensory cues cannot be ruled out.

We recently showed that the virulence of the mites and their capacity to seriously damage the developing bee are influenced by whether the mites harbour replicating DWV, act as biological vectors of DWV and, therefore, are able to transmit fatal DWV infections (Gisder et al., 2009; Möckel et al., 2011; Yue and Genersch, 2005). Here, we clearly demonstrated that the virulence of the mites, as determined by their potential to transmit fatal DWV infections, influenced the removal probability of the parasitised bee pupae: worker bees engaged in hygienic behaviour recognised and removed predominantly mite-parasitised pupae with overt DWV infections. In our experiments the damage caused by mite-transmitted overt DWV infections was again confirmed by evaluating the brood kept in an incubator for later chemical analyses. Significantly, neither the parasite’s presence or reproduction nor the damage caused by parasitisation *per se* (without transmission of virulent DWV) seemed to produce olfactory cues sufficiently strong to elicit hygienic behaviour of the bees used in our study. Bee pupae parasitised by DWVH mites shared no specific odour profile, but were instead characterised by higher proportions of rare compounds or compounds occurring only in single samples. We therefore suggest that it was the fatal DWV infection which induced the bee pupae to release deviant volatile bouquets which were used by worker bees to discriminate between overtly infected (i.e. damaged by mite parasitism) and non-infected, covertly infected (i.e. asymptomatic despite mite parasitism) or non-infested brood. Thus, the discrimination is not based on specific compounds but rather on the quantitative deviation of the profile from that of non-infected, covertly infected or non-parasitised brood.

Based on our results we propose that bees express a damage-dependent hygienic behaviour towards mite-parasitised pupae, reacting to cues coming from the affected larvae like they do in the case of other brood diseases. The overall effect of damage-dependent hygienic behaviour on the health status of mite-infested colonies is unclear at present. The impact of *V. destructor* infestation on colony health is likely to depend on numerous factors such as the number of mites in the colony, the prevalence of virus in the mite population, the virulence of the virus strain, the susceptibility of the bees and the nature and extent of hygienic behaviour (Bowen-Walker et al., 1999; Sumpter and Martin, 2004; Genersch and Aubert, 2010). When parasitised by ‘benign’ mites, e.g. mites not acting as a biological vector of DWV or other viruses, pupae may still develop normally and become able members of the colony’s work force. When parasitised by a virulent mite, e.g. a mite transmitting a fatal DWV infection, the pupa has a high probability of being cannibalised by adult worker bees [so that the brood comb looks ‘spotty’ (Shimanuki et al., 1994)] or developing into a disabled adult that will die soon after emergence (Yang and Cox-Foster, 2007). Both phenomena are likely to result in less efficient brood rearing and subsequently in reduced colony growth. The negative effects of mite parasitisation at both the individual and the colony level therefore seem to result more from the damage caused by vectored viral infections than from nutrient deprivation (Bowen-Walker and Gunn, 1999) or immunosuppression (Yang and Cox-Foster, 2005).

For DWV, the following scenario seems likely: when adult worker bees cannibalise diseased brood, the uptake of DWV particles may cause overt infections (Möckel et al., 2011) suggesting a trade-off for the colony between (a) the removal of mites associated with damaged pupae and overt DWV infections and (b) the health status of adult bees involved in hygienic behaviour. Bees infected with

virulent variants of DWV through cannibalism of infected pupae might also contribute to the transmission of these variants. However, the main route of selection and spreading of virulent DWV variants remains that through DWV-infected mites parasitising pupae. This route is efficiently blocked by the selective hygienic behaviour aimed at pupae with mite-vectored overt DWV infection. The selective hygienic behaviour might not necessarily reduce the total mite population in the hive but could represent a negative selection pressure for more virulent DWV genotypes and *V. destructor* genotypes conducive to DWV replication. Furthermore, when bees show selective hygienic behaviour, pupae not damaged by mite parasitism are saved from being killed, and this could be an important mechanism for mite-infested colonies to avoid an unnecessary reduction in brood-rearing efficiency. Further studies are necessary to analyse the overall effect of damage-dependent hygienic behaviour on the population dynamics and evolution of *V. destructor* and DWV or other more virulent viruses like ABPV (de Miranda et al., 2010) as well as on colony health.

In summary, in our experiments we found that worker bees removed mite-parasitised brood predominantly when the parasitising mites carried replicating DWV and thus had a high probability of transmitting virulent DWV and, consequently, inducing overt infections (Bowen-Walker et al., 1999; Gisder et al., 2009; Möckel et al., 2011). The analyses of the volatile compounds emanating from brood suggest that the odour of brood parasitised by mites vectoring virulent DWV is clearly distinct, while odour of brood parasitised by mites not vectoring virulent DWV might be indistinguishable from odour of non-parasitised brood. Therefore, we propose that worker bees show selective, damage-dependent hygienic behaviour towards mite-parasitised pupae. Instead of indiscriminately cleaning out all mite-infested pupae and thus weakening the colony the bees that are engaged in hygienic behaviour might follow the rule ‘as much as necessary and as little as possible’ and limit their removal behaviour to pupae suffering from mite-associated damage severe enough to produce deviant olfactory cues.

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Supplementary material Table 1

Primers used for strand specific RT-PCR and for virus detection with RT-PCR

Primer	Primer Sequence (5‘- 3‘)	Length of amplicon	Annealing temp.	Reference
DWV F1	CCTGCTAATCAACAAGGACCTGG	354 bp	54.3 °C	Genersch, 2005
DWV B1	CAGAACCAATGTCTAACGCTAACC			
tag-F15	agcctgcgcaccgtggTCCATCAGGTTCTCCAATAACGG	450 bp	54.5 °C	Yue and Genersch, 2005
tag-B23	agcctgcgcaccgtggCCACCCAAATGCTAACTCTAACGC			
Tag	agcctgcgcaccgtgg			
SBV F1	GTGGCAGTGTCAGATAATCC	816 bp	52.0 °C	Yue et al., 2006
SBV B1	GTCAGAGAATGCGTAGTTCC			
ABPV F1	CATATTGGCGAGCCACTATG	398 bp	49.5 °C	Bakonyi et al., 2002
ABPV B1	CCACTTCCACACAACTATCG			
CBPV F1	AGTTGTCATGGTTAACAGGATACGAG	455 bp	55.0 °C	Ribiere et al., 2002
CBPV B1	TCTAATCTTAGCACGAAAGCCGAG			
IAPV F1	GAGCGTCGATCCCCCGTATGG	524 bp	55.0 °C	Maori et al., 2007
IAPV B1	TCCATTACCACTGCTCCGACAC			
KBV F1	GATGAACGTCGACCTATTGA	414 bp	50.5 °C	Stoltz et al., 1995
KBV B1	TGTGGGTTGGCTATGAGTCA			

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Supplementary material Table 2

Mean (± SD) proportion (% total peak area) of volatile compounds in collected from the headspace of pupae parasitised by mites from DWVH colonies (MDWVH; $N = 7$), pupae parasitised by mites from DWVL colonies (MDWVL; $N = 7$), and pupae not parasitised by mites (Control; $N = 6$). The numbers in brackets indicate the number of samples in which the respective compound was identified.

No	Compound	MDWVH N=7	MDWVL N=7	Control N=6
1	1-Methoxy-3-methylbutanol	1.8 ± 4.7 [1]	-	-
2	Acetoin	5.1 ± 13.4 [1]	-	-
3	Propanoic acid	3.3 ± 2.5 [7]	1.5 ± 1.6 [6]	0.8 ± 0.8 [4]
4	3-Methylbut-3-enol	tr [1]	-	-
5	3-Methylbutanol	3.3 ± 8.6 [1]	-	-
6	2-Methylpropanoic acid	0.7 ± 1.0 [4]	tr [1]	-
7	Butanoic acid	1.9 ± 1.2 [7]	0.3 ± 0.4 [3]	0.5 ± 0.6 [3]
8	2,3-Butanediol[1]	6.0 ± 15.7 [1]	-	-
9	2,3-Butanediol[1]	2.0 ± 5.2 [1]	-	-
10	3-Methylbutanoic acid	4.6 ± 7.6 [4]	-	0.8 ± 1.9 [1]
11	2-Methylbutanoic acid	1.6 ± 2.9 [4]	-	0.1 ± 0.3 [1]
12	Pentanoic acid	0.2 ± 0.4 [2]	-	0.1 ± 0.3 [1]
13	Dimethyl sulfone	tr [1]	-	-
14	Isoamyl acetate	0.1 ± 0.2 [1]	-	-
15	α-Pinene	1.7 ± 1.4 [7]	3.5 ± 2.8 [7]	2.2 ± 1.7 [6]
16	Benzaldehyde	1.2 ± 1.2 [7]	1.7 ± 0.8 [7]	1.6 ± 0.6 [6]
17	Hexanoic acid	3.0 ± 1.9 [6]	0.7 ± 0.8 [4]	4.2 ± 2.9 [6]
18	β-Pinene	0.5 ± 0.5 [5]	0.8 ± 0.8 [5]	0.4 ± 0.6 [3]
19	6-Methyl-5-hepten-2-one	1.1 ± 1.4 [5]	1.9 ± 1.3 [7]	1.5 ± 0.7 [6]
20	Myrcene	0.2 ± 0.4 [3]	0.2 ± 0.3 [4]	0.4 ± 0.3 [5]
21	3-Carene	5.6 ± 3.2 [7]	7.0 ± 2.0 [7]	7.0 ± 2.2 [6]
22	*p*-Cymene	1.5 ± 1.3 [7]	1.7 ± 0.3 [7]	1.8 ± 0.4 [6]
23	Limonene	0.6 ± 0.5 [7]	0.9 ± 0.4 [7]	0.8 ± 0.3 [6]
24	*Z*-β-Ocimene	0.6 ± 0.4 [7]	0.5 ± 0.2 [7]	0.6 ± 0.2 [6]
25	*E*-β-Ocimene	40.6 ± 21.8 [7]	62.6 ± 8.6 [7]	61.8 ± 4.8 [6]
26	Heptanoic acid	0.2 ± 0.4 [1]	0.1 ± 0.3 [1]	-
27	γ-Terpinene	1.1 ± 0.9 [7]	1.0 ± 0.6 [6]	1.2 ± 0.6 [6]
28	Ethyl heptanoate	-	1.9 ± 5.1 [1]	0.6 ± 1.5 [1]

29	*p*-Cymenene	0.7 ± 0.5 [7]	1.0 ± 0.8 [5]	0.9 ± 0.5 [5]
30	Methyl benzoate	1.8 ± 2.8 [4]	1.3 ± 1.7 [3]	1.7 ± 1.9 [3]
31	Menthatriene ?	0.5 ± 0.7 [4]	0.4 ± 0.6 [3]	0.5 ± 0.9 [2]
32	Epoxyocimene	1.2 ± 0.8 [6]	3.5 ± 2.4 [6]	2.7 ± 0.7 [6]
33	Monoterpene oxide	0.1 ± 0.4 [2]	0.3 ± 0.7 [1]	-
34	Octanoic acid	3.8 ± 2.6 [7]	3.1 ± 1.0 [7]	3.7 ± 2.6 [6]
35	Hexyl butanoate	-	0.3 ± 0.8 [1]	-
36	Ethyl octanoate	-	0.7 ± 1.9 [1]	-
37	Formylpiperidine	0.7 ± 1.9 [1]	-	1.9 ± 4.7 [1]
38	Methyl nonanoate	0.5 ± 1.3 [1]	-	-
39	Nonanoic acid	2.3 ± 3.3 [5]	2.9 ± 3.0 [6]	2.1 ± 1.4 [6]

tr = traces (mean proportion <0.1%, but that compound was present in at least one sample with ≥ 0.1%)

[1] These are two isomeric forms that were not characterized further.