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Diana Tentcheva, Laurent Gauthier, Leila Bagny, Julie Fievet, Benjamin Dainat, et al.. Comparative analysis of deformed wing virus (DWV) RNA in *Apis mellifera* and *Varroa destructor*. *Apidologie*, 2006, 37 (1), pp.41-50. 10.1051/apido:2005057 . hal-02660547

HAL Id: hal-02660547

<https://hal.inrae.fr/hal-02660547>

Submitted on 30 May 2020

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Comparative analysis of deformed wing virus (DWV) RNA in *Apis mellifera* and *Varroa destructor*¹

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Received 7 March 2005 – revised 16 June 2005 – accepted 17 June 2005

Abstract – A two step quantitative RT-PCR assay was validated to monitor the deformed wing virus (DWV) RNA loads in *Apis mellifera* L. and *Varroa destructor*. A pair of primers hybridising in a conserved domain of the putative DWV RNA polymerase gene region was designed. These primers amplified a 69-nucleotide fragment which was quantified using the SYBR-green chemistry. The experimental validation of the method showed that the RNA extraction and cDNA synthesis steps were responsible for the greatest variability in the results while assays repeated on different PCR plates were reproducible. Quantitative RT-PCR analysis on drone bee prepupae showed that DWV RNA loads were higher in cells parasitized by several mother mites. In workers, DWV prevalence was directly correlated to mite infestation and DWV was detected in all the bee developmental stages except in eggs. Very important DWV RNA loads could be recorded even in absence of clinical sign; however bees emerging with deformed wings were predominantly infected by DWV. In mites collected on emerging bees, the DWV RNA yields varied from 10⁴ to 10⁶ copies per mite but might exceed 10⁸ copies in some cases.

deformed wing virus (DWV) / bee virus / *Varroa destructor* / quantitative PCR

1. INTRODUCTION

A large number of viruses have been described from the domestic bee *Apis mellifera* L. (Bailey and Ball, 1991; Anderson, 1995). The most commonly observed and best known honey bee viruses are 30 nm isometric particles containing a single-stranded positive RNA belonging to Dicistroviridae or Iflaviridae families (Mayo, 2002). Among them, five have been already characterized on a molecular level: acute bee paralysis virus (ABPV), black queen cell virus (BQCV), sacbrood virus (SBV), Kashmir bee virus (KBV) and deformed wing virus (DWV) (Ghosh et al., 1999; Govan et al., 2000; Leat et al., 2000; de Miranda et al., 2004; Fujiyuki et al., 2004). Methods for molecular diagnosis

of these viruses using the polymerase chain reaction technique have already been published (Stoltz et al., 1995; Benjeddou et al., 2001; Grabensteiner et al., 2001; Bakonyi et al., 2002; Ongus et al., 2004; Tentcheva et al., 2004a; Chen et al., 2005). According to epidemiological data, the distribution of these viruses in honey bee colonies appears to be worldwide (Allen and Ball, 1996), a situation resulting most likely from intensive exchanges of honey bee stocks throughout the world. Most of these viruses are prevalent in colonies (Ball and Allen, 1988; Tentcheva et al., 2004b) and are believed to cause persistent, usually unapparent multiple infections in honey bee (Dall, 1985; Anderson and Gibbs, 1988; Hung et al., 1996a), and their association with colony

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¹ Manuscript editor: Klaus Hartfelder

mortalities observed in fields is still controversial (Hung et al., 1996b, Tentcheva et al., 2004b). The close association of viruses with the ectoparasitic mite *Varroa destructor* Anderson and Trueman (2000) is one of the explanations put forward for bee colony breakdown (Ball, 1989). The mite was first detected in Western Europe in the seventies but is now widespread all over the world. Several bee viruses have been identified in the mite, namely KBV, ABPV, SBV and DWV (Allen et al., 1986; Bakonyi et al., 2002; Bowen-Walker et al., 1999; Ongus et al., 2004; Tentcheva et al., 2004 a, b). The mite can act as a vector by directly injecting virus particles in the insect haemolymph or can also trigger replication of viruses already present in bees by a simple mechanical effect of cuticle piercing or by injection of external proteins in the insect haemolymph (Colin et al., 2002). This latter hypothesis is supported by several works reporting the reactivation of unapparent viral infections in insects following experimental inoculations (Dall, 1985; Anderson and Gibbs, 1988).

Deformed wing virus is believed to be associated with signs of wing deformities occurring during the imaginal molt of bees, and also with collapse of bee colonies, particularly in colonies severely infested by *V. destructor* (Bailey and Ball, 1991; Bowen-Walker et al., 1999; Nordström et al., 1999). A recent survey in French apiaries has shown that DWV infections are common both in asymptomatic bees and in mites (Tentcheva et al., 2004b), suggesting a low pathogenicity of DWV for bee colonies. In an attempt to elucidate the complex relationship between DWV, honey bee colonies, and the mite, we have developed a simple and reliable method to quantify this virus using quantitative RT-PCR (for a review on this technique, see Mackay et al., 2002).

2. MATERIALS AND METHODS

2.1. Sample preparation and cDNA synthesis

Samples were collected from a bee colony infested by *V. destructor* mites and were immediately frozen at -20°C before processing. Adult bees were individually crushed in a mortar under liquid nitrogen and were resuspended in 200 μL of TN

buffer (10 mM Tris, 400 mM NaCl, pH 7.5). Larvae, pupae and mites were removed from their cell with a toothpick and were individually homogenized in TN buffer (200 μL for worker larvae and pupae, 500 μL for drone prepupae, 100 μL for mites) in a 1.5 mL microtube using a piston pellet® (Eppendorf). Eggs were collected, pooled and washed once with PBS supplemented with 0.5% Tween 20 and three times with water before homogenisation in RNA extraction buffer.

Total RNA was extracted following the RNA-II® kit (Macherey-Nagel) protocol from 50 μL of the homogenate. An exogenous RNA reference consisting of 5×10^7 copies of Tobacco Mosaic Virus RNA (TMV, strain INRA – personal gift of Dr B. Alliot, ENSAM Montpellier) particles was included during the preparation. The TMV RNA added to each sample allows the efficiency of RNA extraction and cDNA synthesis steps to be monitored and reports the influence of potent inhibitors in quantitative PCR assays.

About 10 μg of total RNA were purified from each sample and 2 μg were used for reverse transcription. The reaction was processed at 25°C for 10 min and at 50°C for one hour using the ThermoScript® RT-PCR kit (Invitrogen) and random hexamers. A master mix was used for cDNA synthesis. The cDNA was diluted 10 fold in water and stored at -20°C or processed for PCR assays.

2.2. Primers design for viral RNA quantification

The following DWV specific primers were designed with the Primer Express® software (Applied Biosystems) from DWV genomic sequence AY224602:

Q-DWV-fwd (5'-GGATGTTATCTCTGCGT-GGAA-3')

Q-DWV-rev (5'-CCTCATTAAGTGTGTCGTT-GATAATTG-3').

For quantification of the exogenous RNA reference (TMV), the following primers were used:

Q-TMV-fwd (5'-CATGCGAACAT-CAGCCAATG-3').

Q-TMV-rev (5'-TGTAGCGCAATGGCGT-ACAC-3').

2.3. Quantitative PCR assays

Quantitative PCR amplifications were done on an ABI PRISM® 7000 apparatus (Applied Biosystems) using a standard protocol (50°C 2 min, 95°C 10 min and 40 cycles: 95°C 15 s, 60°C 1 min). Each quantitative PCR analysis was performed in triplicates, in a 96 well PCR plate (Thermo-fast®,

Abgene) sealed with an optical adhesive cover (Applied Biosystems). Non template controls (water) were included in triplicates in each assay. The Sybr Green I 2X Reaction System[®] kit (Eurogentec) was used, in 25 μ L final volume. The fluorochrome ROX (6 Carboxy-X-Rhodamine, Interchim) was included in the reaction mix at 0.5 μ M final concentration as a passive reference used to normalise the signal between each 96 wells of the PCR plate. For each analysis, 5 μ L of the diluted cDNA was used. After several trials, the primer concentrations were fixed at 0.2 μ M. The specificity of the amplicons synthesised during the PCR run was ascertained by performing a dissociation curve protocol from 60 °C to 95 °C.

For each sample, both DWV and TMV quantitative analysis were performed in separate wells. The DWV RNA values recorded from each sample were invalidated if their corresponding TMV RNA control values were out of the 95% interval of confidence of the TMV RNA population recorded on the PCR plate.

According to the different steps of the method, the following dilution factors were used: 800 for worker (adult, larva or pupa), 2800 for drone pupa, 400 for mite.

2.4. Calibration curve

PCR amplicons containing the DWV 69 nucleotide target sequence were used to set up a calibration curve. These amplicons were obtained after PCR amplification of a 395 nucleotide DNA fragment using primers DWV-T3-fwd (TTTGCAAGATGCTGTATGTGG) and DWV-T3-rev (GTCGTGCAGCTCGATAGGAT) as previously described (Tentcheva et al., 2004a). To establish a calibration curve for the quantification of the TMV RNA reference, a 601 bp TMV DNA fragment was amplified with the primers TMV-fwd (AAAAACAGTCCCAACTTCC) and TMV-rev (AAGGAGGAT-TCTCTCGCTGT) as described (Tentcheva et al., 2004a). After 35 amplification cycles, the DNA fragments were gel purified and aliquots were used for measuring their DNA concentration using a fluorescent assay (Picogreen[®] Molecular Probes). For each quantitative PCR assay, ten fold serial dilutions in water were made and each dilution was processed in triplicates on the same 96 well PCR plate where the samples were deposited.

2.5. Statistical analysis

Statistical analysis were performed using the Sigmatat[™] 2.03 software (SPSS Sciences). The two way analysis of variance was performed to check if the measurement of the number of viral copies is affected, (i) by the plate itself and by the conditions

of the amplification process of DWV and TMV cDNA, (ii) by the total RNA extraction and cDNA synthesis process (DWV and TMV). When one factor affected the measure, a one way analysis of variance was performed, followed by a Tukey test at $P = 0.01$.

3. RESULTS

3.1. DWV primers design

A pair of primers specific for the DWV RNA dependent RNA polymerase domain was designed from the DWV GenBank sequence AY224602 (Tentcheva et al., 2004a) and from the partial sequencing of 6 distinct DWV isolates. Among available DWV sequences registered in GenBank (AY292384 (de Miranda et al., 2003, Unpublished), NC_004830 (Lanzi and Rossi, 2003, unpublished data) and Kakugo virus sequence NC_005876 (Fujiyuki et al., 2004), only one mismatch was found with the latter, in the center of the DWV forward primer. Conversely, 10 mismatches were found when aligning the primers with the *Varroa destructor* virus 1 polyprotein gene sequence (AY251269, Ongus et al., 2004). This sequence is thus unexpected to be amplified using the DWV primers described in this paper.

The DWV primers amplified a fragment of 69 nt. The melting temperature of this 69 nt DNA amplicon was 78.5 °C. The DWV negative samples (water) showed a peak around 75 °C which corresponds to the synthesis of primer dimers during the PCR run. By comparison, the melting temperature of the TMV reference amplicon averages 81.5 °C for a fragment of 55 nucleotides long.

3.2. Limit of quantification

Standard curves were obtained after 40 cycles of PCR run using 6 serial tenfold dilutions of the 395 nt DWV amplicon or of the 600 bp TMV amplicon. The linear range was obtained from 3×10^7 to 300 single strand DNA copies of the amplicon, and the slope averages the value of -3.32 which corresponds to the optimal PCR efficiency (DWV: $y = -3.323x + 41.827$; TMV: $y = -3.334x + 32.99$). Below 300 equivalent DWV RNA copies, positive samples were considered detected but not

quantifiable. Thus, considering the extraction of a single mite in 100 μ L buffer, the quantification threshold of our assay corresponds to 120 000 copies of DWV RNA per mite (300×400 dilution factor). For a worker bee homogenised in 200 μ L buffer, the quantification threshold is 240 000 DWV RNA copies per bee (300×800 dilution factor). For a drone prepupa homogenized in 500 μ L of TN buffer, the quantification threshold is 840 000 DWV RNA copies per prepupa (300×2800 dilution factor).

3.3. Variability in the quantitative PCR method

Two assays were performed to validate the quantitative PCR method, (i) to check the variability observed between the different wells on the same plate and between different plates, (ii) to check the variability originating from the preparation of the cDNA (total RNA extraction and cDNA synthesis steps).

First, the analysis of the same cDNA was repeated 21 times on six different 96 well plates, for the quantification of DWV and TMV. The assays for DWV and TMV were done independently but from the same cDNA batch. The values measured in each plate are presented in Figure 1. The statistical analysis, performed by two way analysis of variance shows that the variability between plates was not significant ($df = 5$, $F = 0.0325$, $P = 0.99$), nor was the interaction between plates and the two different viruses ($df = 5$, $F = 0.0368$, $P = 0.99$). The variability of the values recorded for DWV and TMV is correlated with the initial template concentration ($df = 1$, $F = 731.58$, $P < 0.001$). The mean values for DWV and TMV cDNA templates were 32295 and 2452, respectively. The estimated mean of the true population is included in the interval of the sample mean $\pm 4.63\%$ and $\pm 6.34\%$ for DWV and TMV, respectively (95% confidence level).

Second, 10 different RNA extractions were prepared from a same homogenate of adult bees and corresponding cDNAs were analysed in triplicates on the same 96 well plate. The distribution of the values is presented in Figure 2. Statistical analysis showed that the total RNA extraction and cDNA synthesis steps affect significantly the measure of the number of copies ($df = 9$, $F = 18.53$, $P < 0.001$). The factor “total RNA extraction and cDNA synthesis” was ana-

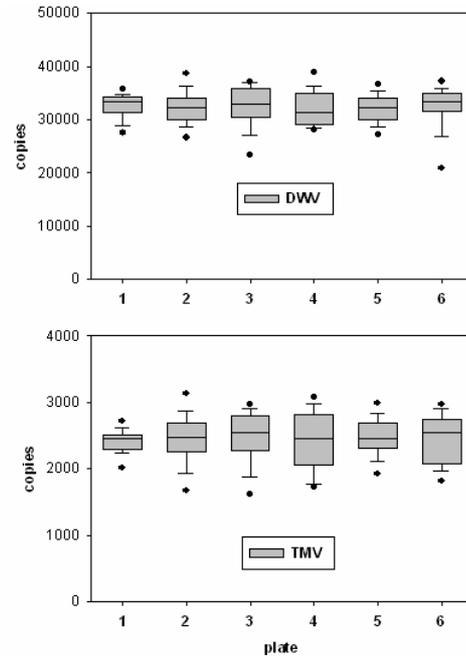


Figure 1. Results showing the repetition of 21 quantitative PCR analysis from a same cDNA on six different 96 well PCR plates, for DWV and TMV detection.

lysed by pairwise comparisons (Tukey test at 0.01) for both viruses separately, the equality of the variances being assumed. For DWV and TMV, the group including the extracts 6 and 8 is significantly different from another group gathering the remaining extracts. For DWV, the mean of the groups including the extracts 6 and 8 (15 650 000) is roughly 60% higher than in the other group (9 750 000). For TMV, the mean number of copies for the extracts 6 and 8 (2851) is roughly two times higher than that of the other group (1367). In this assay, based on triplicates, the estimated mean of the true population is included in the interval of the sample mean $\pm 5.10\%$ and $\pm 9.18\%$ for DWV and TMV, respectively (confidence level of 95%).

3.4. Bees and mites analysis

First, several drone prepupae (5th instar larvae) infested by *V. destructor* mites were collected individually for quantitative PCR analysis. The number of mother mites were counted in each cell and pooled for DWV RNA analysis.

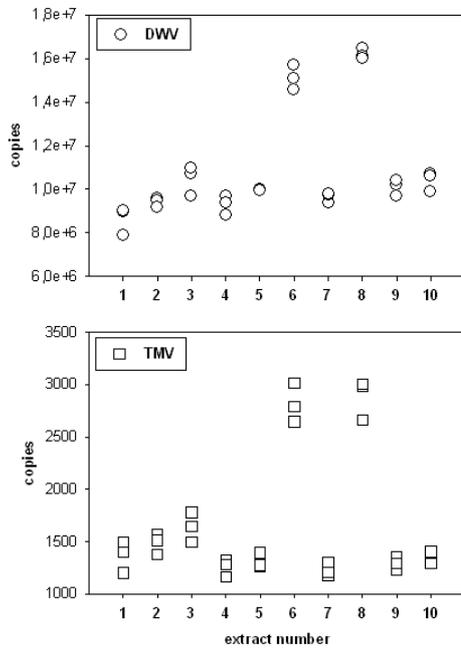


Figure 2. Results showing the distribution of 10 different RNA extractions and cDNA synthesis from a same bee homogenate. Samples were analysed in triplicates on the same 96 well plate, for both DWV and TMV quantification.

Deformed wing virus RNA was detected in 80% of the prepupae samples and in all the mites samples. The DWV RNA loads recorded in prepupae and mite samples were slightly positively correlated with the number of mites per cell (linear regression $R^2 = 0.513$) (Fig. 3).

Second, DWV RNA loads were recorded individually from workers and mites collected on the same frame at various developmental

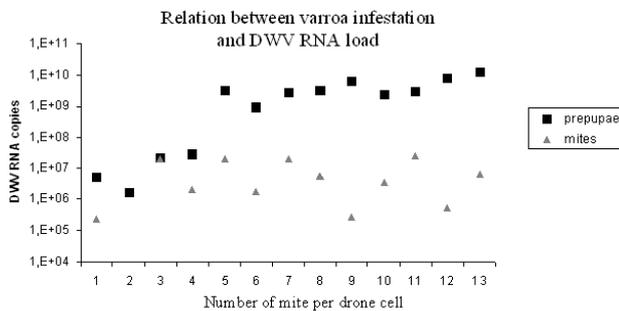


Figure 3. DWV RNA loads recorded from drone prepupae collected from their cell and from the mother mites found within. Samples were analysed in triplicates and results are expressed as mean quantity of DWV RNA per drone prepupa or mite.

stages. As shown in Figure 4A, DWV was detected in the different worker samples except in eggs. In sealed brood, DWV was more prevalent when pupae were parasitized by mites. Adult bees emerging from their cell were all DWV negative in absence of mites. Conversely, parasitized emerging workers were 60% positive for DWV. The bees emerging with deformed wings were shown infected by DWV (100% with and 80% without mite in the cell, respectively). The mite samples were positive for DWV except when collected from a DWV negative bee.

The viral load was examined from the positive samples. As shown in Figure 4B, the DWV infected larvae contained significantly less DWV RNA than pupae and emerging bees. No significant differences were observed between the different mite samples.

4. DISCUSSION

Here we present a reliable two step quantitative RT-PCR method for detecting and quantifying viral RNA in bee and mite samples. A major advantage of the protocol relies on the 96 well format which allows a large number of samples to be screened in a row, since each sample and standards should be analysed at least as duplicates. Our use of SYBR green chemistry was proved cheaper than the Taqman[®] probe system since the record of a dissociation curve at the end of the run easily permitted us to detect non specific amplicons. However, a higher specificity will probably be reached using the Taqman[®] probe system since a single nucleotide mismatch in the probe sequence may block the fluorescence emission.

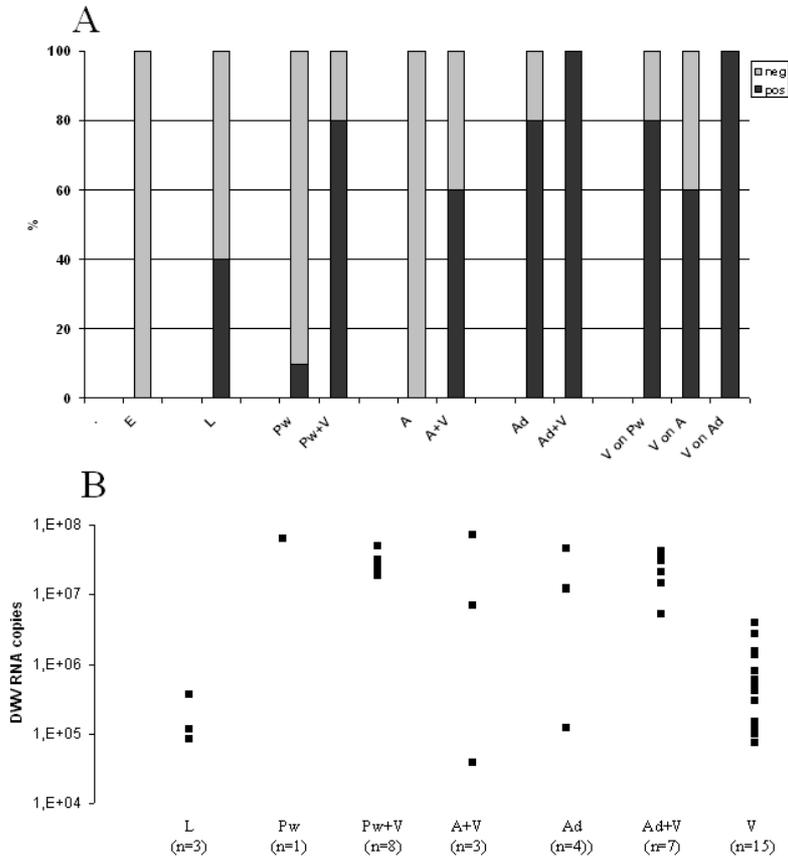


Figure 4. Analysis of groups of ten individual worker bees collected on the same frame at different developmental stages. A, percentages of DWV positive samples in each group. B, quantitative PCR analysis performed on DWV positive individuals. Results are expressed in DWV RNA copies per bee or mite. E: eggs (pool of 40 eggs); L: larva (L3 stage). Pw: pupa (white eye stage); A: adult bee emerging; Ad: adult bee emerging with deformed wings; V: mite; V on Pw: mite collected on pupa; V on A: mite collected on emerging adult bee; V on Ad: mite collected on adult bee emerging with deformed wings.

This last technique might be useful for discriminating viral subspecies, for example. The utilisation of tobacco mosaic virus as an external reference proved valuable and allows PCR quantification of bee viruses from samples such as dead bees collected at the hive entrance, which would not be possible with the use of a classical housekeeping gene (e.g. β -actin gene).

The assay was validated by analysing the variability attributable to each step of the PCR procedure. The results showed first that, whatever the plate and position of the sample on the plate, the estimated number of RNA copies (mean of six values) is not significantly different for both DWV and TMV cDNA templates

(Fig. 1). Second, the total RNA and cDNA synthesis step affects significantly the estimation of the number of copies (see Fig. 2), as two cDNA samples varied significantly from the others (extracts 6 and 8) for both DWV and for TMV. While expensive, a solution to circumvent that problem would be to perform two RNA extractions and two cDNA synthesis for the same sample.

With the protocol presented here, considering the dilution of the samples during RNA purification, cDNA preparation and PCR assays steps, the limits of quantification were found around 240 000, 840 000 and 120 000 copies of DWV RNA per worker, drone pupa and mite,

respectively. This threshold corresponds roughly to one picogram of DWV RNA per bee. However, such conversion probably underestimates the DWV RNA level in samples as the reverse transcription step operates below 100% efficiency. This threshold can be reduced, if needed, by improving the method, such as purifying only polyadenylated mRNA after immunoprecipitation of virions for example. However, the amounts of DWV RNA in samples was found large enough to use a simple total RNA preparation for diagnosis such as the protocol we describe in this paper.

The quantitative PCR technique was first applied to the quantification of DWV RNA load in drone prepupae extracted from their cell and in mother mites found within. The amounts of DWV RNA in drones were found to be dependent on the number of mother mites. The results, while based on few samples, tend to show that multiple infestations increase the probability of DWV infection and DWV replication yields in the drone prepupa. This finding is not in accordance with a previous work (Nordström, 2003) but can be explained by a different proportion of DWV infected mother mites in the colony, knowing that not all the mother mites were tested individually in this assay. The large amounts of DWV RNA recorded in mother mites collected before drone nymphosis suggests that these mites had acquired the virus before entering the cell.

In a second experiment, worker bees were analysed individually at different developmental stages. The results present some similarities with previous works (Ball and Allen, 1988; Nordström et al., 1999). First, a strong proportion of larvae were shown positive for DWV (40%). This suggests that DWV can be transmitted by nurse bees to the larvae. Likewise, DWV was detected in stored pollen collected in combs (not shown) suggesting that this virus might be secreted in saliva. Second, very few pupae were detected positive for DWV in absence of *V. destructor* suggesting that DWV infected larvae died and were removed by workers before capping. In sealed infested cells, the proportion of DWV infected pupae increased dramatically to 80%, suggesting that the mites transmit the virus or reactivate undetectable infectious DWV particles. The fact that DWV negative mites were always found in

our assay on DWV negative pupae or emerging bees tend to show that *V. destructor* acts as a vector of DWV. However, it is still difficult to determine if the mite acts as a passive or as an active vector because the replication of DWV in mites was poorly investigated. Two recent works reporting para-crystal arrangements of viral particles of 30 nm size in mite cells (Kleespies et al., 2000; Ongus et al., 2004) might argue for that possibility. Further experiments should be undertaken to validate these results, such as identifying DWV replicative forms in mite tissues using *in situ* techniques.

Bees emerging from non infested cells with deformed wings were mainly DWV positive and substantial viral loads were recorded from those samples except one. Likewise, the bees parasitized by *V. destructor* had very high DWV titres. However, some DWV positive bees emerging with normal wings had similar DWV RNA yields. An analysis done in parallel on a group of workers collected at the hive entrance showed that the bees displaying deformed wings had approximately 10 times more DWV RNA loads than asymptomatic ones (not shown). These results are in agreement with previous observations showing that bees displaying deformed wings had usually larger amounts of DWV (Chen et al., 2005), but conversely, high titres of DWV are not necessarily linked to wing deformity (Nordström et al., 1999).

The study of viral populations impact on bee colony health and their relationships with the ectoparasitic mite *V. destructor* is complicated by the fact that many different viruses infect bee colonies chronically, without producing reliable clinical signs. Among them, DWV is one of the most prevalent in bee colonies and seems very likely associated with mite infestation of apiaries. However, some data are still missing to understand the relationships between bees, DWV, and the mite, such as the viral loads that bees or mites can hold without displaying physiological damages, or the localization of the virus in bee and mite tissues. The quantitative PCR technique applications will undoubtedly help to understand host parasite relationships. In this way, this technique would be very helpful to follow the outcome of bee virus populations in bee colonies using modified (tagged) virus genomes derived from infectious DNA clone (Benjeddou et al., 2002).

ACKNOWLEDGEMENTS

Diana Tentcheva and Laurent Gauthier contributed equally to this work. We are very grateful to Dr B.V. Ball (Rothamsted Institute, UK) for her help in providing Rothamsted DWV isolate. This work was supported by the EEC and the French Ministère de l'Agriculture et de la Pêche (Règlement CE n° 1221/97, convention n° 2207).

Résumé – Quantification du virus des ailes déformées (DWV) chez *Apis mellifera* et *Varroa destructor*. L'étude des populations virales et de leur impact sur la santé des colonies d'abeilles (*Apis mellifera*) souffre du fait que différents virus infectent simultanément les colonies sans produire de symptômes clairement définis. Le virus des ailes déformées (DWV) est un des virus les plus fréquemment détecté chez *A. mellifera* et sa prévalence est d'autant plus forte que les colonies sont infestées par l'acarien *Varroa destructor*. Afin d'étudier les relations complexes qui existent entre le DWV, l'acarien et l'abeille, nous avons développé une méthode permettant de quantifier ce virus dans les échantillons. Pour cela, un couple d'amorces a été choisi à partir de l'alignement de séquences de plusieurs isolats de DWV. Ces amorces s'hybrident dans la partie codant pour l'enzyme de réplication du virus. La limite de quantification du DWV dans les échantillons a été déterminée respectivement à 120 000, 240 000, 840 000 copies d'ARN du DWV par acarien, ouvrière ou puppe. Les analyses étant réalisées sur des plaques PCR de 96 puits, les résultats de validation ont montré que seules les étapes de purification d'ARN et de synthèse d'ADN complémentaire produisent des variations significatives dans les mesures de quantification. La méthode a été tout d'abord appliquée à la quantification du DWV dans des échantillons de nymphes de mâles (stade pré-pupal) infestées par différentes charges de *V. destructor*. La fréquence du DWV dans les nymphes était de 80 %, contre 100 % dans les acariens. Les résultats ont montré une légère corrélation entre la charge virale mesurée dans les nymphes et le nombre d'acariens par cellule (Fig. 3). Ensuite une série d'analyses individuelles ont été réalisées à partir d'ouvrières prélevées à différents stades larvaires et d'acariens isolés dans les cellules infestées. Les résultats ont montré que le DWV était présent à tous les stades larvaires de l'abeille, excepté dans l'œuf et que le DWV était plus fréquemment détecté dans les nymphes isolées de couvain operculé parasité par *V. destructor* (Fig. 4A). Les imagos émergents de cellules indemnes d'acariens étaient négatifs pour le DWV tandis qu'à l'inverse 60 % des ouvrières parasitées étaient positives pour ce virus. D'autre part les abeilles nées avec des ailes atrophiées étaient infectées par le DWV (100 % des individus infestés par *V. destructor* et 80 % des individus non infestés). Les acariens étaient positifs pour le DWV, sauf ceux isolés de larves DWV négatives. Comme le montre la figure 4B,

les larves contenaient des quantités de DWV plus faibles que les nymphes ou les adultes émergents. Les charges virales relevées dans les acariens étaient statistiquement identiques.

Apis mellifera / *Varroa destructor* / virus des ailes déformées / DWV / PCR quantitative

Zusammenfassung – Vergleichende Analyse des Deformed Wing Virus (DWV) bei *Apis mellifera* und *Varroa destructor*. Die Erfassung viraler Populationen in Völkern der Honigbiene (*Apis mellifera*) und ihrer Effekte auf die Gesundheit der Völker stellt ein Problem dar, wenn es sich um einen simultanen Befall durch mehrere Viren handelt und wenn diese keine klaren klinischen Symptome hervorrufen. Das Deformed Wing Virus (DWV) wird häufig bei *Apis mellifera* gefunden, insbesondere dann wenn Völker von *Varroa destructor* befallen sind. Um die komplexen Beziehungen zwischen Virus, Milbe und der Biene erfassen zu können, haben wir eine Methode entwickelt, die es uns erlaubt den Virusbefall in Proben mittels Real Time RT-PCR zu quantifizieren. Hierzu wurde ein Primer-Paar aus Sequenzvergleichen verschiedener DWV-Isolate ausgewählt. Diese Primer hybridieren mit der für ein virales Replikationsenzym kodierenden Region. Die Detektionsgrenzen lagen bei 120 000 Kopien der viralen RNA bei Proben von Milben, bei 240 000 Kopien in Proben adulter Arbeiterinnen und bei 840 000 Kopien bei Puppen. Da die Methode in 96-well PCR-Platten durchgeführt wurde, validierten wir sie zunächst hinsichtlich ihrer Wiederholbarkeit zwischen Proben auf der gleichen und verschiedenen Platten. Anschliessend testeten wir die Variabilität aufgrund unterschiedlicher Präparationsverfahren. Hierbei zeigte sich, dass lediglich in den Schritten der RNA-Extraktion und der cDNA Synthese signifikante Variationen in den quantitativen Messungen zu verzeichnen waren. Die Methode wurde daraufhin für die Quantifizierung des DWV in Proben von Drohnenlarven (Vorpuppenstadium) eingesetzt, die einen unterschiedlichen Befallsgrad durch *V. destructor* aufwiesen. Das Virus wurde in 80 % der Brut- und in 100 % der Milbenproben detektiert. Die Ergebnisse zeigen eine leicht positive Korrelation zwischen der viralen Dosis und dem Milbenbefallsgrad pro Brutzelle (Abb. 3). Anschliessend wurde eine Serie von Analysen an Arbeiterinnenbrut in unterschiedlichen Entwicklungsstadien und an einzelnen aus Brutzellen entnommenen Milben erstellt. Die Ergebnisse zeigten, dass DWV in allen Brutstadien zu finden war, ausser in Eiern, und dass es am häufigsten in Brut aus gedeckelten und von *Varroa destructor* befallenen Zellen auftrat (Abb. 4A). Imagines, die aus Brutzellen ohne Milben schlüpften, erwiesen sich als virusnegativ, während 60 % der Arbeiterinnen, die aus milbenbefallenen Zellen schlüpften, viruspositiv waren. Im Vergleich hierzu konnten wir bei Bienen mit verformten Flügeln das DWV in 100 % der

Imagines aus milbenbefallenen Brutzellen und in 80 % der Bienen aus milbenfreien Brutzellen nachweisen. Die Milbenproben waren durchweg positiv für DWV, mit Ausnahme einzelner Proben, die aus Zellen mit DWV-negativen Larven stammen. Wie Abbildung 4B zeigt, waren Larven deutlich weniger virusbelastet als Puppen oder schlüpfende Bienen. Die virale Dosis bei Milben hingegeben wies keine statistisch signifikanten Schwankungen auf.

***Apis mellifera* / *Varroa destructor* / Deformed Wing Virus / DWV / quantitative PCR**

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