

Up-regulated pathways in response to Deformed Wing Virus infection in *Apis mellifera* (Hymenoptera: Apidae)

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Received 08 - VI - 2018 | Accepted 21 - II - 2019 | Published 28 - III - 2019

<https://doi.org/10.25085/rsea.780101>

Vías de señalización activadas en respuesta a la infección por el virus de las alas deformes en *Apis mellifera* (Hymenoptera: Apidae)

RESUMEN. La transmisión de virus por *Varroa destructor* Anderson & Trueman a las abejas ha sido identificada como una de las principales amenazas para las colonias de *Apis mellifera* L. También se ha sugerido que la interacción sinérgica entre parásitos y patógenos mediada por la inmunidad de *A. mellifera* puede conducir al colapso de las colonias de abejas melíferas. Analizamos la relación entre la carga viral del virus de las alas deformes (DWV) y los niveles de expresión génica de Toll Wheeler, cactus, domeless, TEPA, óxido nítrico sintasa, himenoptecina, vitellogenina, defensina, abaecina en muestras de abeja invernal de *A. mellifera* con y sin alas deformadas que se obtuvieron de colmenas en el campo con alta infestación de *V. destructor*. En las abejas con alas deformadas encontramos aumento en las cargas virales de DWV y regulación positiva de los genes de la vía de señalización Jak-STAT.

PALABRAS CLAVE. Abeja melífera. Virus. Vías del sistema inmune de insectos. Expresión de inmunogenes. Cargas virales.

ABSTRACT. Transmission of viruses by *Varroa destructor* Anderson & Trueman to bees has been identified as one of the main threats to *Apis mellifera* L. colonies. It has also been suggested that synergistic parasite-pathogen interaction mediated by *A. mellifera* immunity can drive the collapse of honey bee colonies. We analyzed the relationship between deformed wing virus (DWV) viral load and gene expression levels of Toll Wheeler, cactus, domeless, TEPA, nitric oxide synthase, hymenoptaecin, vitellogenin, defensin, abaecin in winter bee samples of *A. mellifera* with and without deformed wings. Samples were collected from hives in the field with high *V. destructor* infestation. In bees with deformed wings we found an increase in DWV titre and upregulation of genes in the Jak-STAT signalling pathway.

KEYWORDS. Honey bees. Virus. Insect immune system pathways. Immunogene expression. Viral load.

INTRODUCTION

Alarming increases in depopulation and the loss of bee colonies have been reported over the last few years globally (Le Conte et al., 2010; Potts et al., 2010). These losses are of great ecological, agricultural and economic relevance as honey bees pollinate natural and agricultural ecosystems worldwide. This critical situation has major impacts on the fragile balance between bees and plants, forcing many scientists to redirect their research areas to tackle the possible factors which cause this problem. Most researchers agree that there is no single explanation for the large losses of colonies observed, but they are the result of a synergistic interaction between different stressors (Hendriksma et al., 2011). Argentina is no exception, with beekeepers across the country reporting cases of depopulation of hives and colony losses. Unfortunately, there is no nationwide data for this available (Maggi et al., 2013).

Transmission of viruses by the mite *Varroa destructor* Anderson & Trueman is considered one of the main threats to *Apis mellifera* colonies (Martin et al., 2012). Viruses replicate within mites (without causing the death of the parasite) and consequently infect the bees with high viral loads, as is the case for the deformed wing virus (DWV) (Gisder et al., 2009; Martin et al., 2012). So far, 24 different viruses have been isolated from the honey bee and it has been demonstrated that some can be vectored by *V. destructor* (Genersch & Aubert, 2010; Runckel et al., 2011; de Miranda et al., 2013; Dalmon et al., 2017). These include Kashmir bee virus, Sacbrood virus (SBV), acute bee paralysis virus (ABPV), Israeli acute paralysis virus (IAPV) and DWV (Genersch & Aubert, 2010). ABPV and DWV have been implicated in colony losses in Germany and in the colony collapse disorder (CCD) observed in the USA (Cox-Foster et al., 2007; vanEngelsdorp et al., 2007, 2008, 2009; Genersch, 2010).

In Argentina, the information about bee viruses is scarce and fragmented. However, Reynaldi et al. (2010) reported the molecular identification of chronic bee paralysis virus (CBPV), ABPV and SBV in Buenos Aires, the main honey-producing province of Argentina. In the study, a lower rate of infection and some cases of co-infection with more than one virus were found when compared to other countries of South America (Antúnez et al., 2006). In 2011, the same authors reported the presence of IAPV by RT-PCR in samples taken from several provinces. These data indicated a high frequency of IAPV in asymptomatic hives in Argentina (Reynaldi et al., 2011).

During 2013 our research group collected and

updated the distribution of SBV, DWV, IAPV and ABPV in colonies of the South-eastern region of Buenos Aires province. Viruses were detected in 33.3% of all samples tested. SBV was identified in pupae, IAPV and DWV was identified in adult bees and in *V. destructor* mites. ABPV was not detected in any of the samples analyzed (Brasesco et al., 2013). High loads of DWV in bees with deformed wings and lower viral loads in bees with normal wing appearance were observed, as previously reported (Yang & Cox-Foster, 2005; Martin et al., 2012). In this manner, the information was expanded to provide new molecular records about the presence of such bee viruses in the country (Brasesco et al., 2013).

Martin and colleagues studied viral load, prevalence and genetic diversity of DWV in four Hawaiian Islands, finding differences in viral load and also variations in DWV strain diversity (Martin et al., 2012). Ryabov et al. (2014) also described the existence of variants of DWV associated with differing virulence.

Some reports have described changes in the expression levels of genes of the immune system of *A. mellifera* in response to pathogen infection, like ABPV and *Paenibacillus larvae* (Siede et al., 2012; Boncristiani et al., 2013). It has also been suggested that synergistic parasite-pathogen interaction mediated by *A. mellifera* immunity can drive the collapse of honey bee colonies (Nazzi et al., 2012). There is considerable interest in understanding the mechanisms mediating non-self-recognition, activation of signal transduction pathways that controls the expression of immune genes, and the identity and mode of action of the biocidal molecules used by insects (Nappi et al., 2000).

Vitellogenin (Vg) is a yolk precursor as well as a pathogen pattern recognition receptor (Zhang et al., 2011). It is a nutritious lipoprotein synthesized by the invertebrate fat body or vertebrate liver, secreted to the hemolymph/blood and taken up by nurse cells and eggs through receptor-mediated endocytosis (Finn, 2007). Vg concentration varies between the members of the honey bee colony, from almost undetectable to 40% of the total hemolymph protein fraction in the functionally sterile helper females -called workers- and it constitutes up to 70% of the hemolymph protein fraction in the egg-laying queens (Barchuk et al., 2002). Vg has an important role in bee's immune response being the carrier of immune elicitors; it binds to bacteria, both the gram-positive *P. larvae*, causing American foulbrood disease, and the gram-negative *Escherichia coli* (Salmela et al., 2015). It also binds to pathogen-associated molecular patterns; lipopolysaccharide, peptidoglycan and zymosan, as demonstrated by Salmela et al. (2015). Furthermore, there have also been previously described changes in

Vg expression in response to microsporidia, for example a decrease after *Nosema ceranae* infection (Antúnez et al., 2009). Therefore, it is interesting to study the implications of this molecule during virus infections in bees.

The major mechanism of antiviral defence in insects is the small interfering RNA pathway that responds through the detection of virus-derived double-stranded RNA to suppress virus replication. However, other innate antimicrobial pathways such as Imd, Toll, and Jak-STAT and the autophagy pathway have also been shown to play important roles in antiviral immunity (Evans et al., 2006; Kingsolver et al., 2013). In insects, Toll receptors are necessary for sensing the invasion by pathogens (Takeda & Akira, 2003). Recent reports have implicated *Drosophila* Toll and Jak-STAT signalling pathways in controlling RNA viral infection (Merkling & van Rij, 2013). Initially characterized for its role in development and hemocyte proliferation, it has been shown that the Jak-STAT pathway also responds to bacterial and viral infections by regulating the production of downstream effector molecules including antimicrobial peptides (AMPs). This pathway is composed of one signal transducer and activator of transcription (STAT92E; hereafter STAT), a Janus kinase (Hopscotch), a receptor (Domeless), and three unpaired-related ligands (Kingsolver et al., 2013).

Also, data from various research groups, using both RNA and DNA viruses in experimental systems, have shown the role for nitric oxide (NO) in insect immune response. The data suggests that NO may inhibit an early stage of viral replication and thus prevent viral spread, promoting viral clearance and recovery of the host (Reiss & Komatsu, 1998). Further, it has been found that in insects, nitric oxide synthase (NOS) expression is also regulated by the STAT pathway (Chen et al., 2002).

A deeper knowledge of the response of selected immune genes to viral infection may help to better understand the complex interactions between host and pathogen, illuminating the phenomenon of colony bee losses. In order to examine this interaction between immune genes and virus, bees with deformed wings that normally carry high viral load (Martin et al., 2012) and bees with normal wings that are expected to show lower viral levels were used to study viral load of the DWV and to analyse the changes in the expression levels of Toll Wheeler, cactus, domeless, TEPA, NOS, hymenoptaecin, Vg, defensin, and abaecin in response to the virus infection. This study was conducted in bee samples with and without deformed wings which were collected during winter from hives in the field with high *V. destructor* infestation.

MATERIAL AND METHODS

Collection of honey bees

Ten adult winter bees with deformed wings (DW) and 10 adult winter bees with normal wings (NW) were

collected from field hives highly infested (5.45% of phoretic *Varroa* prevalence in the apiary), naturally, with *V. destructor* in Santa Paula apiary (37° 56' 00.69" S; 57° 40' 40.53" W). Overwintering losses associated with *V. destructor* have been detected in this region in the past (Eguaras & Ruffinengo, 2006; Invernizzi et al., 2011; Maggi et al., 2013); thus, it is expected to find hives highly infested in winter.

RNA extraction, DNase treatment and reverse transcription

Total RNA from individual adult *A. mellifera* was isolated using TRI-reagent (Biobasic Inc., USA) according to the manufacturer's protocol. DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA) was used to degrade genomic DNA carried over in the RNA extraction by digestion at 37 °C for 30 min. The quantity of the resulting RNA was determined in a Rotor Gene 6000 cyclor (Qiagen, USA) using a Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen, USA). Complementary DNA (cDNA) was synthesized using a reaction mixture containing 1 µg of total RNA, random hexamers (12 ng/µl) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, USA) according to the procedures suggested by the manufacturer. Negative controls, omitting either the RNA or the reverse transcriptase were included during reverse transcription.

Primers

A list of the primers for qPCR (synthesized by Eurofins MWG Operon, USA) used in PCR protocols, specific annealing temperature and melting temperature are summarised in Table I. Primers for the study of Toll wheeler mRNA levels were designed with Primer Premier Software (PREMIER Biosoft International, Palo Alto, USA).

RT-qPCR

To determine whether DWV infection has an effect on *A. mellifera* immune related genes expression, the levels of Toll Wheeler, cactus, domeless, TEPA, NOS, hymenoptaecin, Vg, defensin and abaecin mRNA were quantified. mRNA expression levels of all analyzed genes from DW bees (10) were compared to their expression in NW bees (10).

RT-qPCR reactions were run with KAPA HRM FAST qPCR kit, with EvaGreen as intercalating dye (KAPA Biosystems, USA) and 1 µl of cDNA sample in a final volume of 20 µl. The amplification and detection of the specific products were carried out using a Rotor Gene 6000 cyclor (Qiagen, Germany), with the following amplification conditions: 2 min at 95 °C, 40 cycles of 10 s at 95 °C, 15 s at specific annealing temperature and 15 s at 72 °C. After amplification, a melting curve analysis was performed, which resulted in single product-specific melting curve. In all cases, the experiments were performed in duplicate. Negative controls for

cDNA synthesis and qPCR procedures were included in all cases. The amplification efficiency was determined for each gene using 10-fold dilutions of cDNA.

The results are reported as the mean fold change of DWV and immune system genes transcription levels in DW bees over levels detected in NW bees, which served as the control group (Pfaffl et al., 2002). *Apis mellifera* β -actin gene was used as a reference / housekeeping gene.

DWV analysis

For the determination of DWV levels and strain diversity, primers that amplify a ~250 bp fragment of the RNA-dependent RNA polymerase (RdRp) gene of DWV were used (Kukielka et al., 2008). PCR amplifications were performed in duplicate and a High-Resolution Melting (HRM) analysis was carried out in all 20 samples immediately after the DWV RT-qPCR at temperature increment steps of 0.1 °C from 75 °C to 95 °C. The results were analyzed with Rotor Gene Q software, version 1.7.94. The genotype identification confidence cut off value of 90% was used as the threshold in the genotyping module of the software and PCR reactions of each sample were performed in triplicate.

Sequencing

After HRM analysis of DWV amplifications, the PCR amplicons were resolved on agarose gels and purified using Accuprep Gel Purification Kit (Bioneer, South Korea). Purified fragments were sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) with the ABI Prism 310 Genetic Analyzer (Applied Biosystems). To verify the differences identified by HRM analysis, all sequences obtained were aligned and compared by use of MAFFT software on-line (Kato

et al., 2017). Using ExpASy Translate tool online (Artimo et al., 2012) we obtained and analyzed the amino acid coding sequence of the fragments of the non-structural polyprotein of the DWV, of each of the nucleotide sequences obtained.

Statistical analysis

The relative expression analysis of DWV viral levels and immune related genes was performed using the Relative Expression Software Tool (REST, Qiagen Inc., Valencia, CA, USA), which compares the expression of the target gene in a sample relative to the control and tests the group differences for significance with a pair wise fixed reallocation randomization test (Pfaffl et al., 2002). RT-qPCR efficiency for each gene was determined by a linear regression model, according to the equation: $E = 10[-1/\text{slope}]$.

RESULTS

Viral load

Relative levels of DWV RNA were analyzed and DW bees showed a 178-fold higher viral load compared to NW bees ($p < 0.01$).

Gene expression studies

No statistically significant differences ($p > 0.05$) were found for the expression levels of Toll Wheeler, Vg, defensin and abaecin mRNA in DW bees when compared to NW (Fig. 1). However, significant up regulation of cactus, domeless, TEPA, NOS and hymenoptecin mRNA levels ($p < 0.05$) was found in DW bees when compared to NW (Fig. 1).

DWV analysis

HRM analysis of DWV RT-qPCR products showed that amplifications of DW bees were primarily dominated by a single genotype cluster, which showed the same HRM profile in all analyzed samples.

In the case of NW samples, half of them (5) exhibited different melting profiles, indicating the presence of a range of DWV variant sequences. The differences of five DW and five NW bee samples are shown in a Normalized temperature-shifted difference graph (Fig. 2), in this set of samples all DW bees have the same melting profile, while two NW samples show a different melting profile. To verify the differences identified by HRM analysis, all sequences were aligned and compared by use of MAFFT software on-line (Kato et al., 2017) (Fig. 2). In this way the strain diversity present in NW samples was corroborated at sequence level observing that sample A9 had two SNPs in the middle of the sequence, and sample A10 had one SNP. In the case of A9 a change in the amino acid coding sequence of the RdRp protein could be observed (Fig. 3).

Blast search (Altschul et al., 1990) of these sequences share over 98% of identity to those reported in countries such as United Kingdom, Brazil, and Syria

Primer	5'-3' Sequence	AT (°C)	MT (°C)	Reference
NOS-Fw NOS-Rv	TCCACTCGAGGTACTTTCC TCTGGAGGATCACCAATTTCC3	54	76.7	Gregorc et al., 2012
β -actina-fw β -actina-Rv	ATGCCAACACTGTCTTTCTGG GACCCACCAATCCATACGGA	60	83.2	Yang & Cox-Foster, 2005
Cactus1-fw Cactus1-rv	CACAAGATCTGGAGCAACGA GCATTCTTGAAGGAGGAACG	53	84.7	Evans, 2006
TEPA-fw TEPA-rv	CAAGAAGAACGTGCGTGAA ATCGGGCAGTAAGGACATTG	53	79.8	Evans, 2006
Defensin1-w Defensin1-rv	TGCGTGTAACTGTCTCAG AATGGCACTTAACCGAAACG	59	81.8	Evans, 2006
Hymen-fw Hymen-rv	CTCTTGTGCGGTGCATA GCGTCTCTGTTCATCCATT	60	83	Evans, 2006
Abaecin-fw Abaecin-rv	AGATCTGCACACTCGAGGCTG TCGGATTGAATGGTCCCTGA	58	85	Yang & Cox-Foster, 2005
Toll18W-fw Toll18W-rv	CTCGGTAGCAATCCGTGGTC CAATCGCCGCTGTAAGC	59	91.2	this study
domeless-fw domeless-rv	TTGTGCTCCTGAAAATGCTG AACCTCCAAATCCGCTCTGTG	53	78.5	Evans, 2006
DWV-fw DWV-rv	CTGTATGTGGTGTGCTGGT TTCAAACAATCCGTGAATATAGTGT	58	82	Kukielka et al., 2008
VgMC-fw VgMC-rv	AGTTCGGACCGACGACGA TTCCTCCACGGAGTCC	54	80.5	Simone et al., 2009

Table I. Primer sequences (forward-fw and reverse-rv), annealing temperature (AT), and melting temperature (MT) used in this study (see details on main text).

(GenBank accession: DQ434932, DQ434942, KT733631, KX530470). Using MAFFT software on-line (Kato et al., 2017) a phylogenetic tree was created to visualize the clusters of the sequences obtained with the top hits from the GenBank (Fig. 4).

DISCUSSION

DWV is considered the most widespread bee virus, often associated with *V. destructor* infestation, and the role of this parasite in the transmission of the virus has already been demonstrated (Allen & Ball, 1996). In the present work we studied DWV viral load, strain diversity and *A. mellifera* immunogene expression in samples of bees, with and without deformed wings, collected from field hives highly infected by *V. destructor*.

DW bees showed a 178-fold higher viral load in comparison to NW bees ($p < 0.01$). These results agree with those reported by Yang and Cox-Foster who also described that DW bees had higher virus titers than NW bees (Yang & Cox-Foster, 2005). It is worth mentioning that virus detection does not implicate an established infection (Lanzi et al., 2006) as this virus, as well as several others RNA bee viruses, usually persist as covert infections in the colony without the vectoring capacity of *Varroa* (Locke et al., 2012).

DWV detection was performed amplifying a ~250 bp fragment of the RNA-dependent RNA polymerase (RdRp) gene. RdRp is an essential protein encoded in the genomes of all RNA viruses with no DNA intermediate stage. RdRp catalyzes synthesis of the complementary RNA strand, is highly conserved (Lanzi et al., 2006) and vital for viral replication. Substitutions in

its nucleotide sequence could contribute to variations in the amino acid coding sequence of this protein, hence in viral epidemiology, since any variation in this gene could suggest an alteration in the genetic shift of the rest of the viral genome (Ryabov et al., 2014). Here, we found that the portion of RdRp sequence analyzed from the local viruses share over 98% of identity to those reported in countries such as United Kingdom, Brazil, and Syria (Berényi et al., 2007; Wang et al., 2013).

High-resolution melting (HRM) analysis has been previously used to monitor the relationship between *Varroa* infestation and virus diversity (Martin et al., 2012). We have also used this technology as a virus identification method (Marin et al., 2016). In the present work we analyzed a region of the RdRp gene to exploit the possibility of differentiating virus strains by their slight differences in specific nucleotides, as observed in the alignment of the amplified fragments of DWV RdRp gene fragment. The alignment revealed differences in the 250 bp amplified product, with distinct melting peaks (Fig. 2A); all DWV amplifications of DW bees showed the same melting profile, revealing a unique viral strain in these samples, while five NW samples showed different melting profiles, showing strain diversity in bees without clinical symptoms. Sample A9 (NW bee) had two SNPs in the middle of the sequence, and sample A10 (NW bee) had one SNP. In the case of A9, an alteration of the amino acid coding sequence of the fragment of the RdRp protein analyzed was found (Fig. 3).

The DW honey bees analyzed here were sampled from field colonies that were parasitized by *V. destructor* for the necessary period of time (more than three years of infestation) required for the selection of virus variants

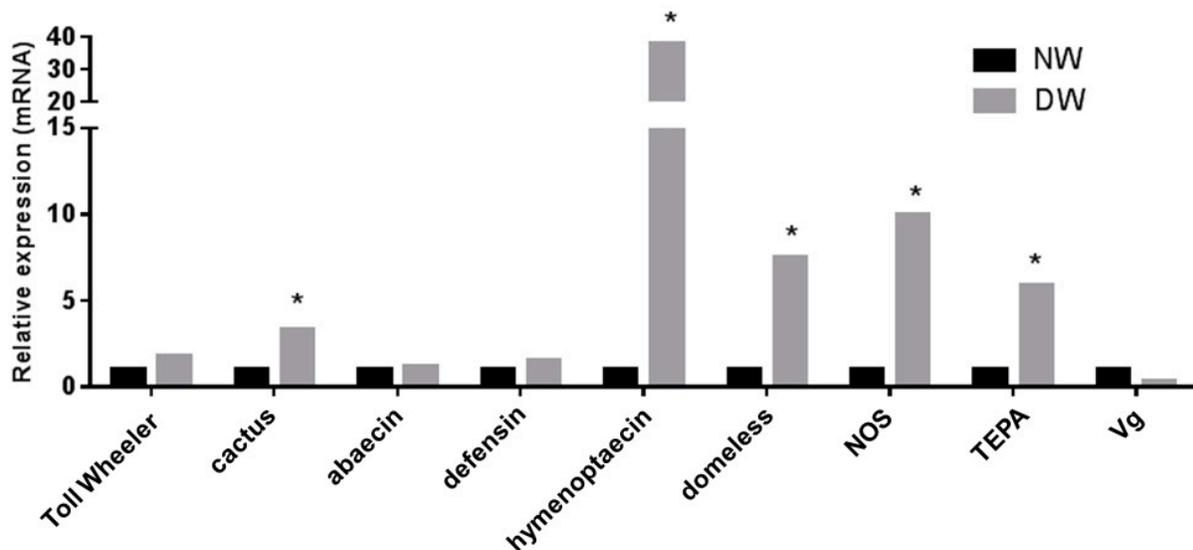


Fig. 1. Relative mRNA expression levels of Toll Wheeler, cactus, abaecin, defensin, hymenoptaecin, domeless, NOS, TEPA and Vg in NW and DW samples Asterisks indicate statistically significant differences ($P < 0.05$) in relation to NW samples (control). mRNA relative levels and statistical significance of the differences in mRNA expression were obtained with the software REST (Qiagen Inc, USA).

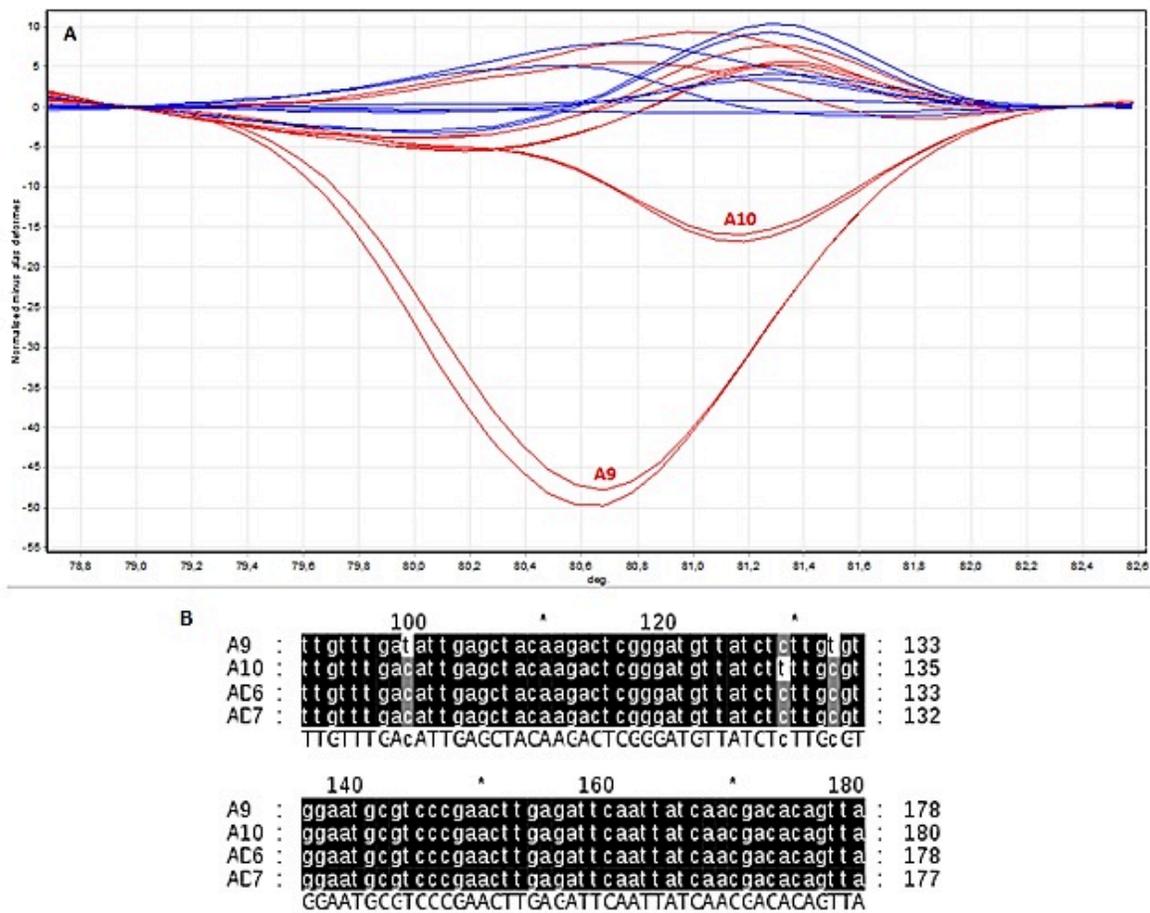


Fig. 2. A. HRM analysis of DWV-RT-qPCR products. Normalized temperature-shifted difference graph, DWV amplicons of 5 DW samples (blue) and 5 NW samples (red) analyzed in duplicate are shown. The melting profile of a DWV RT-qPCR product of a DW bee sample has been chosen as a horizontal baseline. **B.** Partial alignment of DWV- RT-qPCR products between 2 NW samples (A9 and A10) which showed different melting profiles and 2 DW samples (AD6 and AD7).

adapted to mite transmission (Martin et al., 2012).

A large number of polymorphisms were described in a number of regions in the deep sequencing data of DWV by Wang et al. (2013) suggesting that sequence conservation might not necessarily be maintained at the population level. Further studies including complete viral genome sequencing and experimental infections of honey bees with different DWV strains are required for testing virulence.

Some studies have found no correlation between the presence of *Varroa* and changes in host immune responses (Gregory et al., 2005; Navajas et al., 2008). Yet, Marringa et al. (2014) demonstrated changes in the type of hemocytes freed in the hemolymph, suggesting a cellular response to *Varroa* attack and could be the link to induction of the cellular responses pathways. Also, Yang and Cox-Foster (2005) suggest that the increase in DWV titre and reduction in variant diversity cannot be solely explained by *Varroa*-induced immunosuppression of honey bees. Considering this background, here we

studied the expression levels of genes of various effector pathways of the immune system of *A. mellifera* in samples of DW and NW bees to make a contribution to the studies focussed in this host-pathogen interaction.

Antimicrobial peptides expression levels have been previously studied in *A. mellifera* in relation to viral infections. It has been reported that the expression of these peptides is induced by activation of Toll and Imd pathways (Brutscher et al., 2015). In this study, three antimicrobial peptides were analyzed, abaecin, defensin-1 and hymenoptaecin. Only hymenoptaecin mRNA expression levels were affected in DW bees, showing a 38-fold increase in comparison with NW bees. Hymenoptaecin expression was not correlated to either abaecin or defensin, this result could indicate that hymenoptaecin uses a different regulatory pathway than abaecin and defensin as previously suggested (Yang & Cox-Foster, 2005). It was proposed that hymenoptaecin is regulated by the IMD pathway (Schlüns & Crozier, 2007) and the Toll pathway (Evans et al., 2006), whereas

- Blast identification of the complete sequence of the non-structural polyprotein gene of the DWV used for the analysis of the amino acid coding sequence of the RdRp protein fragment.

LOCUS GU109335 10167 bp RNA linear VRL 04-NOV-2009
 DEFINITION Deformed wing virus isolate Warwick-2009 polyprotein gene, complete cds.
 ACCESSION GU109335
 VERSION GU109335.1

- Amino acid coding sequence of the non-structural polyprotein of the DWV.

```
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                /protein_id="ACY38662.1"/translation=
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NLQLSNRPNAQKPEMDNPNPGEDEVELEKDSNVVLTQRDPSTSPAPVSVKSWRSTNDVVDYATITSRWYQIAEFVNSKDDPFDEKLARLLP
RALLSSIEANSDAICDVPNTIPFKVHAYWRGDMVVRVINSKFKVQGLQATWYSDHENLNISSKRSVYGFQSMQDHALISASASNEAKLVIIPKHVYV
FLPTRIVPWTGTGILDMGALNIRVIALPMSATGPTTCNVVFIKLNNEFTGTSSGKFYASQVRAKPEMDRILNLAEGLLNNTIGNNMNDSPYQSP
RHFVPTMGHSLAIGNLVLEPLHALRLDAAGTQHPVGCAPDEDMTVSSIASRYGLIRRVQWKDHAAGSLLQLDADPPVQRIEGRNPIISLWYFAPVG
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NKTPIINADGLYEVILQGVYTYPYHGDGVCGLLSRNLQRPITIGIHVAGTEGLHGFVAEPLVHEMFTGKAIESEREPEYDRVYELPRELEDESIDGL
DITDLPYIGRVDAKLAHQSPSTGIKKTILHGTFDVTRTEPNMSSRDPRIAPHDPLKLGCEKHGMPSCFPFNKHLLELATNHLKELISVVPKINGCKIRS
LQDAVCGVPGVDGFSISWNTSAGFPPLSSLKPPGTSGRKRWLFDIEIQDSGCYLLCMRPELEIQLSTTQLMRKKGIKPHITFTDCLEKDTPLVPEKCRIP
GKTRIFSIPVQFTIPFRQYLDPMASYRAARLNAEHIIGIDVNSLEWTLNATSLSKYGTHTVTDYKNGFGLDSVAASAFETIIDVVLHYTEENDK
DEMRVMTMAQEIILAPSHLCRDLVYRVPCGIPSGSPITDILNFTISNCLLIRLAWLGITDPLSEFSQNVVLCYGGDLMNVSDNMIKFNVAITGKPF
FSQYKMEFTDQKSGNTVWRTLQATATFLKHFGLKHPTRPVFLANLDKVSVEGTTNWTNTHARGLRRTATIEAKQALELAFGWGPEYFNYVRNTIKMAF
DKLGYEDLITWEEMDVRVYASA"
```

- Amino acid coding sequence of the fragments of the non-structural polyprotein of the DWV obtained from the nucleotide sequences of AD6, AD7, A9 y A10 using ExPASy Translate tool online (web.expasy.org/translate/; ExPASy-SIB Bioinformatics Resource Portal).

-AD6 has the sequence that covers more of the complete amino acid coding sequence of the DWV reported.

5'3' Frame 2
 FGPDSISWNTSAGFPPLSSLKPPGTSGRKRWLFDIEIQDSGCYLLRMRPELEIQLSTTQLMRKKGIKPHITFTDCLEK

-AD7 sequence covers the center of the fragment analyzed.

5'3' Frame 1
 ERV-FDILLGANDPESLKSSTGSKNINFDIEIQDSGCYLLCMRPELEIQLSTTQLMRKKGIKPHITFTDCLEK-LTDE-N

-A9 presents a mutation in the center, with a different amino acid to that found in the reported sequence.

5'3' Frame 2
 ERV-FISWNTSAGFPPLSSLKPPGTSGRKRWLFDIEIQDSGCYLLCMRPELEIQLSTTQLMRKKGIKPHITFTDCGE

-A10 sequence covers the center of the fragment analyzed.

5'3' Frame 1
 VEGLLLSWNTSAGFPPLSSLKPPGTSGRKRWLFDIEIQDSGCYLLCMRPELEIQLSTTQLMRKKGIKPHITFTDCLEK-GE

Fig. 3. Amino acid coding sequence analysis of the fragments of the RdRp protein, of the non-structural polyprotein of the DWV, obtained from the nucleotide sequences of AD6, AD7, A9 & A10.

defensin has been reported to be regulated by the Toll pathway (Lourenço et al., 2018). In contrast to our results, Yang and Cox-Foster (2005) described a decrease in the expression of abaecin, defensin-1 and hymenoptaecin in DW bees, relating this immunosuppression with viral amplification. Interestingly, Azzami et al. (2012) reported that bees infected with ABPV produced neither elevated levels of specific antimicrobial peptides, such as hymenoptaecin and defensin, nor any general antimicrobial activity.

In our study no differences were found in NW and DW bees in Vg mRNA levels, which could indicate that this glycolipoprotein is not involved in the immune response induced by DWV.

We also studied the expression of two genes of the Toll pathway by analyzing mRNA levels of Toll wheeler, previously studied in antimicrobial immune defence in *A. mellifera* (Aronstein & Saldivar, 2005) and cactus, the NF

kappa B inhibitor (IκB). According to Evans et al. (2006), following conformational changes of the activated Toll-like receptor, several intracellular death-domain (DD) containing proteins are recruited to form a receptor complex. Activation of this complex leads to the degradation of the NF kappa B inhibitor (IκB) cactus and subsequent nuclear translocation of the NF-κB transcription factor Dorsal. In this study no differences in Toll wheeler mRNA levels were found, but, in concordance to Nazzi et al. (2012) who showed a down-regulation of a honey bee NF-κB transcription factor, a 3.3-fold induction of cactus mRNA levels was detected in DW bees in comparison with NW bees. These results suggest that this pathway is inactivated against high levels of *Varroa* infestation and high viral loads of the DWV. This could indicate that the overexpression of hymenoptaecin discussed above could be related with other signalling pathway other than Toll (see Evans et al.,

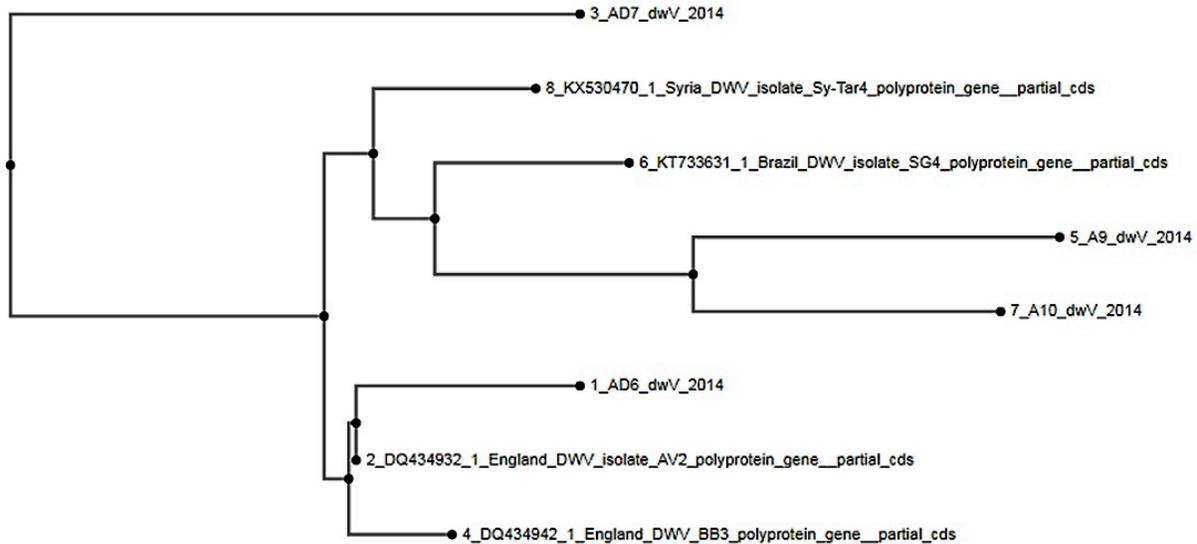


Fig. 4. Phylogenetic tree created using MAFFT software on-line (Kato et al., 2017) to visualize the clusters of the sequences obtained (AD6, AD7, A9 & A10) with the top hits from the GenBank.

2006) or that other Toll receptors (besides Toll wheeler) should be evaluated.

The Jak-STAT pathway has been shown to respond to bacterial and viral infections in insects (Kingsolver et al., 2013; Chen et al., 2014). In this work, two members of the Jak-STAT pathway were studied: the receptor domeless and the effector protein TEPA showing a significant up-regulation in DW bees. These results demonstrate that the Jak-STAT pathway is activated in response to *Varroa* infestation and DWV infection in *A. mellifera*. The Jak-STAT pathway represents an extremely rapid membrane-to-nucleus signalling system. Interferon-g (IFN-g) is well known to play crucial roles in several aspects of the immune responses and one action responsible for its inflammatory function results from activation of the nitric oxide synthase (NOS) promoter and NOS gene expression. To achieve pleiotropic biologic activities, including NOS induction, signalling cascades initiated from receptors associated with Jak-STAT pathway are critical (Chen et al., 2002). Interestingly, in this study the expression levels of the NOS gene were detected in high level at the DW group.

Nitric oxide (NO, the signalling molecule that results from NOS activity) has been described recently as an effector that mediates cellular and humoral immune response in insects (Sadekuzzaman & Kim, 2018). The involvement of NO in the immune response of *A. mellifera* has been previously reported (Negri et al., 2013, 2014) and a role for NO in the immune response to viruses has also been described (Reiss & Komatsu, 1998). Increased NOS has also been shown to be related to bacterial infection (Hillyer & Estévez-Lao, 2010) and induced after blood feeding (Lim et al., 2005) in mosquitoes that transmit intracellular pathogens like

malarial protozoans. In other insects as *Bombyx mori* (Lepidoptera) (Ishii et al., 2013) NOS mediates the expression of AMP (Sadekuzzaman & Kim, 2018), and this particularity could be related to the hymenoptaecin up-regulation observed from our results.

In DW bees we found higher titers of DWV compared with NW bees, and an up-regulation of *A. mellifera* Jak-STAT signalling pathway genes and cactus expression level, while the abaecin and defensin expression levels were not significantly different. In insects, the connection between the RNAi and Jak-STAT pathway has been demonstrated with the NF-κB pathways (Toll and Imd). The explanation of AMPs expression decrease could be explained according to Jak-STAT and the Imd/Toll pathways as a negative interaction, as seen in other insects (Kingsolver et al., 2013). This was related to the inhibitory effect of a complex integrated by STAT that competes with Relish (the NF-κB transcription factor from Imd pathway) inducing the negative regulation of certain AMPs (Kim et al., 2007).

This study shows that DW bees are infected with a virulent DWV strain that not only induces changes in immunogene expression indicating activation of the Jak-STAT pathway, but also shows an increased viral load, in comparison with NW bees. *Varroa*-adapted DWV variants present in our country should continue evolving, and investigations of virus strain differences may explain the different pathologies currently seen in honey bee colonies. Such variants may interact with other pests, pathogens, environmental factors, and regional beekeeping practices, resulting in recent largescale losses of honey bee colonies (Cox- Foster et al., 2007; Maggi et al., 2013). In concordance with this and all the results found in the current study more analyses are

required to target the possible cross talk between Jak-STAT and Toll/Imd pathways, and also the possibility of a relation between NOS expression and AMP production. Analysis of the differences in the modulation of immunogens in response to DWV different variants and viral load between summer and winter bees, with different infestation levels of *Varroa*, is also needed.

ACKNOWLEDGEMENTS

This research was supported by grant and PICT-2011 No. 0162 to M.M. The authors would like to thank the Institute of Analysis Fares Taie, CONICET and the UNMdP for financial support.

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