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Differential viral levels and immune gene expression in three stocks of *Apis mellifera* induced by different numbers of *Varroa destructor*

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ABSTRACT

The viral levels and immune responses of Italian honey bees (IHB), Russian honey bees (RHB) and an outcross of Varroa Sensitive Hygienic bees (POL) deliberately infested with one or two foundress *Varroa* were compared. We found that the Deformed wing virus (DWV) level in IHB inoculated with one or two foundress *Varroa* increased to about 10^3 or 10^5 fold the levels of their uninfested brood. In contrast, POL (10^2 or 10^4 fold) and RHB (10^2 or 10^4 fold) supported a lower increase in DWV levels. The feeding of different stages of *Varroa* nymphs did not increase DWV levels of their pupal hosts. Analyses of their corresponding *Varroa* mites showed the same trends: two foundress *Varroa* yielded higher DWV levels than one foundress, and the addition of nymphs did not increase viral levels. Using the same pupae examined for the presence of viruses, 16 out of 24 genes evaluated showed significant differential mRNA expression levels among the three honey bee stocks. However, only four genes (*Defensin*, *Dscam*, *PPOact* and *spaetzle*), which were expressed at similar levels in uninfested pupae, were altered by the number of feeding foundress *Varroa* and levels of DWV regardless of stocks. This research provides the first evidence that immune response profiles of different honey bee stocks are induced by *Varroa* parasitism.

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1. Introduction

Varroa destructor Anderson and Trueman remains the major cause of deaths of *Apis mellifera* colonies worldwide (Anderson and Trueman, 2000). Significant injuries to individual honey bees largely occur within the capped brood where these mites reproduce. One foundress *Varroa* can produce an average of three offspring in *A. mellifera* worker brood, which usually depends upon the genotype of the honey bees (de Guzman et al., 2008). While inside the cells, both the foundress and her progeny feed on the hemolymph of the developing brood through wounds inflicted by the foundress. Typically, one integumental wound per pupal host is made, which is shared as a feeding site by the foundress with her progeny or with other invading foundresses (Kanbar and Engels, 2005).

As *Varroa* mites feed on host bees, they also can transmit honey bee viruses (Yue and Genersch, 2005; Chantawannakul et al., 2006; Gisder et al., 2009). Infestation by *Varroa* has been associated with infections of multiple honey bee viruses such as Deformed wing virus (DWV), Kashmir bee virus (KBV), and Israeli acute bee paralysis virus (IAPV) (Chen et al., 2004; Tentcheva et al., 2004; Chen and Siede, 2007; Di Prisco et al., 2011; Martin et al., 2012). DWV infection is a prominent result of *Varroa* parasitism which results in wing deformation, decreased body weight and size, and shortened life span (de Jong et al., 1982; de Jong and de Jong, 1983; Chen and Siede, 2007; Annoscia et al., 2012). The wound sites resulting from *Varroa* feeding also permit secondary bacterial infections (Chen and Siede, 2007), which may further impact honey bee health. Thus, the combination of *Varroa* parasitism and its associated pathogens may synergistically affect the host's immune responses and eventually lead to colony collapse.

Honey bees possess both cellular and humoral immune responses (Evans et al., 2006). In general, antibacterial peptides (AMPs) are expressed in response to pathogens and parasites, which directly lyse foreign microbial cells and inhibit activities of enzymes essential for pathogen replication (Otvos et al., 2000; Li et al., 2006). Several studies of immune responses have shown that mites and viruses could alter transcript levels of immunity-related genes in their corresponding hosts. Gregory et al. (2005) observed a positive relationship between the number of *Varroa* mites and

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expression of AMPs (*Defensin* and *abaecin*) in the hosts. Yang and Cox-Foster (2005) reported that DWV titers were negatively correlated with the expression levels of immunity-related enzymes. A recent study has implicated the feeding activities of *Varroa* in the suppression of *dorsal-1A* (NF-κB) which allows unregulated DWV replication (Nazzi et al., 2012).

Honey bees vary in their ability to defend themselves against *V. destructor* infestation (Rinderer et al., 2010). Several stocks resistant to *Varroa* have been developed. Two *Varroa*-resistant stocks, Russian honey bees (RHB) and the bees with *Varroa* Sensitive Hygienic (VSH) trait, are now commercially available in the United States (Danka et al., 2008; Brachman, 2009; Rinderer et al., 2010). In Europe, native honey bee subspecies also have shown some degree of resistance to *Varroa* mites (Büchler et al., 2010). Various mechanisms of resistance to *Varroa* mites have been identified. Both RHB and VSH have a high proportion of infested brood having non-reproductive mites (de Guzman et al., 2007; Harbo and Harris, 2009). RHB colonies also support a low proportion of multiply infested brood (de Guzman et al., 2007) and have a higher proportion of damaged mites or dropped mites (Rinderer et al., 2003, 2013, 2014a). VSH routinely remove *Varroa* infested brood (Danka et al., 2008; Harbo and Harris, 2009).

Studies of the resistance to pathogens by *A. mellifera* were pioneered by Rothenbuhler (1964). Hygienic behavior was observed to be a resistance mechanism to American foulbrood (Rothenbuhler, 1964), Chalkbrood (Gilliam et al., 1983), and then against parasitic mites. However, no study has been conducted to determine whether or not honey bee stocks vary in their responses to pathogens or pathogen-parasite exposures. Gregory et al. (2005) investigated the immune response of Italian honey bees (IHB) to *Varroa* parasitism using different pupal stages infested with different mite loads (= foundress and progeny within a cell). However, the DWV levels of their test bees and the potential effects of virus levels on the expression of different immune genes were not examined. In this study, we compared the immune responses of three honey bee stocks exposed to different numbers of foundress *Varroa* and the presence of viruses that may be affected by mite parasitism.

2. Materials and methods

2.1. Test brood preparation

Three honey bee stocks were compared in this study: (a) Italian honey bees (IHB, $n = 4$ colonies), (b) VSH outcross (POL, $n = 4$ colonies), and (c) Russian honey bees (RHB, $n = 4$ colonies). For each test colony (one colony per stock), age-controlled eggs were obtained by caging the queen with a push-in cage on an empty frame for 24 h. The grafting technique was employed (de Guzman et al., 1995), which was performed when larvae were less than 24 h old. Royal jelly may be a source of virus infection for the developing brood, and gamma irradiation has been shown to inactivate viruses (Sullivan et al., 1971; White et al., 1990). To minimize virus exposure, dry grafting (no priming of cells with royal jelly) was employed and irradiated combs were used to receive the grafted larvae ($n = 150$ larvae per stock/trial). At the middle of the frame, a section comprising 15 rows of 30 cells was created to receive the grafted larvae. Five rows were assigned randomly for each stock per comb (trial). Each trial involved one colony each from the three honey bee stocks. Four trials, each using different colonies, were conducted. Thereafter, the test frame was placed in the middle of the brood chamber of a host colony. Three host colonies (with three medium boxes) having very low levels of *Varroa* infestation (2.5 ± 0.29%) were used. Host colonies were fed with pollen patties and all had plenty of honey.

2.2. Mite inoculation

The transfer technique was used in this study (Garrido and Rosenkranz, 2003; Kirrane et al., 2011; Khongphinitbunjong et al., 2012). Inoculum female *Varroa* mites were collected from newly sealed larvae of five highly infested colonies. For each trial, 10 inoculum mites were collected from each mite source colony and mixed into a holding container with fresh larvae. Newly sealed grafted larvae (L5) received one of the following treatments: (a) no mite inoculation (control), (b) capping opened and closed without mite inoculation (O/C), (c) one foundress *Varroa*, and (d) two foundress *Varroa*. The locations of each treatment within each brood section were recorded. After inoculation, the test frame was kept in an incubator (34 °C, 50% RH) to prevent brood removal by bees and small hive beetle infestation. Eight days after mite inoculation, all tan-bodied pupae were collected and individually placed in microcentrifuge vials. For the mite-inoculated (and few naturally infested) brood, the numbers of mites infesting each pupa were counted, stages differentiated and collected. Both the infested pupa and its corresponding mite/s were kept in one microcentrifuge vial. All samples were then stored in a −80 °C freezer until RNA extraction.

2.3. Molecular analyses

A total of 188 pupae (IHB = 64, POL = 60, RHB = 64) and 140 corresponding mite samples were analyzed for this study. One mite sample comprised either the inoculated foundresses only (one or two) or together with their respective nymphs (1–5). Total RNA was isolated from individual bees using the Maxwell® nucleotide purification system with the LEV simplyRNA tissue kit (AS1280) (Promega, WI). The concentration (ng/μl) and purity (A260/280) of total RNA was determined using a spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE). Reverse transcription reactions for cDNA synthesis were performed using a QuantiTect Reverse Transcription Kit (Qiagen Inc., Valencia, CA). A spike in exogenous RNA was added into the RNA reaction to act as an external control (Alien Reference RNA qRT-PCR Detection Kit Agilent Technologies Inc., Santa Clara, CA). cDNA conversion was done in accordance to the manufactures protocol. Double-Stranded cDNA was synthesized with 600 ng poly-A RNA template.

The incidence of honey bee viruses and the expression of targeted genes (Table 1) were determined by qPCR using CFX96™ Real-Time PCR (BioRad, Inc.) The 24 target genes were selected from published literatures because they showed interactions between mite infestation and pathogen infection, and cover immune responses such as Toll, Imd, JNK, JAK/STAT pathways. Amplification was performed in 10 μl reaction volumes, consisting of 4 μl SsoFast Eva Green SMX (Biorad), 0.5 μl of 10 μM of each primer, 4 μl of nuclease free water and 1 μl cDNA. Reactions were run for 95 °C for 30 s, 40 cycles of 95 °C for 1 s, 59 °C for 5 s followed by a melt-curve dissociation analysis. All reactions included three technical replicates. The qPCR data were expressed as the threshold cycle (C_t) values and were normalized using geometric averaging of multiple reference genes (*β-actin*, *rp49 and the exogenous Alien control*) to the target genes (ΔC_t) (Vandesompele et al., 2002). To compare viral and gene expression levels across treatments, the qPCR data were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schnittgen, 2001; Chen et al., 2005; Chaimanee et al., 2012; Boncristiani et al., 2013). The treatment with the lowest viral or expression level was used as the calibrator (Chen et al., 2005).

2.4. Statistical analyses

For each stock, the viral level (ΔC_t converted to $\log_{10}$) and relative gene expression data (from Section 2.3) were analyzed separately using a one-way analysis of variance (ANOVA) (SAS

Table 1
Oligonucleotide primers used in this study for the immune responses of three honey bee stocks.

Primer	Pathway/target	Forward	Reverse	References/Gene ID
abaecin	Toll/Antimicrobial peptide	CAGCATTCGCATACGTACCA	GACCAGGAAACGTTGGAAAC	Evans et al. (2006) (GB18323)
Apidaecin	Toll/Antimicrobial peptide	AGTCGCGGTATTTGGGAAT	TTTCACGTGCTTCATATTCTTCA	Evans et al. (2006) (GB17782)
apisimin	Toll/Antimicrobial peptide	TGAGCAAAATCGTTGCTGTC	AACGACATCCACGTTCGATT	Evans et al. (2006) (GB19468)
Basket	JNK	AGGAGAACGTGGACATTTGG	AATCCGATGGAAACAGAACG	Boncristiani et al. (2012) (GB16401)
Defensin	Toll/Antimicrobial peptide	TGTCGGCCTTCTCTTCATGG	TGACCTCCAGCTTTACCCAAA	Yang and Cox-Foster (2005) (GB41428)
domeless	JAK/STAT	TTGTGCTCCTGAAAATGCTG	AACCTCCAAATCGCTCTGTG	Evans et al. (2006) (GB12159)
Dorsal	Toll	TCACCATCAACGCCTAACAA	AACTAACACCACGCGCTTCT	Evans et al. (2006) (GB19066)
Dscam3-7	Down syndrome cell-adhesion molecule	TTCAGTTCACAGCCGAGATG	ATCAGTGTCCCGCTAACCTG	Boncristiani et al. (2012) (GB15141)
EGFlike 1		CATTTGCCAACCTGTTTGT	ATCCATTGGTGCAATTTGG	Evans et al. (2006) (GB14654)
hem	JNK	CACCTGTTCAGGGTGGATCT	CCTTCGTGCAAAAGAAGGAG	Evans et al. (2006) (GB17167)
hexam10869	JAK/STAT	GGACAATTGGATCTGCTCGT	GTGTTGCTTCCGCTTTTCAG	Gregorc et al., 2012 (GB10869)
Hopscotch	JAK/STAT	ATTCATGGCATCGTGAACAA	CTGTGGTGGAGTTGTTGGTG	Evans et al. (2006) (GB16422)
Hymenoptaecin	Toll/Antimicrobial peptide	CTCTTCTGTGCCGTTGCATA	GCGTCTCCTGTCATTCCATT	Evans et al. (2006) (GB17538)
imd	Imd	TGTTAACGACCGATGCAAAA	CATCGCTCTTTTCGGATGTT	Evans et al. (2006) (GB18606)
Kenny	Imd	GCTGAACCAGAAAGCCACTT	TGCAAGTGATGATTGTTGGA	Evans et al. (2006) (GB17106)
Lys-1	Toll/Antimicrobial peptide	GAACACACGGTTGGTCACTG	ATTTCCAACCATCGTTTTCG	Evans et al. (2006) (GB10231)
Myd88	Toll	TCACATCCAGATCCAACTGC	CAGCTGACGTTTGAGATTTTTG	Evans et al. (2006) (GB12344)
PGRPSC	Imd	TCCGTCAGCCGTAGTTTTTC	CGTTTGTGCAAATCGAACAT	Gregorc et al. (2012) (GB19301)
PPOact	Toll	GTTTGGTCGACGGAAGAAAA	CCGTCGACTCGAAATCGTAT	Evans et al. (2006) (GB18767)
Relish	Imd	GCAGTGTTGAAGGAGCTGAA	CCAATTCTGAAAAGCGTCCA	Evans et al. (2006) (GB13742)
rp49	House keeping	AAGTTCATTCGTCACCAGAG	CTTCGAGTTCCTTGACATTATG	de Miranda and Fries (2008) (GB47227)
Spaetzle	Toll	TGCACAAATTGTTTTTCCTGA	GTCGTCCATGAAATCGATCC	Evans et al. (2006) (GB15688)
Tak-1	Imd	ATGGATATGCTGCCAATGGT	TCGGATCGCATTCAACATAA	Evans et al. (2006) (GB14664)
TepA	JAK/STAT	CAAGAAGAAACGTGCGTGAA	ATCGGGCAGTAAGGACATTG	Evans et al. (2006) (GB18789)
Toll	Toll	TAGAGTGGCGCATTGTCAAG	ATCGCAATTTGTCCCAAAAC	Evans et al. (2006) (GB18520)
β-actin	House keeping	TTGTATGCCAACACTGTCCTTT	TGGCGCGATGATCTTAATTT	Simone et al. (2009) (GB17681)
DWV	Deformed wing virus (DWV)	GAGATTGAAGCGCATGAACA	TGAATTCAGTGTCGCCCATA	Boncristiani et al. (2012) (AY292384.1)
BQCV	Black queen cell virus (BQCV)	TTTAGAGCGAATTCGGAAACA	GGCGTACCGATAAAGATGGA	Boncristiani et al. (2012) (HQ655494.1)
IAPV	Israeli acute paralysis virus (IAPV)	GCGGAGAATATAAGGCTCAG	CTTGCAAGATAAGAAAGGGGG	Boncristiani et al. (2012) (EF219380.1)
KBV	Kashmir bee virus (KBV)	TGAACGTCGACCTATTGAAAAA	TCGATTTTCCATCAAATGAGC	Boncristiani et al. (2012) (AY275710.1)

JMP version 10.0, SAS Corp.). For the viral level comparisons, two separate analyses were performed: one to show the effect of the number of inoculated foundresses on DWV levels, and the other to determine the effect of the number of actively feeding mites (foundress plus their nymph progeny) on viral levels. Where differences were found, means were compared using a Tukey-HSD with a 95% confidence. A *t*-test was used to compare DWV levels in pupae infested with one or two *Varroa* foundresses.

3. Results

3.1. DWV levels of three honey bee stocks as affected by the number of inoculated foundress

For all honey bee stocks, the levels of DWV increased with an increasing number of introduced foundress *Varroa* (IHB, $F = 55.29$; $P < 0.0001$; Pol; $F = 22.97$; $P < 0.0001$; RHB, $F = 24.16$; $P < 0.0001$) (Fig. 1). Initial DWV levels of *Varroa*-free pupae from IHB colonies were similar to that of RHB and were higher to that of POL ($F = 13.32$; $P < 0.0001$). However, the amount of increase in DWV varied among stocks. When one foundress *Varroa* was introduced, a similar increase of DWV levels was observed in IHB (10^3 fold increase in the ΔC_t values) while POL and RHB had about 10^2 fold more than the uninfested brood ($F = 14.39$; $P < 0.0001$). The same trend was observed when two foundress *Varroa* mites were introduced. The increase of DWV levels in RHB (10^4 fold) and POL (10^4 fold) were similarly lower than IHB which supported an increase of about 10^5 fold more than the levels of their uninfested brood ($F = 13.17$; $P < 0.0001$). However, these DWV levels among honey bee stocks when infested with one ($F = 0.92$; $P = 0.402$) or two mites ($F = 3.06$; $P = 0.055$) were not statistically different.

3.2. Virus levels of three honey stocks as affected by the number of actively feeding Varroa

The effect of the number of actively feeding mites (foundress and nymphs excluding eggs) on the levels of DWV in both pupal

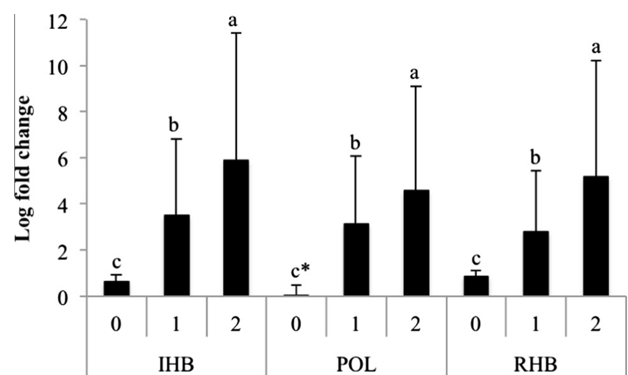

Fig. 1. Log fold change of DWV levels in pupae of three honey bee stocks inoculated with different numbers of foundress *Varroa*. For each stock, bars with different letters are significantly different ($P < 0.001$). IHB = Italian honey bees, POL = VSH outcross, RHB = Russian honey bees. 0 = pupae with no mite inoculation, 1F = pupae inoculated with one foundress *Varroa*, 2F = pupae inoculated with two foundress *Varroa*. * Calibrator with the lowest DWV level.

hosts and their corresponding mites was investigated. In this study, not all inoculated mites reproduced. Those mites that reproduced had 1–5 progeny. Hence, data from all stocks were pooled and grouped as follows: (a) 0 = uninfested (control + O/C), (b) 1F = 1 foundress without nymphs (1F), (c) 1F + P = 1 foundress with 1–5 nymphs, (d) 2F = 2 foundress without nymphs, and (e) 2*F* + *P* = 2 foundress with 1–5 nymphs. Analyses revealed that the levels of DWV both in pupal hosts ($F = 53.23$; df = 4; $P < 0.001$) and their corresponding mites ($F = 8.89$; df = 3; $P < 0.0001$) varied among the mite treatment groups (Fig. 2). Nevertheless, the presence of actively feeding nymphs within the brood cells did not enhance the positive effect of increasing number of foundress on DWV levels in both pupae and their corresponding mites.

When analyzed for other viruses only 9.04% (17 out of 188) of our bee samples were infected with low levels of Black queen cell virus (BQCV) ($C_t = 33.17$ to $C_t = 37.27$). There was no significant

($P > 0.05$) difference between uninfested ($C_t = 39.88 \pm 0.20$) and infested ($C_t = 39.61 \pm 0.18$) pupae. For the mite samples, 67.86% (65 out of 140) were infected with varying levels of BQCV ($C_t = 19.29$ to $C_t = 36.73$). There was no significant ($P > 0.05$) difference between one ($C_t = 34.35 \pm 0.60$) and two ($C_t = 33.77 \pm 0.77$) foundress mites. All pupae and their corresponding mites were found negative for Israeli acute paralysis virus (IAPV) and Kashmir bee virus (KBV).

3.3. Effects of Varroa infestation on immune gene expression

The expression of 24 genes was compared between pupae that were uninfested and infested with 1 or 2 foundress mites in three stocks IHB, RHB and POL. Overall 16/24 genes were differently expressed between treatments across all stocks (Fig. 3). In IHB, 8/24 genes were differently expressed among treatments. Four genes were found to be up-regulated with mite inoculation (*Defensin, lys-1, hexam* and *kenny*) and four down-regulated (*domeless*, *Dscam, PPOact* and *imd*). In POL, 2/24 genes were differentially expressed (*Defensin* and *relish*) in response to mite inoculation and both were down-regulated. For RHB, 9/24 genes were differentially expressed among treatments. All these nine genes (*basket*, *domeless*, *hem*, *Myd88*, *tepA*, *toll*, *imd*, *PGRP* and *spaetzle* were down-regulated in response to mite inoculation.

Across all three stocks, only four genes (*Defensin*, *Dscam*, *PPOact* and *spaeztle*) displayed a correlative (positive or negative) relationship with mite infestation levels in one or more stocks (Fig. 4). The gene *Defensin* displayed a significant positive relationship with the increased number of inoculated mites in both IHB and POL stocks indicating bees' immune response to mite feeding. The gene *PPOact* displayed a negative relationship with increased number of inoculated mites in the IHB stock.

4. Discussion

Varroa and the pathogens they transmit negatively impact honey bee health worldwide. In order to combat *Varroa* mites, a number of strains of bee have been bred for *Varroa* resistance. Each bee strain has unique characteristics that help the bee colony survive against the mite. For example both RHB and VSH bees have been selected to have behavioral traits that improve aspects of mite removal, therefore reducing the overall numbers of mites in a colony (de Guzman et al., 2007; Rinderer et al., 2010; Danka et al., 2013). What is not known is how individual bees of these stocks differ in their immunity response to *Varroa* feeding. In our experiments, we compared individual bees from multiple stocks [IHB, RHB, and POL (VSH outcross)] in their immunity response to *Varroa* feeding.

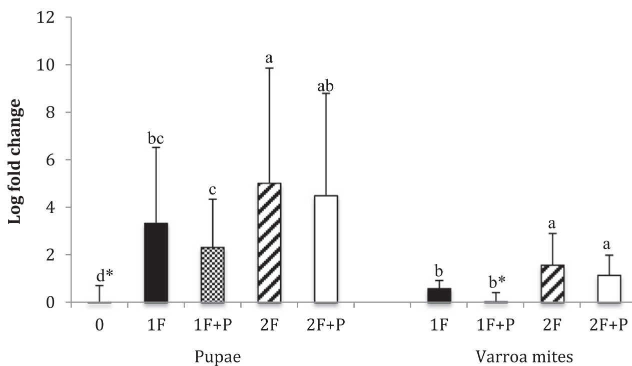

Fig. 2. Log fold change of DWV levels in pupae and their corresponding mites as affected by the number of actively feeding mites. For each group, bars with different letters are significantly different from each other ($P < 0.001$). 0 = uninfested, 1F = 1 foundress *Varroa*, 1F + P = 1 foundress *Varroa* with 1–5 nymphs, 2F = 2 foundress *Varroa*, 2F + P = 2 foundress *Varroa* with 1–5 nymphs. * Calibrator with the lowest DWV level.

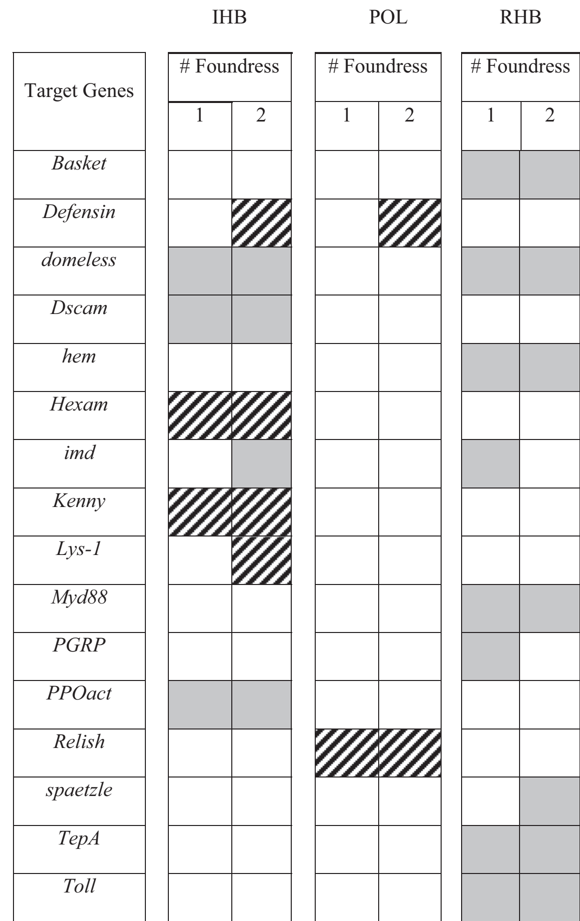

Fig. 3. Immune profile summary of significant changes in pupae inoculated with one or two foundress *Varroa* as compared to uninfested pupae (no *Varroa* inoculation) in three honey bee stocks. IHB = Italian honey bees, POL = VSH outcross, RHB = Russian honey bees.

Low levels of DWV have been observed in contemporary *Varroa*-free colonies (Martin et al., 2012) and were also present before the invasion of mites into the US. DWV and bees with the deformed wing phenotype are more prevalent, however, in colonies with high *Varroa* levels (Yang and Cox-Foster, 2005). Because of the prevalence of DWV, it is difficult to find colonies that have no detectable levels of DWV. Therefore, our experiments looked at the increase in DWV after mite feeding.

In our research, the DWV levels in multiply infested pupae were higher than those with a single infesting mite, regardless of stock. This observation corroborated the findings of Nazzi et al. (2012) who reported that the increased numbers of *Varroa* foundresses trigger higher levels of DWV in Italian honey bees. The contrasting effect of one versus two foundress-infestations on viral levels may be associated with wound size inflicted by the mites, or just the increased amounts of virus transmitted because of multiple feeding foundresses. According to Kanbar and Engels (2005), the size of wounds increased with increasing numbers of foundress mites while generally only one wound site is found. Larger wounds resulting from increased hemolymph feeding may accelerate virus

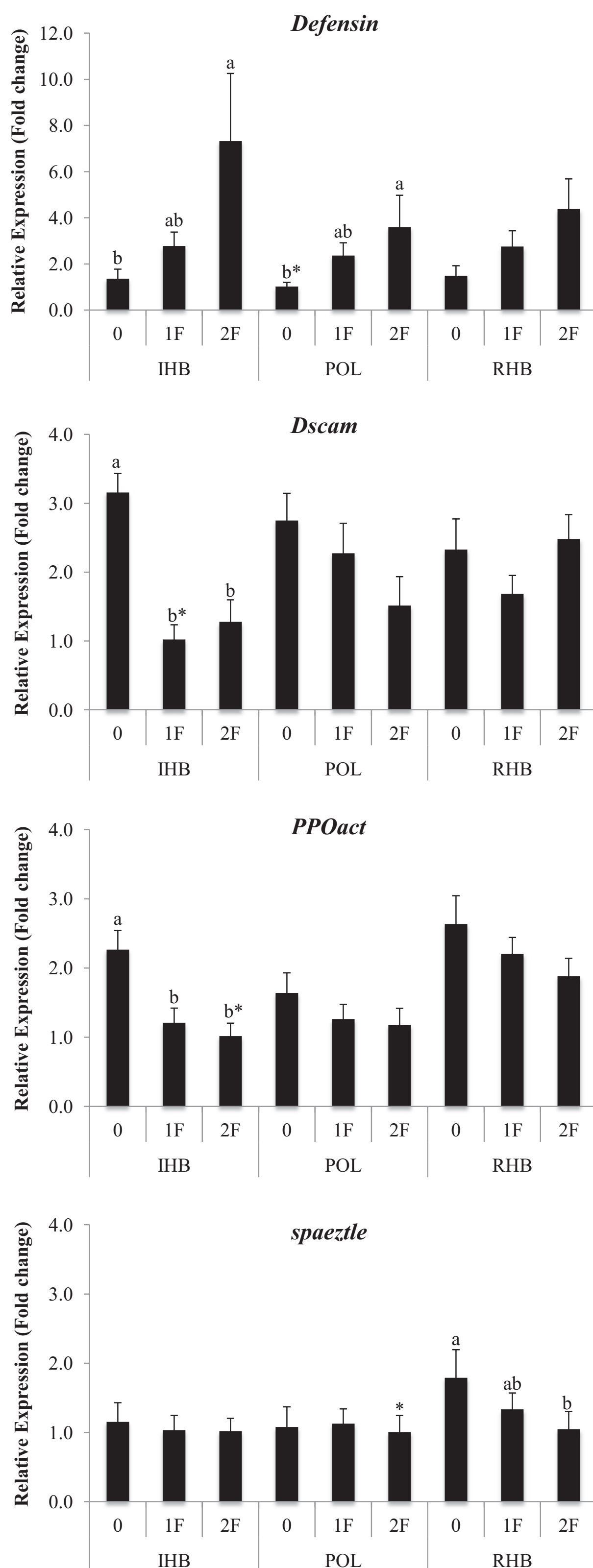

Fig. 4. Mean expression levels for *Defensin*, *Dscam*, *PPOact* and *Spaetzle* gene in pupae inoculated with different number of foundress *Varroa*. For each group, bars with different letters indicate significantly different transcript expressions for selected genes in honey bee stocks ($P < 0.05$). IHB = Italian honey bees, POL = VSH outcross, RHB = Russian honey bees. 0 = pupae with no mite inoculation, 1F = pupae inoculated with one foundress *Varroa*, 2F = pupae inoculated with two foundress *Varroa*. * Calibrator with the lowest expression level.

transmission to the pupal host and among infesting mites. Further, larger wounds increase the risk of bacterial or fungal infections. *Melissococcus plutonius*, causative agent of European foulbrood, has been isolated from wounds inflicted by *Varroa* mites (Kanbar and Engels, 2003). Another possibility, as suggested by Nazzi et al., is that mite feeding interferes with the immune response of bees and permits virus replication which leads to an increase in DWV levels.

Although *Varroa* mites are known vectors of DWV (Shen et al., 2005; Chen et al., 2006), our results indicate that the presence of nymphs did not intensify viral levels in their honey bee hosts, but rather, only the feeding of foundresses increases infection levels. It is possible that during feeding, any virus that the nymphs introduce into the feeding site has undetectable effects on the virus titers in the bees. Perhaps the protonymphs are not mature enough to support DWV replication or affect the host immunity. All of the inoculum mites were infected with DWV, therefore variation among DWV levels in stocks could not be influenced by the variation in the mites.

In nature, they rarely have multiple infestations of *Varroa* mites but the introduction of one or two foundress mites resulted in large DWV level increase indicating that while the bee stocks are resistant to *Varroa* (Danka et al., 2013; Rinderer et al., 2014b) they are still susceptible to DWV infection. However, other behavioral mechanisms against *Varroa* parasitism (e.g. brood removal) may indirectly contribute to low level of the overall virus infections by keeping low mite population in the colonies, and reduce the infected brood which can serve as the reservoir of the virus.

The expression of immune genes has been measured in response to *Varroa* feeding (Gregory et al., 2005; Yang and Cox-Foster, 2005; Nazzi et al., 2012), but the expression patterns have never been compared between mite resistant honey bee stocks. Twenty-four target genes were measured in response to inoculated mite feeding. Results here suggest that the different honey bee stocks have different immune responses to *Varroa* parasitism. Overall, 16 of the 24 genes tested were significantly different among stocks or treatments. In the IHB, eight target genes were either up- or down-regulated with mite feeding. The POL stock had two genes that were up-regulated. Finally, the RHB stock exhibited nine genes that were significantly down-regulated. The expression seen among the stocks may be indicative of stock variations in several genetically controlled immune responses.

Of all of the genes tested, only four genes (*Defensin, Dscam, PPO-act and spaetzle)* had significant differences in the levels of gene expression that correlated with mite numbers in one or more stocks (Fig. 4). Only *Defensin* expression increases with increased numbers of feeding foundress mites across all three stocks, but only significantly in IHB and POL. This up-regulation coincided with the findings of Kuster et al. (2014) which reported that pupae (240 h post capping) infested with three to five *Varroa* (regardless of mite stage or these are all foundress) had higher levels of *Defensin* as compared to that of the control (uninfested pupae). These results indicate that the expression of the antimicrobial peptide *Defensin* is turned on in response to mite feeding, perhaps as a defense mechanism. However, our data did not fully corroborate the findings of Gregory et al. (2005) which demonstrated that pupae infested with low numbers of *Varroa* (2–4 mites) showed a significant down-regulation of *Defensin*, but the suppression of these transcripts disappeared when bee pupae had higher numbers of mites feeding (5–6 mites). This discrepancy may due to the differences in the stages of mites considered in the two studies. Gregory et al. (2005) considered both foundresses and nymphs while this study only considered the number of foundress *Varroa*. Also, Gregory et al. (2005) did not measure DWV levels in their samples. In this study, all stocks were inoculated with DWV-infected *Varroa* mites. Thus, the increase of the transcription levels

observed in this study may be due to this concurrent exposure of pupae to *Varroa* and DWV. Further, the entry of other microorganisms through the wound sites from mite feeding may have triggered the up-regulation of *Defensin*.

The gene *PPOact* was down-regulated in IHB pupae inoculated with either one or two foundress *Varroa* and exhibited a similar but non-significant pattern in the other stocks. Prophenoloxidaes (PPOs) are important enzymes in the melanization process in insects, especially during microbial defense and wound healing. During feeding, mites inhibit melanization around the feeding site through an uncharacterized mechanism. However, the decreased expression of *PPOact* in infested brood suggests that the mites directly inhibit the regulation of the known melanization mechanism in the honey bee which may later increase the risk of microbial contamination or the spread of pathogens which in this case was DWV. In addition, *spaetzle* was down- regulated only in RHB pupae infested with two foundress *Varroa*. Our data corroborated the findings of Ryabov et al. (2014) which suggested that down-regulation of this gene occur as a response to both the presence of *Varroa* and an increase in DWV levels.

In conclusion, there is a considerable diversity of stress factors that can interfere with the immune system. These different stresses may account for the multitude of putative possible causes suggested in honey bee colony loss. By studying the immune responses of different honey bee stocks to various stressors, we can identify mechanisms that enhance survival and then select for these mechanisms in breeding programs. Research presented here suggests that not all honey bee stocks react to *Varroa* parasitism in similar ways. Thus, further studies on the interactions among *Varroa*, DWV and honey bee genetic traits may provide valuable information on immunity and resistant mechanisms of the honey bee against pests and parasites.

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References

Anderson, D.L., Trueman, J.W.H., 2000. *Varroa jacobsoni* (Acari: Varroidae) is more than one species. Exp. Appl. Acarol. 24, 165–189.

Annoscia, D., Piccolo, F.D., Nazzi, F., 2012. How does the mite *Varroa destructor* kill the honeybee *Apis mellifera*? Alteration of cuticular hydrcarbons and water loss in infested honeybees. J. Insect Physiol. 58, 1548–1555.

Boncristiani, H., Underwood, R., Schwarz, R., Evans, J.D., Pettis, J., Dennis vanEngelsdorp, D., 2012. Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. J. Insect Physiol. 58, 613–620.

Boncristiani, H.F., Evans, J.D., Chen, Y.P., Pettis, J., Murphy, C., Lopez, D.L., Simone-Finstrom, M., Strand, M., Tarpy, D.R., Rueppell, O., 2013. In vitro infection of pupae with Israeli acute paralysis virus suggests disturbance of transcriptional homeostasis in honey bees (*Apis mellifera*). PLoS One 8 (9), e73429.

Brachman, B., 2009. Up and running: Russian honey bee breeders. Bee Culture 137, 46–47.

Büchler, R., Berg, S., Le Conte, Y., 2010. Breeding for resistance to *Varroa destructor* in Europe. Apidologie 41, 393–408.

Chaimanee, V., Chantawannakul, P., Chen, Y.P., Evans, J.D., Pettis, J., 2012. Differential expression of immune genes of adult honey bee (*Apis mellifera*) after inoculated by *Nosema ceranae*. J. Insect Physiol. 58, 1090–1095.

Chantawannakul, P., Ward, L., Boonham, N., Brown, M., 2006. A Scientific note on the detection of honeybee viruses using real-time pcr (taqman®) in varroa mites collected from a Thai honeybee (*Apis mellifera*) apiary. J. Invertebr. Pathol. 91, 69–73.

Chen, Y.P., Siede, R., 2007. Honey bee viruses. Advances in Virus Research, vol. 70. Academic Press, pp. 33–80.

Chen, P., Pettis, J.S., Evans, J.D., Kramer, M., Feldlaufer, M.F., 2004. Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*. Apidologie 35, 441–448.

Chen, Y.P., Higgins, J.A., Feldlaufer, M.F., 2005. Quantitative real-times reverse transcription- PCR analysis of Deformed Wing Virus infection in the honeybee (*Apis mellifera* L.). Appl. Environ. Microbiol.. http://dx.doi.org/10.1128/AEM.71.1.436-441.2005.

Chen, Y., Evans, J.D., Feldlaufer, M., 2006. Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. J. Invertebr. Pathol. 92, 152–159.

Danka, R., Harris, J., Ward, K., Ward, R., 2008. Status of bees with the trait of Varroa-sensitive hygiene (VSH) for Varroa resistance. Am. Bee J. 148, 51–54.

Danka, R.G., Harris, J.W., Villa, J.D., Dodds, G.E., 2013. Varying congruence of hygienic responses to *Varroa destructor* and freeze-killed brood among difference types of honeybees. Apidologie 44 (1). http://dx.doi.org/10.1007/s13592-013-0195-8.

de Guzman, L.I., Rinderer, T.E., Lancaster, V.A., 1995. A short test evaluating larval attractiveness of honey bees to *Varroa jacobsoni*. J. Apic. Res. 34, 89–92.

de Guzman, L., Rinderer, T.E., Frake, A., 2007. Growth of *Varroa destructor* (Acari: Varroidae) populations in Russian honey bee (Hymenoptera: Apidae) colonies. Ann. Entomol. Soc. Am. 100, 187–195.

de Guzman, L., Rinderer, T., Frake, A., 2008. Comparative reproduction of *Varroa destructor* in different types of Russian and Italian honey bee combs. Exp. Appl. Acarol. 44, 227–238.

de Jong, D., De Jong, P.H., 1983. Longevity of Africanized honeybees (Hymenoptera: Apidae) infested by *Varroa jacobsoni* (Parasitiformes: Varroidae). J. Econ. Entomol. 76, 766–768.

de Jong, D., De Jong, P.H., Goncalves, L.S., 1982. Weight loss and other damage to developing worker honeybees from infestation with *Varroa jacobsoni*. J. Apic. Res. 21, 165–167.

de Miranda, J.R., Fries, I., 2008. Venereal and vertical transmission of deformed wing virus in honeybees (*Apis mellifera* L.). J. Invertebr. Pathol. 98, 184–189.

Di Prisco, G., Pennacchio, F., Caprio, E., Boncristiani Jr., H.F., Evans, J.D., Yanping Chen, Y., 2011. *Varroa destructor* is an effective vector of Israeli acute paralysis virus in the honeybee, *Apis mellifera*. J. Gen. Virol. 92 (1), 151–155.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L., Jiang, H., Thompson, G.J., Zou, Z., Hultmark, D., 2006. Immune pathways and defence mechanisms in honey bees *Apis mellifera*. Insect Mol. Biol. 15, 645–656.

Garrido, C., Rosenkranz, P., 2003. The reproductive program of female *Varroa destructor* mites is triggered by its host, *Apis mellifera*. Exp. Appl. Acarol. 31, 269–273.

Gilliam, M., Taber, S.I.I.I., Richardson, G.V., 1983. Hygienic behavior of honey bees in relation to chalkbrood disease. Apidologie 14, 29–39.

Gisder, S., Aumeier, P., Genersch, E., 2009. Deformed wing virus: replication and viral load in mites (*Varroa destructor*). J. Gen. Virol. 90, 463–467.

Gregorc, A., Evans, J.D., Scharf, M., Ellis, James D., 2012. Gene expression in honey bee (*Apis mellifera*) larvae exposed to pesticides and Varroa mites (*Varroa destructor*). J. Insect Physiol. 58, 1042–1049.

Gregory, P.G., Evans, J.D., Rinderer, T., de Guzman, L.I., 2005. Conditional immune-gene suppression of honeybees parasitized by *Varroa* mites. J. Insect Sci. 5, 1–5.

Harbo, J.R., Harris, J.W., 2009. Responses to Varroa by honey bees with different levels of Varroa Sensitive Hygiene. J. Apic. Res. 48 (3), 156–161.

Kanbar, G., Engels, W., 2003. Ultrastructure and bacterial infection of wounds in honeybee (*Apis mellifera*) pupae punctured by Varroa mites. Parasitol. Res. 90, 349–354.

Kanbar, G., Engels, W., 2005. Communal use of integumental wounds in honey bee (*Apis mellifera*) pupae multiply infested by the ectoparasitic mite *Varroa destructor*. Genet. Mol. Res. 4, 465–472.

Khongphinitbunjong, K., de Guzman, L.I., Burgett, D.M., Rinderer, T.E., Chantawannakul, P., 2012. Behavioural responses underpinning resistance and susceptibility of honeybees to *Tropilaelaps mercedesae*. Apidologie. http://dx.doi.org/10.1007/s13592-012-0129-x.

Kirrane, M.J., de Guzman, L.I., Rinderer, T.E., Frake, A.M., Wagnitz, J., Whelan, P.M., 2011. Asynchronous development of honey bee host and *Varroa destructor* (Mesostigmata: Varroidae) influences reproductive potential of mites. J. Econ. Entomol. 104, 1146–1152.

Kuster, R.D., Boncristiani, H.F., Rueppell, O., 2014. Immunogene and viral transcript dynamics during parasitic *Varroa destructor* mite infection of developing honey bee (*Apis mellifera*) pupae. J. Exp. Biol. 217, 1710–1718.

Li, W.F., Ma, G.X., Zhou, X.X., 2006. Apidaecin-type peptides: biodiversity, structure-function relationships and mode of action. Peptides 27, 2350–2359.

Livak, K.J., Schnittgen, T.D., 2001. Analysis of relative gene expression data using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25, 402–408.

Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E., Powell, M., Nikaido, S., Schroeder, D.C., 2012. Global honey bee viral landscape altered by a parasitic mite. Science 336 (6086), 1304–1306.

Nazzi, F., Brown, S.P., Annoscia, D., Del Piccolo, F., Di Prisco, G., Varricchio, P., Vedova, G.D., Cattonaro, F., Caprio, E., Pennacchio, F., 2012. Synergistic parasite–pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. PLoS Pathog. 8, e1002735.

Otvos, L.J., Insung, O., Rogers, M.E., Consolvo, P.J., Condie, B.A., Lovas, S., Bulet, P., Blaszczyk-Thurin, M., 2000. Interaction between heat shock proteins and 973 antimicrobial peptides. Biochemistry 39, 14150–14159.

Rinderer, T.E., de Guzman, L.I., Delatte, G.T., Harper, C., 2003. An evaluation of ARS Russian honey bees in combination with other methods for the control of Varroa mites. Am. Bee J. 143, 410–413.

Rinderer, T.E., Harris, J.W., Hunt, G.J., de Guzman, L.I., 2010. Breeding for resistance to *Varroa destructor* in North America. Apidologie 41, 409–424.

Rinderer, T.E., de Guzman, L.I., Frake, A.M., 2013. Associations of parameters related to the fall of *Varroa destructor* (Mesostigmata: Varroidae) in Russian and Italian honey bee (Hymenoptera: Apidae) colonies. J. Econ. Entomol. 106 (2), 566–575.

Rinderer, T.E., de Guzman, L.I., Frake, A.M., Tarver, Matthew R., Khongphinitbunjong, K., 2014a. An evaluation of the associations of parameters related to the fall of *Varroa destructor* (Mesostigmata: Varroidae) from commercial honey bee (Hymenoptera: Apidae) colonies as tools for selective breeding for mite resistance. J. Econ. Entomol. 107, 516–522. http://dx.doi.org/10.1603/EC13477.

Rinderer, T.E., Danka, R.G., Johnson, S., Bourgeois, L., Frake, A.M., Villa, J.D., de Guzman, L.I., Harris, J.W., 2014b. Functionality of varroa-resistant honey bees (Hymenoptera: Apidae) when used for western U.S. honey production and almond pollination. J. Econ. Entomol. 107 (2), 523–530. http://dx.doi.org/10.1603/EC13419.

Rothenbuhler, W.C., 1964. Behaviour genetics of nest cleaning in honey bees. IV. Responses of F1 and backcross generations to disease-killed. Am. Zool. 4, 111–123.

Ryabov, E.V., Wood, G.R., Fannon, J.M., Moore, J.D., Bull, J.C., Chandler, D., Mead, A., Burroughs, N., Evans, D.J., 2014. A virulent strain of deformed wing virus (DWV) of honeybees (*Apis mellifera*) prevails after *Varroa destructor*-mediated, or in vitro, transmission. PLoS Pathog. 10 (6), e1004230.

Shen, M.Q., Yang, X.L., Cox-Foster, D., Cui, L.W., 2005. The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. Virology 342, 141–149.

Simone, M., Evans, J.D., Spivak, M., 2009. Resin collection and social immunity in honey bees. Evolution 11, 3016–3022.

Sullivan, R., Fassolitis, A.C., Larkin, E.P., Read Jr., R.B., Peeler, J.T., 1971. Inactivation of thirty viruses by gamma radiation. Appl. Microbiol. 22, 61–65.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F., Colin, M.E., Bergoin, M., 2004. Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France. Appl. Environ. Microbiol. 70, 7185–7191.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 (research0034).

White, L.A., Freeman, C.Y., Hall, H.E., Forrester, B.D., 1990. Inactivation and stability of viral diagnostic reagents treated by gamma radiation. Biologicals 18, 271–280.

Yang, X., Cox-Foster, D.L., 2005. Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proc. Natl. Acad. Sci. U.S.A. 102, 7470–7475.

Yue, C., Genersch, E., 2005. RT-PCR analysis of Deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). J. Gen. Virol. 86, 3419–3424.