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Multiple benefits of breeding honey bees for hygienic behavior

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ABSTRACT

Honey bee colonies are prone to invasion by pests and pathogens. The combination of the parasitic mite *Varroa destructor* (*Varroa*) and the multiple viruses it vectors, is a major driver of colony losses. Breeding for hygienic behavior to reduce *Varroa* populations is considered a sustainable way to reduce the impact of *Varroa* on honey bee health. However, hygienic behavior may have a cost to the health of individual bees, both in terms of viral infection risk and immune function. To determine whether selection for hygienic behavior at the colony level is associated with trade-offs in honey bee viral infection and immune function, we compared *Varroa* populations, viral loads, and individual immune function between honey bee colonies that were bred for high and low hygienic behavior. Specifically, we measured *Varroa* infestation, Deformed wing virus DWV-A, DWV-B, Acute bee paralysis virus (ABPV), and Israeli acute paralysis virus IAPV viral genome levels in bee samples from artificially inseminated queens in our bi-directional selection program for hygienic behavior in Israel. In addition, we evaluated the expression of 12 genes from the Jak-STAT, Toll, IMD and RNAi immune pathways. We found significantly lower *Varroa* infestation and DWV loads in highly hygienic colonies than in colonies exhibiting low hygienic behavior. In addition, workers of the hygienic colonies had significantly higher expression of the immune genes *PGRP-S2* and *hymenoptaecin* compared to workers from low hygienic colonies. These results indicate no trade-offs in breeding for hygienic behavior. Hygienic honey bees were associated with reduced *Varroa* populations and reduced DWV prevalence or load at the colony level. Individual immunity of hygienic bees was increased, which could also contribute to lower virus levels, although lower *Varroa* levels due to social immunity presumably contributed as well. In sum, we demonstrate multiple health benefits of breeding for honey bee hygiene.

1. Introduction

The European honey bee (*Apis mellifera*) is an important pollinator of many essential crops (Klein et al., 2007; Potts et al., 2010). The honey bee populations in North America, across Europe, and in the Middle East are experiencing considerable annual losses (Gray et al., 2019, 2020; Lee et al., 2015; Soroker et al., 2011; van Engelsdorp & Meixner, 2010). These losses can be attributed to a combination of factors including parasites, pathogens, pesticide use (particularly insecticides), poor nutrition due to loss of habitat, invasive species, and climate change (Goulson et al., 2015; Sánchez-Bayo & Wyckhuys, 2019).

In particular, the combination of the parasitic mite *Varroa destructor* (*Varroa*) and the viruses it vectors, is a major driver of colony mortality (Martin et al., 2012; Traynor et al., 2020). Most *Varroa*-vectored viruses can exist asymptomatically in honey bee colonies, as covert infections. However, symptomatic viral infections can be induced by replication of the viruses inside the *Varroa* followed by their direct injection into bee hemolymph during *Varroa* feeding (Bowen-Walker et al., 1999; di Prisco et al., 2011; Kuster et al., 2014; Santillán-Galicia et al., 2010; Gisder and Genersch, 2021). We focus our study on the most prevalent *Varroa* transmitted viruses from two groups: deformed wing virus (DWV-A and DWV-B, *Iflaviridae*) and acute paralysis viruses (ABPV and IAPV, *Dicistroviridae*), both of which are associated with winter losses (Highfield et al., 2009; Berthoud et al., 2010). Four master variants have been

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described for DWV, namely DWV-A, DWV-B [previously designated at *Varroa* destructor virus 1 (VDV-1)], DWV-C, and DWV-D (de Miranda et al., 2022; Yañez et al., 2020). We focus on the most common DWV variants in Israel – DWV-A and DWV-B (de Miranda et al., 2022; Levin et al., 2016). Both of these variants are capable of infecting and replicating in the same bee (Dalmon et al., 2017; Gisder & Genersch, 2021; Mordecai et al., 2016a; Mordecai et al., 2016b) but with different degrees of virulence (Kevill et al., 2017). Honey bee infection by the members of the acute paralysis virus cloud (ABPV and IAPV) (Baker & Schroeder, 2008) follow a classic acute-type infection rapidly translated into overt symptoms of paralysis and ultimately honey bee death (Maori et al., 2007).

While there are no treatments against honey bee viruses, most beekeepers strive to reduce *Varroa* levels in their colonies as a means of reducing the source of virus transmission. However, conventional chemical treatments to reduce *Varroa* infestation are problematic due to their low efficacy, their susceptibility to the evolution of *Varroa* resistance, and their negative impact on bees and bee products (Tihelka, 2018). A more sustainable strategy to control *Varroa* and viruses is to utilize the honey bees' own innate defenses, including social and individual immune mechanisms.

Social barriers to pests and pathogens involve mechanical, physiological, and behavioral traits (Evans & Spivak, 2010). One important form of social immunity for honey bees is hygienic behavior (HB) (Evans & Spivak, 2010; Spivak & Danka, 2021; Wilson-Rich et al., 2009), a heritable trait in which bees detect and remove diseased, mite-parasitized, and dead brood (larvae and pupae) from the colony. Though results may vary by selection method, HB has been shown to be effective against *Varroa*, *Paenibacillus larvae* (the bacteria that causes American foulbrood), and *Ascosphaera apis* (the fungus that causes chalkbrood disease), but not against *Melissococcus plutonius* (the bacteria that causes European foulbrood) (Robertson et al., 2014; Spivak & Danka, 2021). Only few studies have investigated the hygienic response to virus-infected brood (Schöning et al., 2012; Roberson et al., 2014; Toufailia et al., 2014). Recently, Posada-Florez et al. (2021) showed that cannibalism associated with hygienic handling of *Varroa* and virus-infested brood can increase transmission of tagged DWV between workers via subsequent interactions of the hygienic nurse bees with nest mates. These findings raise the possibility that hygienic behavior may increase colony-level virus infection. Alternatively, individual and social immune mechanisms in hygienic colonies may be able to prevent or reduce viral transmission in the colony.

In addition to social immunity, honey bees, like other invertebrates, also rely on individual innate immune responses to defend against disease. Although operating both, social and individual immune mechanisms, may be evolutionary costly, findings by Harpur et al. (2014) indicated that there is no genetic tradeoff between social and individual immunity. However, the relation between social and individual immunity with respect to the protection of colonies against viral infections is not yet clear. Immune defense mechanisms include cellular responses like phagocytosis and encapsulation, which are mediated by hemocytes, as well as humoral immune responses. We focus on the humoral immune responses in four prominent pathways: Jak/STAT, Toll, IMD and RNAi. The last three are considered most significant for the defense against viruses (Barroso-Arévalo et al., 2019; Brutscher et al., 2015; Larsen et al., 2019) and include many genes that are upregulated in brains of DWV-infected workers (Pizzorno et al., 2021). Moreover, RNAi treatments proved effective against IAPV and DWV infections (Desai et al., 2012; Maori et al., 2009).

Elucidating the relationship between social and innate immunity is a critical step towards understanding the role of hygienic behavior in intracolonial transmission of *Varroa*-vectored viruses. Here, we compare *Varroa* infestation, honey bee viral load and prevalence, and immune gene expression between high and low hygienic colonies to determine whether colony level selection for hygienic behavior is associated with trade-offs in viral infection and immune function. The relationships between honey bee hygiene, virus infection, and immune response have important implications for honey bee health and the future of breeding for hygienic behavior.

2. Materials & methods

2.1. Honey bees

This research was conducted in the experimental apiary at the Volcani Center, Agricultural Research Organization (ARO), Israel in 2020 and 2021. The source population of honey bees was a local mixed population mainly composed of *Apis mellifera ligustica*, that has been subjected to a bidirectional selection program for high and low hygienic response to pin-killed brood assay (PKB) since 2012 (Selzer et al., 2022). At the beginning of this study (February to April 2020), 12 colonies were selected as parental colonies, and used to produce queens and drones for artificial insemination. High ("H") and low ("L") hygienic parent colonies differed by at least 30% in PKB tests as described in Selzer et al. (2022). The H colonies were defined as colonies in which above 75% uncapping occurred, and colonies were classified as L if <45% uncapping occurred after 24 h, in two consecutive PKB tests.

All the colonies in the apiary were regularly treated against *Varroa* by Amitraz loaded strips introduced twice a year (August and November). In April 2020, we crossed within the H colonies and within the L colonies via artificial insemination as in Seltzer et al. (2022). In brief, 73 nucleus colonies were created from random non selected colonies with equal bee population. Colonies contained one brood comb, two honey combs, and a virgin queen descendant from either an H or L colony ($n = 45$ and $n = 28$, respectively). At the age of 7–9 days, each queen was inseminated with 7–8 μl of sperm from genetically unrelated drones of the same phenotype. Only colonies with a successfully inseminated queen (31 HXH (H) and 12 LXL (L)) were used in the experiment. Experimental colonies were monitored for colony condition, hygienic behavior, *Varroa* infestation, virus load, and immune gene expression.

2.2. Colony condition

Colony condition was assessed by monitoring the number of populated frames and the presence of an egg-laying queen. Hygienic behavior was tested in colonies using a 24 h PKB assay at least 8 weeks after brood capping of the first produced brood, to ensure that the progeny of the inseminated focal queen was tested (at the end of June and four weeks later in July). A slightly modified 'pin test' was used (Büchler et al., 2013) - marking an area of 100 cells with pink eye pupae, which were approximately seven days old, and piercing them with a #2 entomological pin size. Cell uncapping and cleaning was assessed 24 h later and the percent of manipulated cells in the assay area was calculated. A cell was counted as uncapped when it was more than half opened and cleaned when no pupae remains were visible in the cell. In July, only the performance of 20 H and 12 L well-populated colonies were tested by pin test.

2.3. Varroa infestation

Varroa infestation was monitored using two methods: Counting natural mite fall onto sticky bottom board once a week for four weeks and performing an alcohol wash to quantify mites on workers. A sample of workers (70 on average) collected at the end of July from external frames of each surviving colony (19H and 10L) was washed in 96% ethanol and the number of *Varroa* dislodged from the bees was counted for each sample.

2.4. RNA extraction and cDNA preparation

A sample of ten workers per colony from the alcohol washes was

macerated as a pool for RNA extraction. Total RNA was extracted using TriReagent as described in (Zioni et al., 2011) and cDNA was prepared using RevertAid Reverse Transcriptase (Thermo Fisher Scientific Waltham, MA, USA) with oligo-dT and random primers according to the manufacturer's instructions. One thousand nanograms of RNA template were used to synthesize cDNA. For reverse transcription, RNA and primers were incubated at 65 °C for 5 min, followed by the addition of buffer containing 50 mM of Tris-HCl (pH 8.3), 75 mM of KCl, 2 mM of MgCl2, 5 mM of DTT, 4 units of RNase inhibitor Ribolock® (Thermo Fisher Scientific Waltham, MA, USA), and the reverse transcriptase (200 units; Thermo Fisher Scientific Waltham, MA, USA) in a 20 µl volume, and further incubation at 45 °C for 45 min. The reaction was terminated by heating at 70 °C for 10 min.

2.5. Virus and immune gene analysis

Virus and immune gene analysis were performed via qPCR as described before (Erez & Chejanovsky, 2020; Hou et al., 2014) using a PikoReal 96 machine (Thermo Fisher Scientific, Waltham, MA, USA) with a standard protocol (95 °C for 2 min; 40 cycles of 95 °C for 10 *sec*, 60 °C for 20 *sec*, and 72 °C for 20 *sec*). Each quantitative PCR analysis was performed in triplicate. Non-template controls (water) were included in triplicate for each assay. The KAPA SYBRR FAST qPCR Master Mix (5 µl) Universal (Kapa Bio-systems, Wilmington, MA, USA) was used in a 10 µl final volume. For each analysis, 2 µl of the diluted cDNA (250 ng/µl) were used (dilution factor = 4). For virus analysis, IAPV 7965F and 8191R, DWV-A 6138F and 6326R, DWV-B 6111F and 6299R, and the reference gene primers RPL8 F and RPL8 R were used as described previously (Evans, 2006; Hou et al., 2014; Zioni et al., 2011). For ABPV, the primers ABPV 5′-ACATTCTTTGATTCTGATGACGCT-3′ and 5′-TGCCGTTTTTGT GTTAGGTGG-3′ were used. For gene expression analysis, we tested 12 honey bee immune genes from four different pathways and vitellogenin, as described in Supplementary Table S1.

The expression of immune gene transcripts was calculated in two steps: first relative to gene expression of the reference gene: ΔCt = Delta Ct target gene-Delta Ct reference gene. At the second step, the number of gene copies were quantified based on the standard curves only for transcripts that showed significantly different expression between high and low hygienic colonies. Standard curve was prepared with amplicons of 100–220 bp containing the virus/gene target sequence. The amplicons were obtained by performing PCR with each set of primers. Then a 10-point standard curve was prepared from four-fold serial dilutions of the amplicons with known concentrations: from 4 pg (Ct = 10) and up to $7.6*10^{-6}$ pg (Ct = 25–28) (Erez & Chejanovsky, 2020). The specificity of the amplicons synthesized during the PCR run was ascertained by performing a dissociation curve protocol from 60° to 95°C. The efficiency of the DWV-A PCR reaction was E = 99%, R^2 = 0.9979, and the slope = 3.349. For DWV-B, the PCR reaction was E = 102.5%, R^2 = 0.9998 and the slope = -3.26. For ABPV, the PCR reaction was E = 99.98%, R^2 = 0.9986 and the slope = -3.32. For IAPV, the PCR reaction was E = 99.86%, R^2 = 0.9994 and the slope = -3.32. For *PGRP-S2*, the PCR reaction was E = 93.99%, R^2 = 0.9978 and the slope = -3.48. For *hymenoptaecin* the PCR reaction was E = 100%, R^2 = 0.9828 and the slope = -3.32. For *defensin1* the PCR reaction was E = 95.42%, R^2 = 0.9964 and the slope = -3.44. For reference gene, the PCR reaction was E = 99.787%; R^2 = 0.9986 and the slope = 3.31.

The virus loads/immune genes of one thousand nanogram total RNA extracted from the samples were calculated by plotting the Ct values against the logarithm of the RNA copy number using the PikoReal TM Software 2.2 (Thermo-Fisher Scientific). These values were used to calculate the genomic copy number in the RNA extracted from the pool samples after normalization to the reference gene. The genome copies were log transformed. For hygienic and non-hygienic groups, the total virus prevalence was calculated as number of positive tests (above 10^4 genome copies) out of the total number of colonies tested for each virus. To evaluate the impact of viruses on immune gene expression and hygienic behavior, we divided the colonies of both genotypes into the following categories – non-infected by viruses, and infected by both, paralysis virus complex (ABPV or/and IAPV) and deformed wing complex (DWV-A or/and B).

2.6. Statistical analysis

Statistical analysis was performed using JMP 16.0 for PC. ANOVA was used to compare the expression of different immune genes and viral loads (log transformed) between H and L colonies and between colonies that were highly infected or uninfected with the studied viruses. For ANOVA repeated measures analysis, rates of hygienic behavior (proportions of cells uncapped and cleaned) were subjected to arcsin transformation, while *Varroa* counts were transformed by SQRT transformation. Correlation analysis was performed between *Varroa* parameters (*Varroa* fall and ethanol wash), percentage of hygienic behavior (uncapped and cleaned), between viral load (log transformed) and immune gene expression (ΔCt) (using Multivariate function).

3. Results

3.1. Hygienic behavior

Eight weeks after queen establishment (first capped brood of the inseminated queen), hygienic PKB tests revealed a significant difference between the colonies from high and low origins in both cell uncapping and cleaning (ANOVA: Test 1- uncapped: $F_{(1,24)} = 10.55$, $p = 0.0035$, cleaned: $F_{(1,24)} = 21.83$, $p = 0.0001$, Test 2- uncapped: $F_{(1,25)} = 36.18$, $p < 0.0001$, cleaned: $F_{(1,25)} = 99.4$, $p < 0.0001$, Fig. 1). Hygienic behavior, uncapping and cleaning were highly correlated between the two tests (uncapping $r = 0.82$, $p < 0.001$, cleaning $r = 0.8$, $p < 0.001$). The second test, revealed more pronounced differences with low hygienic colonies (n = 10) uncapping and cleaning 0.47 ± 0.06 and 0.26 ± 0.05 of cells respectively, whereas high hygienic colonies (n = 19) uncapped and cleaned 0.94 ± 0.04 and 0.92 ± 0.04 of cells, respectively. The second test was more likely representative of the inseminated queen progeny and in close proximity to bee sample collection and thus used in the subsequent analyses.

3.2. Varroa infestation

Monitoring *Varroa* infestation by counting natural mite fall on bottom boards for 24 h (Fig. 2) revealed that colonies of high and low hygienic groups differed consistently and almost significantly in their

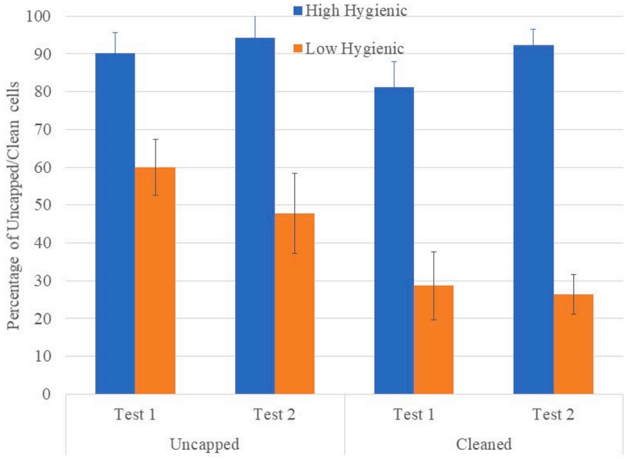

Fig. 1. The differences in hygienic behavior in high hygienic and low hygienic colonies measured with two consecutive pin-killed brood tests. Data represent average (+SE) percentages of cell uncapped and cleaned in June (test 1) and July (test 2).

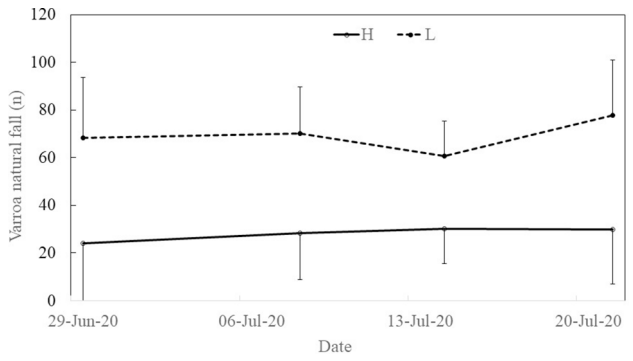

Fig. 2. *Varroa* infestation in High (H) and Low (L) hygienic colonies as assessed by natural mite fall within 24 h. The data represent averages (±SE) of 31 H and 12 L colonies.

Varroa load (Repeated measures ANOVA: $F_{(1,26)} = 4.18$, $p = 0.051$), with fewer *Varroa* in the H group. Correspondingly, the alcohol wash results, and last bottom board counts were significantly lower in H vs L colonies (T-test: alcohol wash: $t = 2.45$, $p = 0.021$, last bottom board count: $t = 2.19$, $p = 0.037$). These two parameters were also significantly correlated (Pearson correlation, $r = 0.6$, $p < 0.001$, Fig. 3A). When comparing both measures of *Varroa* levels (ethanol wash and *Varroa* natural fall) and the extent of two measures of hygienic behavior we found a significant negative correlation with proportion of cell cleaned, (Pearson correlation, ethanol wash: $r = -0.42$, $p = 0.029$ (Fig. 3B) and *Varroa* fall: $r = -0.41$, $p = 0.039$ (Fig. 3C)), but not with proportion of the uncapped cells.

3.3. Virus infection

All four measured viruses (ABPV, IAPV and DWV-A and B) were found in H and L hygienic colonies (Table 1). However, the distribution of the infection varied between the genotypes. In particular, 100% of the L colonies were infected by DWV-A, 90% by DWV-B, and 50% were also infected by one of the paralysis viruses. In one third of H colonies, none of the tested viruses were detected (Supplementary Fig. S1.). Contingency analyses found differences in viral infections between H and L colonies (Table 1). In H colonies we found significant positive correlation between ABPV and IAPV, and between DWV-A and DWV-B (Table S2). Interestingly, in the L colonies we found a positive significant correlation between ABPV and IAPV and a negative significant correlation between ABPV and DWV-A. Quantitative differences in viral load between the H and L colonies were merely close to significance (MANOVA: $F_{(1,27)} = 3.32$, $p = 0.08$). However, the prevalence of DWV-A was significantly lower in high hygienic colonies relative to low hygienic colonies ($\chi 2 = 5.28$, $p = 0.022$) and the viral loads (genomic copies) of DWV-B were significantly lower in H than in L hygienic colonies (ANOVA, $F_{(1,27)} = 4.48$, $p = 0.04$; Table 1).

3.4. Immune gene expression

The expression of *vitellogenin* and 12 genes from four different immune pathways was evaluated by the immune pathways considering genotype, the interaction between viral loads and immune genes in each of the genotypes (Table S2) and the interaction between immune genes in H infected vs uninfected and L infected colonies (Figs. 4 and 5, tables S3-S6).

In the Toll pathway, significant differences between H and L hygienic genotypes were detected only for *PGRP-S2* expression (ANOVA, $F_{(1,27)} = 4.36$, $p = 0.042$). The number of genomic copies of *PGRP-S2* was higher in H colonies than in L hygienic colonies (Average ± SE, H colonies: 3.84*10^6 ± 6.65*10^5 vs L colonies: 2.03*10^6 ± 9.17*10^5 genome copy number). We did not find a significant expression of *PGRP-S2* between H virus-infected and uninfected groups (Fig. 5). Within the pathway, we found positive significant correlation between *PGRP-S2* and *defensin1* only in H infected colonies (Fig. 4 and table S4). Between pathways, a positive significant correlation was found between *PGRP-S2* and *vago*, *hymenoptaecin* and *vitellogenin* in H uninfected and only with *PGRP-LC* in H infected colonies. (Fig. 4 and tables S3-S4). *PGRP-S2* was also significantly positive correlated with the rate of brood cleaning (Pearson correlation, $r = 0.4$, $p = 0.04$). Suggestive evidence for higher genome copy number in H than L hygienic colonies was also found for the antimicrobial peptide (AMP) *defensin1* (Average ± SE, H colonies:

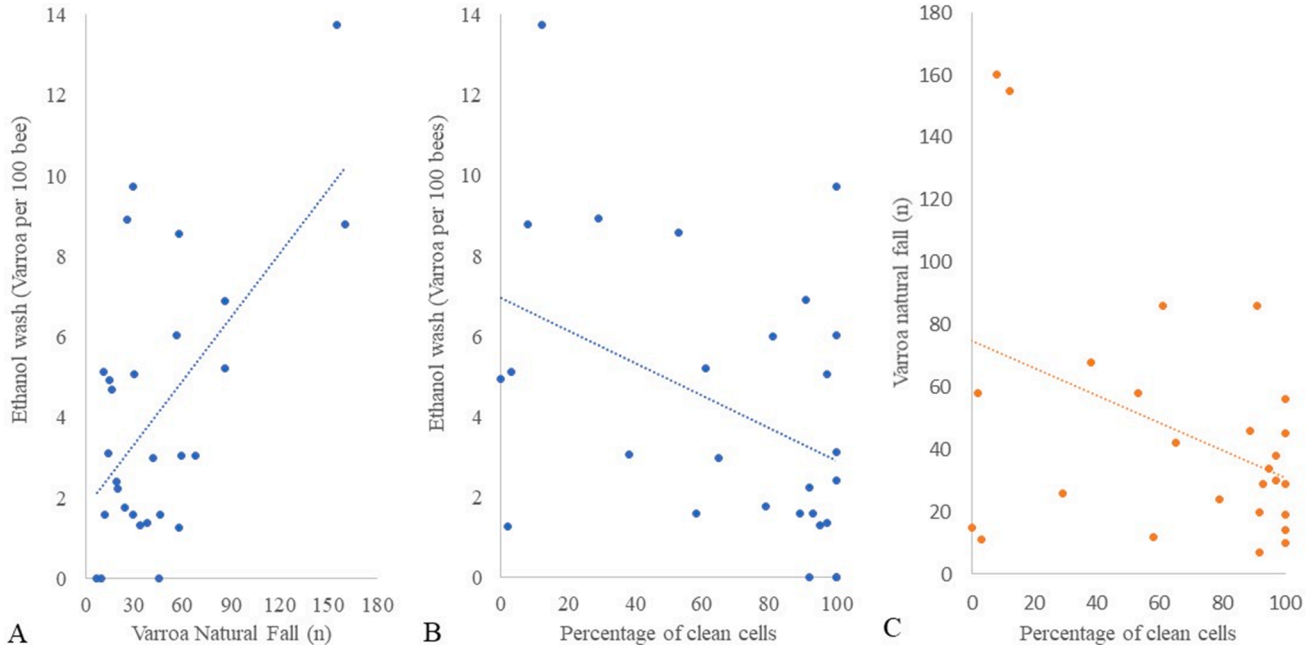

Fig. 3. Correlations between *Varroa* infestation levels, measured by natural mite fall and ethanol wash. and cell cleaning. (A) Positive relation between the two measures of *Varroa* levels ($r = -0.6$, $p < 0.001$). Cell cleaning performance, measured by PKB tests, was negatively correlated to mite levels, determined by ethanol wash (B; $r = -0.42$, $p = 0.029$) and natural mite fall (C; $r = -0.41$, $p = 0.039$).

Table 1
Differences in viral load and prevalence in H and L hygienic colonies, quantified by qPCR. Prevalence is shown as number of infected colonies out of total number of colonies tested.

Genotype	ABPV		IAPV		DWV-A		DWV-B	
	Prevalence	Load Mean Log ± SE	Prevalence	Load Mean Log ± SE	Prevalence*	Load Mean Log ± SE	Prevalence	Load Mean Log ± SE**
High hygienic	31.6% (6/19)	2.2 ± 0.76	26.0% (5/19)	1.4 ± (0.56)	73.7% (14/19)	5.8 ± 0.84	63.2% (12/19)	3.1 ± 0.59
Low hygienic	50.0% (5/10)	3.5 ± 1.1	40.0% (4/10)	2.1 ± (0.86)	100.0% (10/10)	7.3 ± 0.43	90.0% (9/10)	5.1 ± 0.66

* $X^2 = 5.28$, $p = 0.022$.
** $F_{(1,27)} = 4.48$, $p = 0.044$.

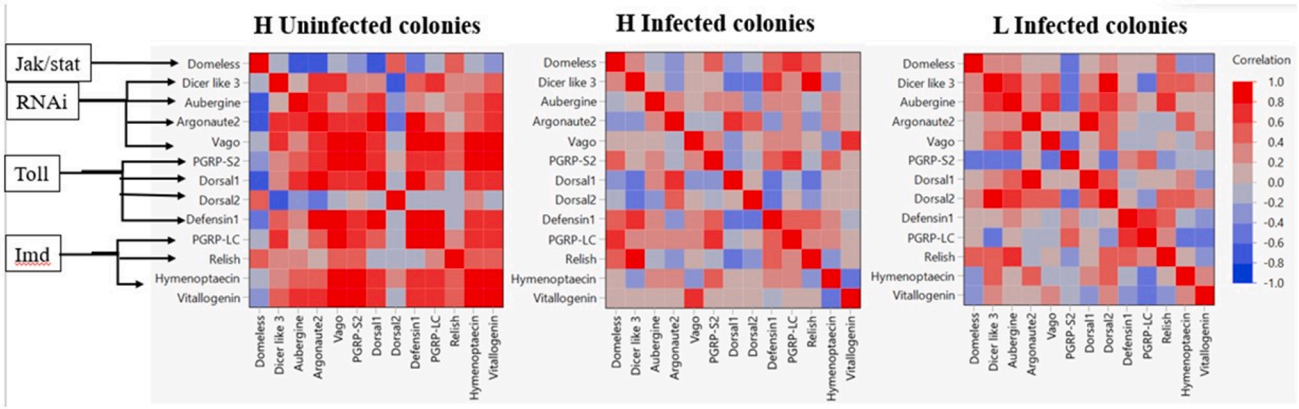

Fig. 4. Heat map of correlations between the relative expression of immune genes in uninfected (on the right; n = 5) and infected colonies (in the middle; n = 14) of H and L genotypes (on the left; n = 10).

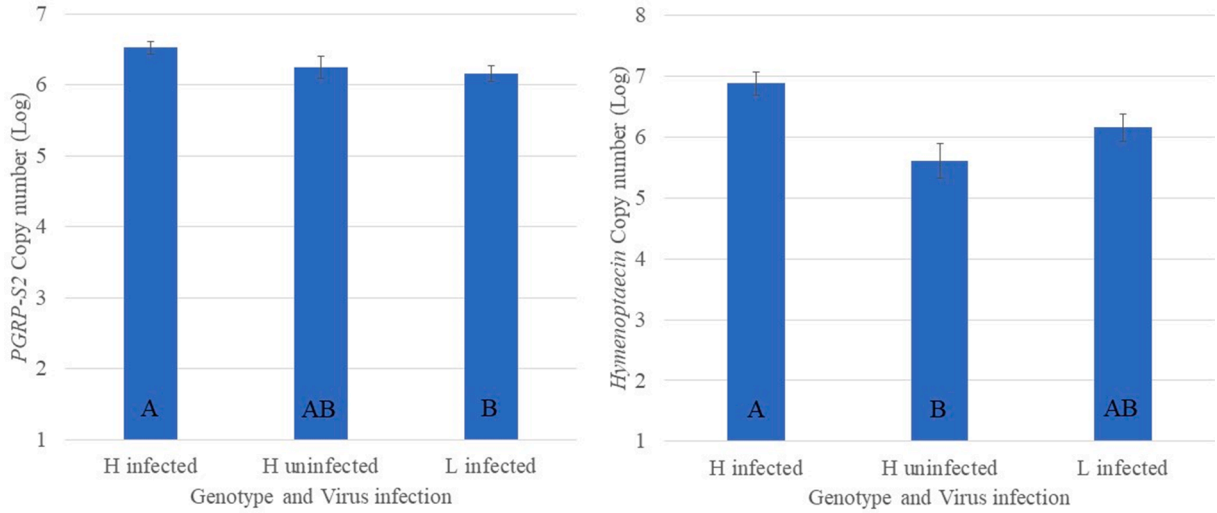

Fig. 5. Expression levels of *PGRP-S2* (on the left) and *hymenoptaecin* (on the right) in H uninfected and H infected and L virus infected colonies. The data are mean + SE log of genomic copies. Different letters indicate groups that are significantly different in ANOVA followed by Tukey HSD, $p < 0.05$.

4.57*10^6 ± 1.39*10^6 vs L colonies: 1.11*10^6 ± 1.19*10^6 genome copy number; ANOVA, $F_{(1,27)} = 2.74$, $p = 0.1$). Correlations between immune genes from the Toll pathway and viral loads in H an L colonies revealed that in H colonies all significant correlations were positive: *PGRP-S2* and DWV-A, *dorsal1* and IAPV, and *defensin1* and DWV-B, while in L colonies the significant correlations were not consistent: a positive significant correlation was found between *PGRP-S2* and DWV-A as opposed to negative significant correlations between *dorsal2* and DWV-A (Table S2(. Correlation patterns between and within immune gene from different pathways visually differ between uninfected and infected H and between infected L colonies (Fig. 4. and tables. S3, S4 and S5 respectively). Within the Toll pathway, in H virus-infected colonies *defensin1* and *dorsal1* and *dorsal2*, were significantly negative correlated, whereas in H uninfected colonies *defensin1* and *dorsal1* had significant positive correlation. No correlation within the Toll pathway was found in L infected colonies (Fig. 4 and table S6). Between pathways, H uninfected colonies had only positive significant correlation between *defensin1* and *vago*. H virus-infected colonies had negative significant correlation between *dorsal1* and *relish* and between *dorsal2* and *dicer-like3*. Positive significant correlation was found between *defensin1* and

domeless and *dicer-like3* and between *dorsal1* and *argonaute2*. Similarly, L infected colonies had positive significant correlation between *dorsal1* and *argonaute2*, but also and between *dorsal2*, *dicer-like3* and *aubergine*, and between *defensin1* and *PGRP-LC* (Fig. 4 and tables S3-5).

In the IMD pathway, a significant difference in expression of the AMP *hymenoptaecin* was found among H hygienic colonies: Higher genome copy number was found in H infected colonies (Fig. 5; ANOVA, $F_{(1,17)} = 11.72$, $p = 0.0038$) than in uninfected colonies. No such comparison was possible for L genotype since all the colonies were infected by at least one virus (Table 1). No significant correlations were found between the genes from the IMD pathway, in all the three groups (Fig. 4. and table S6). Regarding IMD immune genes and viral loads in H and L colonies, *relish* and ABPV, IAPV in H colonies were negatively significant correlated as well as *relish* and IAPV in L colonies. Whereas *hymenoptaecin* had only positive significant correlations with DWV-A, DWV-B and ABPV in H colonies and with DWV-A in L colonies (Table. S2). Correlations between pathways were highest in the H infected group, as we found positive significant correlation between *PGRP-LC* and *domeless*, *PGRP-S2* and *defensin, Relish* and *domeless*. A negative significant correlation was found between *dorsal1* and *dicer-like3* (Table. S4). H uninfected colonies had positive significant correlations between *PGRP-LC* and *vago* and between *hymenoptaecin* and *PGRP-S2* (Table. S3). L infected colonies were positive significantly correlated only between *PGRP-LC* and *defensin1* (Table S5).

Within the RNAi pathway we found positive significant correlations only in the L infected colonies, between *aubergine, dicer-like3* and *vago* (Table S6). Negative correlations between RNAi immune genes and viral load in H colonies were found between *dicer-like3* and IAPV, and in L colonies between ABPV and *dicer-like3* and *aubergine* (Table S2). In H infected and uninfected groups, we only found a positive significant correlation between *vago* and *vitellogenin*. Other than that, H uninfected colonies had positive significant correlations between *vago*, *PGRP-LC*, *defensin1* and *PGRP-S2*, and H infected colonies had positive correlations between *dicer-like3*, *defensin1* and *relish*, and between *argonaute2* and *dorsal1*. H virus-infected colonies had significant negative correlations between *dicer-like3* and *dorsal2* (Tables S3 and S4) opposed to positive significant correlation in L infected colonies. had also positive significant correlation between *aubergine* and *dorsal2* and between *argonaute2* and *dorsal1* (Table S5). Correlations between RNAi immune genes and viral load were positive and significant between *vago* and DWV-B. A negative significant correlation was found between cleaning behavior and *dicer-like3*, *aubergine* (Pearson correlation, *dicer-like3*: $r = -0.53$, $p = 0.005$, aubergine: $r = -0.53$, $p = 0.005$; Table S2).

Domeless, from the Jak-STAT pathway was non-significantly correlated with any immune genes, virus loads or genotype.

4. Discussion

Hygienic behavior, the uncapping and cleaning of infested or diseased brood cells is an important social immune mechanism of honey bees. Although costs of breeding for hygienic behavior on colony health have been recently postulated (Posada-Florez et al., 2021), our experimental evidence does not support this claim. Here we show that increased hygienicity of colonies is associated with lower levels of *Varroa,* reduced DWV load or prevalence, and with upregulation of some immune genes. This is consistent with previous findings indicating a lack of trade-offs in colony size or honey production associated with hygienic behavior (Leclercq et al., 2017; Selzer et al., 2022). Hygienic behavior protects more generally against a range of brood diseases (Spivak & Danka, 2021) and our study also shows that hygiene is associated with the reduction of at least one virus at the colony level. This finding contrasts with the idea that hygienic behavior may lead to the spread of viruses throughout the colony via cannibalism of the removed brood (Posada-Florez et al., 2021). It was suggested that hygienic behavior is directed particularly towards virus-damaged brood, which emit stimuli eliciting hygienic behavior (Schöning et al., 2012; Wagoner et al., 2019). However, we do not find increased viral titers or a suppression of individual immunity in workers from hygienic colonies.

Focusing on *Varroa*-vectored viruses of two major groups relevant for honey bee health, we found lower viral load and lower prevalence of DWV-B and DWV-A, respectively in hygienic colonies. This reduction in DWV could be a direct result of lower *Varroa* populations as shown before (Roberson et al., 2014; Emsen et al., 2015; O'Shea-Wheller et al., 2022), and in fact hygienic colonies in our study were also less infested with *Varroa.* As well, we found a positive significant correlation between cleaning behavior and *Varroa* infestation. At the population level, *Varroa* vectoring has a major impact on the prevalence and genotypic composition of DWV (Yañez et al., 2020). In addition, the H colonies may also be less affected by DWV due to an upregulated individual immune system. This could be a result of constitutively upregulated immune system in H colonies or of its highly effective induction due to higher rate of exposure to pathogens (Holmes & Johnston, 2021) during high hygienic activity. Both mechanisms could also act together to reduce DWV infection. The infections with paralysis viruses detected in hygienic colonies were also somewhat lower, but this decrease was not statistically significant. Potentially, a similar logic applies here but our dataset may not have been powerful enough to demonstrate the effect on IAPV or ABPV due to their overall lower prevalence. However, these highly virulent acute paralysis viruses may depend less on *Varroa* vectoring than DWV and therefore could be less affected by lower *Varroa* levels. In addition, the spread of DWV may rely more on brood infection (de Miranda & Genersch, 2010) than that of IAPV and ABPV (Chen et al., 2014) and therefore be more directly targeted by hygienic behavior. In order to show the impact of upregulated components of individual immunity on worker susceptibility to viruses, one useful approach can be the manipulation of immune gene expression via CRISPR/Cas9 or gene silencing (Li et al., 2016). Another approach can be to compare the ontogenesis of immune gene expression between the two genotypes under conditions of artificial infection with viruses.

To assess the interaction between social and individual immunity we measured the activity of the four most relevant immune pathways in both high and low hygienic bees, the Toll, IMD, Jak-STAT and RNAi pathways. Our results indicate significantly high expression of the gene *PGRP-S2* in high hygienic relative to low hygienic colonies, particularly in response to virus infection. *PGRP-S2* is a pattern recognition receptor (PRR) which recognizes pathogen associated molecules like peptidoglycan (PGN), functions in Toll-pathway activation (Brutscher et al., 2017; Wang et al., 2019) and has been identified as responsive to DWV infection before (Nazzi et al., 2012). Further indication of higher Toll pathway activity in highly hygienic colonies came from the enhanced expression of the AMP *defensin1*, although it deserves further investigation. The Toll pathway is generally known to be involved in a response to gram positive bacteria and fungi (Holmes & Johnston, 2021; Wang et al., 2019). Higher activation of the Toll pathway in high hygienic colonies may contribute to lower American foulbrood and chalkbrood diseases (Spivak & Danka, 2021). Within the Toll pathway we found significant correlations between four transcripts (*PGRP-S2, dorsal1, dorsal2, defensin1*) in H colonies, whereas in L colonies only *dorsal1* and *dorsal2* were significantly correlated. Interestingly, there were opposite correlations between the expression of *defensin1* and *dorsal*1 in uninfected colonies (positive correlation) and in infected colonies (negative correlation). This could be an indication of virus mediated down-regulation of *dorsal1* expression, as was found previously (Nazzi et al., 2012). Taken together, our findings indicate that the Toll pathway is up regulated in high hygienic colonies and could be involved in the honey bee immune response against viruses as was recently suggested (Brutscher & Flenniken, 2015). The significance of the Toll pathway during complex viral infection in honey bees requires further study because we found inconsistent relationships between the expression of two transcripts *dorsal1* and *2* and the load of acute paralysis viruses: significant negative correlation between *dorsal2* and ABPV and significant positive correlation between *dorsal1* and IAPV.

Another pathway that was affected by viral infection in the high hygienic genotype was the IMD pathway, as indicated by higher expression of the AMP *hymenoptaecin*. Similar effects of viral infection on *hymenoptaecin* expression were also reported previously (Brutscher et al., 2017; Kuster et al., 2014). Overall, the viral load was significantly correlated with expression of 8 out of 13 immune transcripts. Interestingly, the significant correlations of Toll and IMD transcripts with loads of DWV variants were all positive, while Toll, IMD and RNAi transcripts were mainly negatively correlated with the ABPV variants load. This might indicate that ABPV variants down-regulate these pathways. These findings are surprising since previously only DWV has been reported to suppress honey bee immune responses (di Prisco et al., 2016; Nazzi et al., 2012).

It is noteworthy that the correlations among immune genes are consistently stronger in virus-free H colonies than both the H and L colonies infected with virus. This result indicates overall that the induction and/or repression of immune genes by different viruses are nuanced and require further investigation overall. It is possible that not only the constitutively higher expression of *PGRP-S2* and *hymenoptaecin* contributes to the individual immunity of highly hygienic bees, but also a stronger induction of anti-viral pathways. Confirming this hypothesis will require *in vitro* infection of H and L colonies with a purified inoculum of specific viruses. Better individual immunity of honey bees selected for hygienic behavior may counteract the risk of individual infection and spreading of viruses through hygienic behavior and cannibalism (Posada-Florez et al., 2021). However, the colony-context also provides other mechanisms of reducing the spread of viruses, such as social isolation of virus-infected workers (Geffre et al., 2020) and self-removal of sick individuals (Rueppell et al., 2010) that are not available to bees in cage studies such as those of Posada-Florez et al. (2021). To disentangle the full interactions between hygienic behavior, individual- and colony-level virus loads, and individual immunity, future studies focused directly on colonies from hygienic lines with workers performing hygienic behavior on infected pupae are still needed.

In conclusion, our study demonstrates that breeding for hygienic behavior not only improves honey bee health by reducing the levels of *Varroa*, but also by reducing DWV at the colony level. The mechanisms of virus-reduction could be due to the reduced levels of the mite vector, however our data suggest that individual immunity may play an important role as well. Furthermore, we provide evidence against the theory that there is a trade-off between individual and colony-level immunity. Thus, our study results highlight multiple benefits of hygienic breeding for the improvement of honey bee health.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2022.107788.

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