Multi-tiered Analyses of Honey Bees That Resist or Succumb to Parasitic Mites and Viruses

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Research Article

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Posted Date: March 13th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-291062/v1

Multi-tiered analyses of honey bees that resist or succumb to parasitic mites and viruses

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Abstract

Background

Varroa destructor mites, and the numerous viruses they vector to their honey bee hosts, are among the most serious threats to honey bee populations, causing mortality and morbidity to both the individual honey bee and colony, the negative effects of which convey to the pollination services provided by honey bees worldwide. Here we use a combination of targeted assays and deep RNA sequencing to determine host and microbial changes in resistant and susceptible honey bee lineages. We focus on three study sets. The first involves field sampling of sympatric western bees, some derived from resistant stock and some from stock susceptible to mites. The second experiment contrasts three colonies more deeply, two from susceptible stock from the southeastern U.S. and one from mite-resistant bee stock from Eastern Texas. Finally, to decouple the effects of mites from those of the viruses they vector, we experimentally expose honey bees to DWV in the laboratory, measuring viral growth and host responses.

Results

We find strong differences between resistant and susceptible bees in terms of both viral loads and bee gene expression. Interestingly, lineages of bees with naturally low levels of the mite-vectored Deformed wing virus, also carried lower levels of viruses not vectored by mites. By mapping gene expression results against current ontologies and other studies, we describe the impacts of mite parasitism, as well as viruses on bee health against two genetic backgrounds. We identify numerous genes and processes seen in other studies of

stress and disease in honey bee colonies, though we find novel genes and new patterns of expression too.

Conclusions

We provide evidence that honey bees surviving in the face of parasitic mites do so through their abilities to resist the presence of devastating viruses vectored by these mites. By revealing responses to viral infection and mite parasitism in different lineages, our data identify candidate proteins for the evolution of mite tolerance and virus resistance.

Background

Parasitic mites present the single greatest threat to managed and wild honey bee (*Apis mellifera*) colonies in much of the world. The mite *Varroa destructor* strongly impacts honey bee colonies on all continents except Australia and Antarctica [1]. These mites directly impact honey bee health [2] and transmit a range of devastating RNA viruses within and across colonies. Among the mite-vectored viruses, the Deformed wing viruses (DWVs) are implicated in bee colony losses in Europe [3] and North America [4]. Understanding the genetic patterns behind resistance and tolerance to parasitic mites and the viruses they transmit is key for effective breeding programs aimed at reducing the risks and management costs of these threats.

We conducted three experiments to examine differences in gene expression patterns and virus infection levels in populations of honey bees with distinct genetic backgrounds and phenotypes - those tolerant of *Varroa* and resistant to honey bee viruses (R), and those susceptible to *Varroa* and/or viruses (S). Experiment 1 assessed natural virus infection loads and immune gene expression in 30 colonies in the field, using quantitative PCR to characterize differences between 15 R colonies, and 15 S colonies. Experiment 2 explored RNA sequencing data for gene-expression differences and pathogen levels distinguishing mite-infested bees of the R and S genetic backgrounds from sister bees that were verified to be mite-free. RNA sequencing data revealed notable transcriptional differences between mite-infested and un-infested honey bees, and between Resistant and Sensitive phenotypes. Reasoning that laboratory injections of bees with viruses would allow us to decouple gene

expression patterns attributable to mite infestation from differences resulting from viral infection in field colonies, we conducted Experiment 3: injecting *Varroa*-free bee pupae in the laboratory with Deformed wing virus (DWV) or a phosphate buffer solution, and subsequently collecting RNA for sequencing. DWV injection evoked gene expression patterns that differed from bees injected with only PBS. Transcriptional profiles of virus-resistant bees injected with DWV were markedly different from virus-sensitive bees, while other gene expression contrasts emerged with buffer injection. The salient gene expression patterns that distinguish R and S bees in the field and laboratory, under a variety of experimental conditions, demonstrate important differences between the two genetic backgrounds in their response to *Varroa destructor* parasitism (mite-infested or un-infested) and Deformed wing virus infection. Equally important, our results allow differentiation of honey bee gene expression signals associated with viral infection in the presence and absence of mite parasites. These results have bearing on programs to understand host-parasite coevolution in a social insect and might be applied toward more sustainable strategies for reducing the impacts of parasitic mites on bees.

Methods

Experiment 1: Natural responses in susceptible and resistant lineages

To assess the impacts of mite parasitism on gene expression and virus loads, 30 colonies were used. These colonies were on a migratory beekeeping path, spending fal, winter, and spring in Texas, and late spring through summer in Montana (where they were sampled).. A total of 15 mite-resistant (R) and 15 mite-susceptible (S) cohorts were sampled. R colonies were headed by queens bred from resistant stock in Texas while queens heading

80 the S colonies were originally from California. Colonies of both R and S bees were
81 managed without application of acaricides or utilization of other beekeeping practices for
82 Varroa control and mite resistance was established based on long-term survivorship and
83 the absence of viral pathologies. Sections of capped honey bee brood were cut from these
84 30 colonies. Worker honey bees were collected as they emerged from brood cells and these
85 cells were simultaneously screened for the presence of *Varroa destructor* mites. Only bees
86 from mite-free cells were utilized. Total RNA was extracted from individual bees using
87 TRIzol® (ThermoFisher) following manufacturers' protocol, generating *ca*. 50 µg total
88 RNA per bee at 1 µg/µl . First-strand cDNA was generated from 1µl of this RNA using
89 random hexamer primers and Superscript II (ThermoFisher) following manufacturers'
90 protocol. Targets were screened using quantitative real-time PCR and appropriate primers
91 for honey bee immune genes Hymenoptaecin and Eater, for viral pathogens Deformed
92 wing virus, Black queen cell virus, and Kashmir bee virus, and for an endogenous control
93 gene (*b-Actin*) as described in [5].
94
95 *Experiment 2: Impacts of Varroa mites and natural DWV infection in susceptible and*
96 *resistant bees*
97 Sets of 30 parasitized and 30 mite-free bees were collected from two mite-susceptible
98 honey bee colonies maintained at the USDA-ARS BRL, Maryland, and from a single
99 colony from a mite-resistant population (Navasota, TX). Total RNA was extracted from
100 individual bees, as above. Pools of RNA were generated using equimolar extracted RNA
101 from the bees of each colony and approximately 8µg total RNA was used directly to

generate libraries for ILLUMINA paired-end 150 base-pair sequencing at the University of Maryland Institute for Genome Sciences.

Experiment 3: Impacts of Deformed wing virus on Resistant and Susceptible Bees

Intact honey bee brood frames were collected from each of 10 colonies maintained in apiaries near Navasota, Texas. 30 white-eyed pupae were removed from mite-free brood cells in brood comb. Bees were injected with 1µl of PBS alone, or PBS containing ca. 10^7 DWV viral copies. DWV suspensions for injection were prepared by extracting hemolymph from adult worker honey bees from the USDA-ARS Bee Research Laboratory apiaries (Beltsville, MD) showing the pathology of DWV infection, deformed wings. After virus injection, pupae were allowed to develop on folded Whatman paper in petri dishes incubating at 34°C with controlled relative humidity. Total RNA was extracted from 15 bees either injected with PBS or DWV. Quantitative PCR was performed on aliquots of RNA from individual bees using primers for Hymenoptaecin, Eater and DWV, as above. RNA from bees of two colonies showing higher mean DWV levels after injection, and RNA from bees of two colonies with stable mean DWV levels after injection were pooled for RNA sequencing. Pools of RNA were generated using equimolar extracted RNA from the bees of each colony, and approximately 8µg total RNA was used directly to generate libraries for ILLUMINA sequencing, as above.

Raw sequence reads in FastQ format were trimmed using Trim Galore. Remaining adapter sequences were removed and sequences with quality scores < 25, as well as reads < 35bp, were also removed. Trimmed FastQ files were aligned to the Honey Bee (*Apis mellifera*) Genome Amel HAv3.1 using HiSAT2 [6] or STAR [7]. Features (genes,

125 transcripts and exons) were counted using featureCounts [8] and StringTie [9] using the
126 GCF_003254395.2_Amel_HAv3.1 gene annotations [10]. Reads not aligning to the honey
127 bee genome were subsequently aligned to the HolobeeBar microbial database
128 (https://data.nal.usda.gov/dataset/holobee-database-v20161) using Bowtie 2 [11]. Basic
129 pairwise differential expression analysis was performed using EdgeR [12] and DESeq2
130 [13].

131 Differential gene expression patterns were derived from RNA sequencing data
132 using read alignments and read counts from the DESeq2 package. Genes showing up-
133 regulation or down-regulation were compiled for all pairwise comparisons of genetic
134 background (R v. S) and biological condition, mite-infested or mite-free and virus-injected
135 or buffer-injected. Results were filtered for all evaluated contrasts by excluding any gene
136 that had a False Discovery Rate (FDR) greater than or equal to 0.05. Increased gene
137 expression levels (UP) or decreased gene expression levels (DOWN) compared to an
138 expression standard (*b-Actin*) are limited to those genes with a FDR<0.05 and that have a
139 log2 Fold Change (logFC) of > +1.5, or <-1.5 for UP-regulated expression and DOWN-
140 regulated expression, respectively.

141 Unfortunately, many honey bee genes remain poorly characterized, and while gene
142 expression responses to the environmental and experimental conditions examined here are
143 inevitably complex, these factors impede interpretation of our differential gene expression
144 results. Thus, to enrich insights gleaned from our differential gene expression results, we
145 employed HymenopteraMine [14], a sophisticated genomic data analysis environment.
146 HymenopteraMine facilitated exploitation of gene annotation information, simplified
147 comparison of genes sets differentially expressed in the many combinations of genetic

lineage and experimental conditions, and enabled easy gene ontology enrichment (GO enrichment) analyses.

We engaged gene ontology (GO) annotations and the GO biological process, molecular function and cellular compartment enrichment widgets (GO BP, GO MF and GO CC) available at HymenopteraMine to analyze the genes UP and DOWN in S_mite v. R_mite, genes UP or DOWN in S_virus v. R_virus, and genes differentially expressed in other sample comparisons. When GO enrichment results were available, they were used to provide insights into the gene expression patterns that distinguished resistant and susceptible bees under the biological conditions we evaluated using RNA sequencing data - natural mite infestation and latent viral infection, and laboratory injection of DWV virus or a phosphate buffer solution. We displayed results using available tools (e.g., ReviGO for visualizing Gene Ontology patterns, http://revigo.irb.hr/) and custom developed scripts.

Results

Experiment 1: Evaluation of Susceptible (S) and Resistant (R) colonies for virus loads and immune gene expression levels

Colonies from both R and S stock both lacked pathologies typical oif heavily infected honey bees. To determine whether these lineages differed in gene expression and viral loads, colonies were screened using quantitative-PCR (qPCR). Comparing qPCR data of bees from R colonies to data of bees from S colonies, we observed that R bees exhibited much lower natural levels of Deformed wing virus (DWV), as expected (Figure 1). These bees also showed decreased levels of Black queen cell virus (BQCV), a virus not

171 transmitted by mites, and Kashmir bee virus (KBV) than did S bees. S bees also expressed
172 much higher levels of the cellular immunity genes Eater and Nim2C than did R bees.
173 Strikingly, resistant bees showed higher levels of the gene encoding the antimicrobial
174 peptide Hymenoptaecin and this was a major driving force in separating the two sample
175 classes by cluster analysis (Figure 2).
176
177 *Experiment 2: Gene expression in R and S genetic backgrounds, with and without Varroa*
178 *mites.*
179 We used RNA sequencing to compare gene expression levels between R and S bees
180 collected from field colonies under natural conditions, comparing transcriptional profiles
181 of R bees with and without Varroa mites to S bees with and without mites. Gene-expression
182 signals were analyzed for statistically meaningful differential expression using DESeq2.
183 Increased gene expression levels (UP) or decreased gene expression levels (DOWN)
184 compared to an expression standard (*b-Actin*) are limited to those genes with a FDR<0.05
185 and that have a log2 Fold Change (logFC) of > +1.5, or <-1.5 for UP-regulated expression
186 and DOWN-regulated expression, respectively. We sequenced RNA of bees from R and S
187 colonies, both from bees with mites present (R_mites; S_mites), and from bees without
188 mite infestation (R_control; S_control). Strikingly, 89% of the variance in differential gene
189 expression among bees from S and R colonies, under the two biological conditions of
190 Experiment 2 (with and without mites), was explained by Principal Component 1 (74%)
191 and Principal Component 2 (15%) in a Principal Component Analysis. See Figure 3 below.
192 Transcriptome-wide analyses tend to cluster both mite and control samples from resistant
193 bees, while showing a sharp disparity in gene expression between the two conditions for

194 the susceptible colonies, highlighting the impacts of mite presence on those lines (Figure
195 S1).

196 In total, 699 honey bee genes were expressed at higher levels in S bees with mites
197 than in R bees with mites (UP in S_mite v. R_mite), including miR-3726 and miR3729
198 with 9.51 and 13.87 log fold elevation in S_mite over R_mite, respectively. Several
199 immune effectors were among those overexpressed genes in S_mite v. R_mite samples,
200 including the effectors Apidaecin, Defensin-1, Defensin-2, and Hymenoptaecin, and two
201 peptidoglycan recognition proteins (GB47805 and GB47804). Differentially expressed
202 genes up-regulated in S bees exposed to mites compared to R bees exposed to mite have
203 GO enrichment results for the Gene Ontology biological processes *cell adhesion*, *cell*
204 *surface receptor signaling* and *biological adhesion*, as well as *aminoglycan metabolism*
205 and *glucosamine compound metabolism*. Cell adhesion molecules and other cellular
206 membrane components are widely implicated as receptors for viral entry into host cells,
207 and subsequent cell receptor signaling aids viral internalization and hijacking of cellular
208 machinery for virus replication [15, 16]. Some aminoglycan derivatives can serve as
209 receptors for virus entry as well as cell and membrane adhesion molecules. Chitin, a
210 principal component of the exoskeleton, is a structural aminoglycan, and Varroa mites must
211 penetrate chitin with their mouthparts to feed on honey bees, and bees must repair that
212 damage to avoid desiccation, infection by microbial species and death. The increased
213 expression of genes involved in these biological processes in mite-susceptible and virus-
214 sensitive S bees have obvious implications in promoting vulnerability to virus infection
215 and compensating for damage inflicted by mite parasitism.

The 699 genes DOWN in R_mite v. S_mite depicted in Figure 5(a) above (equivalent to genes UP in S_mite v. R_mite) have GO enrichments for cell adhesion, aminoglycan, chitin and glucosamine metabolic processes and cell surface receptor signaling pathways, as well as chitin binding, extracellular matrix constituents and structural cytoskeleton functionalities. The complete list of GO enrichments is found in Additional File 1, sheet 1, Excel workbook "GO enrichments for genes UP & DOWN in R_mite v. S_mite"; Sheet 2 "GO DOWN in R_mite v. S_mite".

The 832 genes UP-regulated in R_mite v. S_mite depicted in Figure 5(b) above (equivalent to genes DOWN in S_mite v. R_mite) have GO BP enrichments for nucleic acid metabolism, RNA processing, non-coding RNA metabolism, gene expression, DNA metabolism, DNA repair and cellular response to DNA damage stimulus, mitochondrial gene expression, peptide metabolism and peptide biosynthesis, translation, methylation and cellular response to stress. 24 genes related to cellular response to DNA damage and DNA repair that are elevated in R_mite v. S_mite are apparent homologs or orthologs of dipteran and mammalian genes involved in all distinct DNA repair mechanisms: mismatch base repair, nucleotide and base excision repair, interstrand crosslink repair, non-homologous end-joining and homologous recombination; as well as genes affecting cell cycle arrest and DNA damage checkpoints. 10 of these 24 genes are also UP in R_mite over R_control expression levels. This suggests that DWV and Varroa radically disrupt normal gene expression activity and may inflict substantial damage to host DNA. At least one positive-strand RNA virus in humans - Hepatitis C virus - causes significant DNA lesions in hepatocytes, and there are many more examples of viruses generally with oncogenic/mutagenic activity. Fundamental metabolic changes differentiating R and S

bees affected by Varroa and DWV, especially gene expression program alterations, nucleic acid synthesis, and DNA repair processes, that could compensate for the various pathogenic disruptions of DWV and Varroa, may be key to the enhanced viral and Varroa resistance of R bees. The complete list of GO BP enrichments from genes UP in R_mite v. S_mite is given in Additional File 1, Sheet 2: "GO from genes UP and DOWN in R_mite v. S_mite", Sheet 1: "GO UP R_mite v. S_mite".

Genes up-regulated in S_control v. R_control, where mites are absent also produce many GO BP enrichments, some of the more interesting indicate that energy metabolism, ribonucleoside and nucleotide synthesis, and translation of mRNA to protein are all elevated in S over R bees without mites. The GO Biological Process enrichments are listed in Additional File 1, Sheet 3. For 532 genes UP in R_control v. R_mite, GO enrichments include regulation of transcription, regulation of macromolecule biosynthetic process, regulation of gene expression, cellular adhesion processes, regulation of RNA metabolic processes, and transcription factor activity among others, and are described in Additional File 1, Sheet 4. GO annotations from the 651 genes DOWN in R_control v. R_mite (equivalent to genes up-regulated in R_mite v. R_control) reveal enrichment for DNA biosynthesis, metabolic processes including noncoding RNA processing, peptide processing, cellular response to stress, DNA repair and response to DNA damage, as more fully depicted in Additional File 1, Sheet 5.

Surprisingly, there are very few genes differentially expressed when comparing S_control to S_mite, and in fact there are none UP in S_control over S_mite; so, a general down-regulation of gene expression by Varroa finds no support in our evidence. There are only 16 OGSv3.2 genes DOWN in S_control v. S_mite, yielding no GO enrichment results

for that comparison. The most notable genes down-regulated in S_control v. S_mite - that is, genes with elevated expression in S when Varroa are present are: Cytochrome P450 6A1, Fibroin heavy chain, IL-1 receptor, lactate dehydrogenase, and one of five genes identified as a homolog or paralog of protein lethal(2) essential for life - GB45910 (724367) , plus Hymenoptaecin, and bone morphogenetic protein 2-B.

Another contrast worth consideration is the asymmetric difference of DOWN in R_control v. R_mite MINUS DOWN in S_control v. S_mite, potentially focusing on the differential expression that is unique between R bees with no mites and R bees with mites and natural DWV loads, eliminating any genes that show expression differences when comparing mite-free S bees to mite-infested S bees with natural DWV infection levels. This contrast produces 607 genes with some intriguing GO enrichment results. Among the more interesting are GO BP enrichment for nucleic acid metabolic processes, noncoding RNA metabolic process, ncRNA processing, primary metabolic processes, cellular response to stress, cellular response to DNA damage stimulus, gene expression, response to stress, cellular biosynthetic processes, macromolecule biosynthetic process and methylation; and GO MF enrichment for terms as follows: catalytic activity acting on RNA, nuclease activity, methyltransferase activity, catalytic activity acting on DNA, RNA methyltransferase activity, ligase activity and DNA polymerase activity.

Experiment 3: Differential gene expression of resistant and sensitive bees after injection with DWV or a saline control.

Viral loads and immune gene expression differed significantly across colonies for bees injected with live DWV and those injected with PBS (Figure 6). Pupae from some

285 colonies showed high viral loads with either condition (e.g., sources B4, G3, G4, and G7),
286 suggesting high existing levels of viral infections that were arguably amplified regardless
287 of the addition of new viral copies. Others (B2, B3, B5, G5) showed significant increases
288 in viral loads primarily in the bees injected with live virus. Two sources (B5 and G2) held
289 relatively low average viral titers with both control and live virus injections.

290 Samples of pooled half-sisters from colonies that exhibited little or no increase in
291 mean DWV titers after DWV injection (virus resistant (R)), were compared to samples of
292 pooled half-sisters that demonstrated increases in mean viral loads after DWV injection,
293 (virus sensitive (S)). Again, strong contrasts were revealed when examining comparative
294 responses to virus injection in R and S genetic backgrounds, and other disparate gene
295 expression differences emerged when comparing the response to PBS injection. Both the
296 identity of genes exhibiting expression level changes, and the direction of expression level
297 change (elevated or reduced expression - UP or DOWN) differed among Varroa-free bees
298 drawn from Resistant and Sensitive colonies, as shown by Principal component analyses
299 (Figure S2).

300

301 *Genes expressed more in R_virus than in S_virus*

302 192 genes were UP in R_virus over S_virus, and produced GO BP enrichments for defense
303 response, oxidation-reduction processes, immune response, innate immune response and
304 immune system process (Figure S3; Additional File 1, Sheet 6). However, 75 of these genes
305 had no GO BP annotation. The genes producing the GO BP enrichment for immune
306 response, immune system process and defense response included Defensin-1 (GB41428),
307 peptidoglycan recognition protein 1 (GB47804), peptidoglycan recognition protein-2

(GB47805), leucine-rich repeat-containing protein 26 (GB44192), Hymenoptaecin (GB51233), and GB54506, an uncharacterized protein having scavenger receptor activity, binding acetylated and oxidized LDLs, bacteria, apoptotic cells, and advanced glycation end products, and delivering those ligands into the cell. The genes expressed at higher levels in R bees injected with virus than S bees injected with virus also yield GO molecular function enrichment for endopeptidases and other functional enzymes (Additional File 1, Sheet 7).

Many interesting genes were expressed at higher levels in R bees injected with virus. These genes included Vg or Vitellogenin (GB49544); Pla2 or Phospholipase a-2 (GB48228) a venom component; PGRP-S2 (GB47805) encoding a peptidoglycan recognition protein; SP34 (GB48510), a venom serine protease; and Est-6 or carboxylic ester hydrolase (GB53756) - all are expressed at much higher levels in R_virus than S_virus. AGLU2 (GB43248) and Hbg2 (GB54549) two of three honey bee alpha-glucosidases, Obp-3 (GB53371), and Apid-1 (GB47546), or Apidaecin, are also among the top 50 more elevated in R_virus v. S_virus with log2 Fold Change greater than 3. Defensin-1 (GB41428) and Ecdysis triggering hormone (GB40094) were also among the more highly expressed in R bees compared to S bees injected with virus, both with log FC > 2.9. PPO or Prophenoloxidase (GB43738) and Cyp4g11 (GB51536) a Cytochrome P450 4 g 11 ortholog, and nAChRb2 (GB53428), plus Mcdp (GB40696) or mast cell degranulating peptide, Odp15 - an odorant binding protein (GB46224) and CPR16 (GB52920), a cuticular protein, are also among the top 100 most differentially expressed and elevated in R_virus over S_virus. Another Cytochrome P-450 (GB52023), Obp19, and Melittin-2 (GB44112), another key venom protein, were also

elevated in R bees injected with virus. GB44367 (LOC724436) that produces a phospholipase A 2-like peptide is UP in R_virus v. S_virus, and UP in R_virus v. R_PBS. Lastly, Argonaute-2 (GB50995) is UP in R_virus v. S_virus, as is GB41545 (LOC409187), or MD-2-related lipid recognition protein involved in cholesterol transport, and both have been implicated in anti-viral defenses in honey bees [17].

Genes strongly down-regulated in R_virus v. S_virus injected bees

Surprisingly, the majority of the genes DOWN in R_Virus v. S_Virus lack GO annotations. Nevertheless, many of these proteins have been noted as responsive to honey bee parasitism. Notably, Apidermin-3 like protein has recently been implicated as an outlier protein suppressed by DWV but induced by Varroa parasitism in a proteome study [18]. In that report Apidermin appears to be down-regulated by DWV infection, but up-regulated by Varroa and DWV+Varroa. We find that DWV injection suppresses Apidermin-3 like protein GB53110 (409716) in R bees that are mite-free and in S bees that are mite-free. That is, GB53110 is DOWN in R_virus v. R_PBS and DOWN in S_virus v. S_Control. But Apidermin-3 is also DOWN in R_virus v. S_virus - in substantial agreement with [18]. In contrast, where mite infestation is a variable condition, GB53110 expression is UP in S_mite compared to R_mite, UP in S_mite v. R_control and also UP in S_control v. R_control, and UP in R_control v. R_mite, where S_mite and S_control have elevated natural DWV levels compared to R_control and R_mite. So Apidermin 3 (GB53110) may be up-regulated in S bees generally despite mite infestation and/or DWV infection. Alternatively, Varroa and Varroa+DWV may up-regulate Apidermin-3 only in S bees.

353 The MAGE-like protein (LOC102654246) encoding gene, GB42910, is another
354 example of one of the most DOWN in the R_virus v. S_virus contrast, and is often involved
355 in stress response in other species. Similarly, the gene encoding the *Ctenidin 1-like* protein
356 (LOC102656669) is another gene most DOWN regulated in R_virus v. S_virus, and codes
357 for a glycine-rich protein orthologous to an antimicrobial peptide first described in spiders
358 and scorpions. Interestingly, *kakusei*, a gene transcribed into a non-coding RNA, known to
359 be an Immediate Early Gene (IEG) and previously characterized as involved in associative
360 learning and memory and other neural activity, is also a gene DOWN in R_virus v. S_virus.
361 IEGs are also recognized as one of the key mediators of links between events at the cellular
362 membrane and the nucleus, presumably related to neural activity.
363 The Hexamerin 110 encoding gene GB44996 is also one of the most DOWN in
364 R_virus v. S_virus. Hex110 is an amino acid storage protein used to fuel the drastic changes
365 that occur during metamorphosis, and Hexamerin 110 may have a role in regulating the
366 expression of the ribosomal RNA gene cluster in the nucleolus. Hex70a, Hex70b and
367 Hex70c are also DOWN in R bees relative to S bees injected with virus, though not so
368 dramatically as Hex110. Other notable genes DOWN in the R_virus v. S_virus contrast
369 include Kr (GB41483) or Krueppel, and Usp (GB42692) or Ultraspiracle. Krueppel is a
370 chromatin-associated gap class segmentation protein that is involved in negative regulation
371 of transcription and developmental processes, including hemocyte proliferation, trunk
372 segmentation and neurogenesis. Ultraspiracle is a nuclear steroid hormone receptor,
373 binding ecdysone and ecdysone response elements, and is probably involved in honey bee
374 metamorphosis. GB50662 (Vhdl), a larval-specific very high-density lipoprotein, is also
375 Down in R compared to S bees injected with virus.

376

377 *153 Genes DOWN in R_virus v. R_PBS*

378 45 genes with lower expression in resistant bees injected with DWV had products that
379 localized to the cell membrane and produced GO cellular component enrichments for
380 intrinsic and integral components of the membrane. 10 genes DOWN in DWV injected R
381 bees compared to Pbs injected R bees produced proteins constituting elements of the cuticle
382 and generated GO molecular function enrichment for structural constituent of the cuticle
383 while 13 DOWN genes yielded GO MF enrichment for structural molecule activity.
384 Another 7 genes DOWN in R_virus v. R_PBS had endopeptidase and hydrolase activity
385 and produced GO enrichment for those molecular functions.

386

387 *33 Genes UP in R_PBS v. S_PBS injected bees*

388 GB49544 - or Vitellogenin - is one of the genes up-regulated in R bees compared to S bees
389 injected with PBS, as is GB50290 - inactive serine protease scarface (LOC724917);
390 GB49648 codes for a protein with ecdysis-triggering hormone activity and is involved with
391 ecdysis and chitin cuticle formation is also UP in R relative to S after PBS injection.
392 Cuticular Protein 2 (GB48823), and GB48342 (LOC100576193) a keratinocyte proline-
393 rich protein, plus a second keratinocyte proline-rich protein encoding gene, GB46585
394 (LOC100578514) and Cuticular Protein 3 (GB48832), as well as a structural cuticular
395 protein encoding gene GB46298, and GB41015 (Grp) a glycine-rich cuticle protein, and
396 yet another cuticular protein, GB46591 (LOC725089) are UP in R_PBS relative to S_PBS
397 too. GB47383 (LOC409689) is also UP in R compared to S injected with PBS, and codes
398 for a U4/U6 small nuclear ribonuclear protein. In addition, GB45986 (LOC413408) that

399 produces a scavenger receptor protein; a gene producing a pro-resilin-like peptide
400 (GB43298); and GB40681 (LOC100578829), coding for a protein that elongates very long
401 chain fatty acids are all UP in R v. S with PBS injection. A gene that produces a circadian
402 clock-controlled protein GB42702 (LOC727010 is UP, and GB41707, encoding the
403 Tubulin beta subunit of microtubules, is also elevated in R_PBS v. S_PBS.
404
405 *Genes at the Intersection of UP in R_virus v. S_virus AND UP in S_mite v. R_mite*
406 Examining the *Intersection* of: (1) the set of genes up-regulated in R bees injected with
407 DWV; and (2) the set of genes upregulated in S bees infested with mites compared to R
408 bees infested with mites, yields a total of 51 genes (recall that S_mite bees carried higher
409 naturally occurring DWV loads than did R_mite bees). The 51 genes UP-regulated in
410 R_virus v. S_virus and UP in S_mite over R_mite (with natural virus infection levels) show
411 GO BP enrichment for immune response, defense response and innate immune response.
412 This GO enrichment emerged despite 15 of the 51 genes having no GO BP annotations.
413 The 51 UP regulated in both R_virus v. S_virus and UP in S_mite v. R_mite also had GO
414 MF enrichment for serine endopeptidase, serine hydrolase and peptidoglycan binding, even
415 with 19 of the 51 genes having no GO MF annotations. Natural, pre-existing DWV virus
416 infections were present in the S_mite and R_mite honey bees studied in Experiment Two,
417 as in Experiment One. Importantly, we documented higher natural DWV virus levels in
418 both the S_mite and S_control samples than in the R_mite and R_control honey bees.
419 Perhaps more surprising, while virus levels were elevated in S_mite bees compared to
420 S_control bees - as expected and previously reported in several studies - mite infestation

had little effect on virus loads in R bees. If anything, mean DWV levels in R_mite bees were slightly lower than DWV levels in R_control bees. See Figure 4, above.

Even when GO enrichment results are limited it is possible to glean other distinctive differences between R and S bees and their respective response to mites and DWV. For instance, the 51 genes UP in R_virus v. S_virus and UP in S_mite v. R_mite included not only Apidaecin GB47546, Defensin-1, GB41428 , Hymenoptaecin GB51223 and a gamma interferon inducible thiol reductase like protein (GILT-1), GB40261, but also genes encoding Vitellogenin GB49544 and Malvolio GB54097, the venom components Mellitin-2 GB44112, Phospholipase A-2 GB48228 , a venom serine protease GB44120 and Apamin (GB40697) a neurotoxin. The peptidoglycan recognition proteins GB47805 and GB47804, a cell matrix adhesion molecule, GB40210, various cellular membrane components and transmembrane transporters, e.g., GB50447, lipid recognition proteins, e.g., GB41545 a MD-2-related lipid recognition protein previously implicated in antiviral defenses in honey bees, and involved in cholesterol transport, a protein with DNA transcription factor activity GB52620, a protein with scavenger receptor activity GB54506, other cholesterol and lipid transport proteins, e.g., GB42053, glucose dehydrogenase GB51814, alpha glucosidase GB54549, alpha amylase GB49854, cytochrome P-450 9e2 GB43713, two trypsins GB41097, a two chymotrypsin inhibitors GB45614, GB50116, a methyltransferase GB44871, and a putative multivitamin transporter GB48560 were all elevated in R bees injected with virus.

Conclusions

Honey bees battle their parasites with both individual and group defenses. Honey bees that survive long-term exposure to *Varroa* mites without the help of human interventions are exceedingly rare. These surviving lineages could resist mites themselves or the devastating viruses that mites vector within the colony. These three experiments focused on signals for both traits, namely genetic responses by bees that reduce the impacts of mites and mite-vectored viruses.

Experiment One showed that natural viral loads in a genetically distinct population of honey bees ([19]; 5 TX managed colonies) exhibiting a Varroa tolerant phenotype were markedly lower than natural viral loads in another population of bees susceptible to Varroa. This trend of lower virus levels, including a virus not vectored by *Varroa* mites, suggests that the results reflect a general resistance to viruses. Expression levels of the cellular immunity genes *Eater* and *Nim2C* were elevated in susceptible bees compared to resistant bees. These results led us to more closely examine differences between these two populations, and their respective responses to *Varroa destructor* parasitism and virus infection.

In Experiment Two, we generated RNA sequencing data from R and S bees kept under natural conditions for commercial beekeeping and queen-rearing purposes. These data confirmed the results of Experiment One - that R bees harbor lower covert viral loads, including DWV, than do S bees. Our results also indicate that mite infestation appears not to elevate viral titers in R bees, while mite infestation is associated with significantly higher virus levels in S bees. Previous work has shown that Varroa parasitism consistently results in markedly elevated DWV levels and manifestations of DWV pathology (*See, e.g.,* [20]).

467 This suggests that the Varroa tolerance of R bees may be explained at least in part by R
468 bees suppressing the viral load enhancement that usually follows parasitism by Varroa.
469 Alternatively, the virus resistance of R bees may be related to their ability to cope with
470 Varroa infestation without suffering pathological effects, including aberrant immune
471 system function, and increased mortality usually associated with mite-induced viral load
472 enhancement. Finally, the Varroa tolerance and lower viral loads in R bees compared to S
473 bees may be driven by a combination of enhanced resistance of R bees to both Varroa and
474 viruses, with distinct expression differences conferring resistance both to pathogens and a
475 pathogen-vectoring parasite.

476 Especially intriguing in Experiment 2 are the GO enrichments for RNA processing,
477 including transcription and transcriptional regulation, splicing, nuclear and mitochondrial
478 gene expression, translation and peptide biosynthesis, plus DNA damage repair. The
479 fundamental metabolic changes that distinguish the response of R and S bees to DWV and
480 Varroa mites may better enable R bees to cope with the insults of Varroa and viruses and
481 their deleterious effects on transcription, translation, gene expression programs and nucleic
482 acid processing. The differential responses to Varroa and DWV also encompass extensive
483 activation of DNA damage repair pathways by R bees. Overall, R bees have higher levels
484 of expression for genes implicated in DNA and RNA processing, especially splicing of
485 RNA, gene expression and nucleobase and ribonucleoprotein metabolic processes. R_PBS
486 bees also express Argonaute-2 GB50955 at higher levels than S_PBS bees (DOWN in
487 S_PBS v. R_PBS). This protein is also up-regulated in R_virus v. S_virus samples.
488 Argonaute-2 is a key component of the RNA interference response, a key antiviral pathway

489 [21], and this pathway has been shown to be responsive to viral infections recently by
490 Rutter et al. [17].

491 Experiment Three was designed to decouple the direct effects of mite parasitism
492 from the effects of transmitted viruses. Specifically, we examined differentially expressed
493 genes from mite-free bees of both R and S phenotypes and from common locales, by
494 directly injecting DWV or PBS into bees of both genetic backgrounds. When comparing
495 the changes in gene expression elicited by virus injection, the results clearly differentiate
496 R and S bees. UP regulation of immune response genes in R_virus v. S_virus suggests that
497 elevated expression levels for genes involved in defense response and immune response
498 may be an effective response to viral infection, conferring some degree of protection
499 against DWV, at least in honey bees free of Varroa infestation. Elevated expression of
500 immune response genes differentiate R bees from S bees in response to direct DWV
501 injection and may be an important advantage contributing to the relative resistance of R
502 bees to DWV. However, as Experiment 2 results reveal, when Varroa mites are present,
503 turning up expression of immune system genes alone may not be sufficient for coping with
504 Varroa and natural virus infection. Our GO enrichment results show that S bees infested
505 with mites, and carrying elevated DWV loads compared to R bees with mites, do
506 upregulate some immune genes. But if up-regulation of immune genes was sufficient to
507 confer a virus and mite resistant phenotype, then we might expect to see immune gene
508 expression generally elevated in R_mite v. S_mite, while the GO enrichment data show
509 the opposite. A more complex response to mites + virus, selectively modulating expression
510 of many genes but enhancing expression of only specific immune genes, may contribute to
511 increased mite-tolerance and virus resistance in R bees.

512 Many interesting GO terms emerged from genes with elevated expression after
513 DWV injection, as well as from genes up-regulated in association with natural DWV
514 infection, contrary to the complete absence of GO enrichment among genes elevated by
515 pathogen infection in Doublet, et al. [22]. In an especially striking example, our
516 experiments show that genes involved with immune function were expressed at higher
517 levels in samples with higher DWV loads - a result at odds with the meta-analysis of
518 Doublet, et al. [22], where immune genes, metabolic genes and regulatory genes were all
519 suppressed by pathogen infection. Most notably, we find immune genes and defense
520 response genes were highly over-represented among genes UP in R bees after virus
521 injection, and were also UP in S bees with mites. Enhanced expression of immune defense
522 genes elicited by higher DWV load is one explanation for our results: S bees with mites
523 harbored higher levels of natural DWV infection than R bees with mites or R bees without
524 mites. Equally important, R bees expressed immune defense genes at higher levels but
525 developed lower DWV loads after DWV injection. These results offer intriguing
526 correlations with the differential response of R and S bees to DWV injection, as well as
527 their response to natural DWV infection in conjunction with Varroa infestation. Elevated
528 expression of select immune genes may represent an effective anti-viral response to DWV
529 infection, albeit one modulated by Varroa or of reduced impact when mites are present.
530
531 *Integrating differential gene expression results of bees with natural DWV infection but*
532 *variable Varroa infestation from expression differences after DWV injection of mite-free*
533 *bees.*

534 The disparate patterns of elevated gene expression between R and S bees naturally
535 infected with DWV and infested by Varroa mites, compared to differential expression of
536 genes in R and S bees infected in the laboratory with DWV, potentially provide clues to
537 the mechanisms of resistance to virus and tolerance of mites. The totality of the evidence
538 suggests that R bees devote significant energy to buffering against cellular and metabolic
539 stress that emanate from concomitant virus and parasite insults, and exhibit differential
540 gene expression patterns that provide metabolic, oxidative and developmental stress
541 protections - not simply elevated immune gene expression profiles. While elevated immune
542 system gene expression may be necessary for successfully resisting viral infections,
543 increased expression of immune genes alone may be insufficient to provide protection,
544 especially in bees with both DWV infection and *Varroa destructor* infestation.
545
546 *Genes also differentially expressed in previous studies*
547 We found concordance in differentially expressed genes between our study and
548 four RNASeq analyses conducted on honey bees and their disease responses, as detailed in
549 Additional File 2 and Table S1. On the other hand, we find important differences between
550 our results and prior reports of particular genes being best correlated with DWV infection
551 or antiviral response. In fact, in several instances genes previously identified as key anti-
552 virus responses were not differentially expressed in our study [17, 18, 22-28].
553 The salient gene expression patterns that distinguish R and S bees in the field and
554 laboratory demonstrate important differences between the two genetic backgrounds in their
555 response to *Varroa destructor* parasitism and DWV infection. The gene expression patterns
556 associated with mite infestation and DWV infection provide additional insights into

important transcriptomic changes elicited either by direct virus injection and infection, or by Varroa mite parasitism and natural viral infection. Equally important, our results show different honey bee gene expression signals are elicited by viral infection depending upon the presence or absence of mite parasites. We recovered a robust signal of differential gene expression when comparing bees from two genetically distinct populations exposed to DWV and Varroa mites, despite sampling polyandrous bees with the intra-colony genetic diversity typical of natural hives. Our data may also reveal responses to viral infection and mite parasitism that confer selective advantages in bees that exhibit mite-tolerant and virus-resistant phenotypes, and may suggest mite tolerance or virus-resistance mechanisms. This work should allow us to begin identification of gene expression patterns associated with mite tolerance and viral resistance in populations or lineages that exhibit those traits, and begin to define virus and mite resistance mechanisms at the genomic and transcriptomic level – all of which merit additional investigation.

Declarations

Ethics approval and consent to participate

Not Applicable, invertebrate species

Consent for publication

All authors have approved the content and plan for publication

Availability of data and materials

We have provided all sequences and reagents for the constructs and methods. All RNA sequences are deposited under project PRJNA431793 in the NCBI Sequence Reads Archive.

Competing interests

DW carries out genetic research involving honey bees with colleagues at UT-Southwestern and USDA but also owns a family business breeding and selling honey bees and pollinating crops. The described honey bees are not an advertised breed from his company and we are conscious that we cannot advertise this as pitting his bees over other varieties. The remaining authors have no competing interests and no authors have non-financial competing interests.

Funding

JDE and DL were supported by USD-ARS intramural funds and USDA-NIFA grant 12438043. The remaining authors were supported by their institutions (BC and CE) or self-funded (DW).

Authors' contributions

DW and JDE conceived the project and collected samples, BC and CE helped with bioinformatic analyses, DL helped with experiments, all authors wrote the manuscript.

Acknowledgements

We thank Radhika Khetani for her guidance during this project.

Figure Legends

Figure 1. Natural virus loads of Deformed wing virus (DWV), Black queen cell virus (BQCV), Kashmir bee virus (KBV), and expression levels of three immune genes: the antimicrobial gene, Hymenoptaecin, and the cellular immunity genes, Eater, and Nim2C. Diamond plots show means, plus one and two standard deviations from the mean for each virus and gene.

Figure 2. Clustering Diagram of Susceptible (VS) and Resistant (VR) samples from Experiment One, showing higher virus loads in VS bees than in VR bees and higher levels

617 of Eater expression in VS bees, contrasted with slightly higher levels of Hymenoptaecin
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619
620 Figure 3. Principal Component Analysis of Gene Expression Data produced by DESeq 2
621 for VS1, VS0, VT1 and VT0 samples. VS1 = S_mite; VS0 = S_control; VT1 = R_mite;
622 and, VT0 = R_control. Note that PC1 captures 74% of the total variance between the
623 samples, and shows the extreme divergence of R_mite from S_mite and S_control. PC2
624 provides differentiation of R_control from R_mite, revealing much of the gene expression
625 differences attributable to mite infestation of the R genetic background.
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627 Fig. 4. Quantitative PCR estimates of Deformed wing virus (d-CT) for the R_control,
628 R_mite, S_control, and S_mite samples, showing mite-infested Susceptible honey bees
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630 bees (two sources) had markedly higher levels of DWV than the Resistant bees, regardless
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633
634 Figure 5. Scatterplot of enriched Biological Process Gene Ontology terms (GO BPs). a)
635 Up-regulated in S bees with mites compared to R bees with mites (UP in S_mite v. R_mite),
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a Benjamini-Hochberg multiple testing correction. The GO enrichment test dataset is comprised of genes with FDR < 0.05 and with expression level differences showing a log10 fold change greater than 1.5 (logFC>1.5). Scatterplot generated by ReviGo [29].

Figure 6. qPCR relative levels of Deformed wing virus and two immune-related genes, Eater and Hymentopaecin. Blue dots reflect bees injected with DWV, red dots reflect bees given a PBS control injection. 'B' colonies were predicted to be more susceptible while 'G' colonies were predicted to be resistant.

Figure S1. Heatmap of the sample-to-sample distance in R_mite v. R_control and S_mite v. S_control samples compiled using the R package DESeq2 with a customized map function, (http://bioconductor.org/packages/release/bioc/vignettes/DESeq2/inst/doc/DESeq2.html#heatmap-of-the-sample-to-sample-distances).

Figure S2. Principal Component Analysis of Differential Gene Expression data, contrasting R bees injected with virus, R_Virus, S bees injected with virus, S_Virus, and R & S bees injected only with a phosphate buffer solution, R_PBS and S_PBS; R_Virus and S_Virus are divergent in RNA sequencing data that comprise principal component 1; Principal component 2 captures the different responses to virus versus control injection in the R samples, and differences between the two S samples in response to buffer injection.

Figure S3. a) Scatterplot in semantic space of GO BP enrichment for UP in R_virus v. S_virus, FDR<0.05 and logFC>1.5. b) Scatterplot in semantic space of GO MF enrichment from HymenopteraMine set operation of Asymmetric Difference of UP in R_virus v. S_virus *MINUS* UP in R_virus v. R_PBS, genes with FDR < 0.05 and logFC >1.5.

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757

Figures

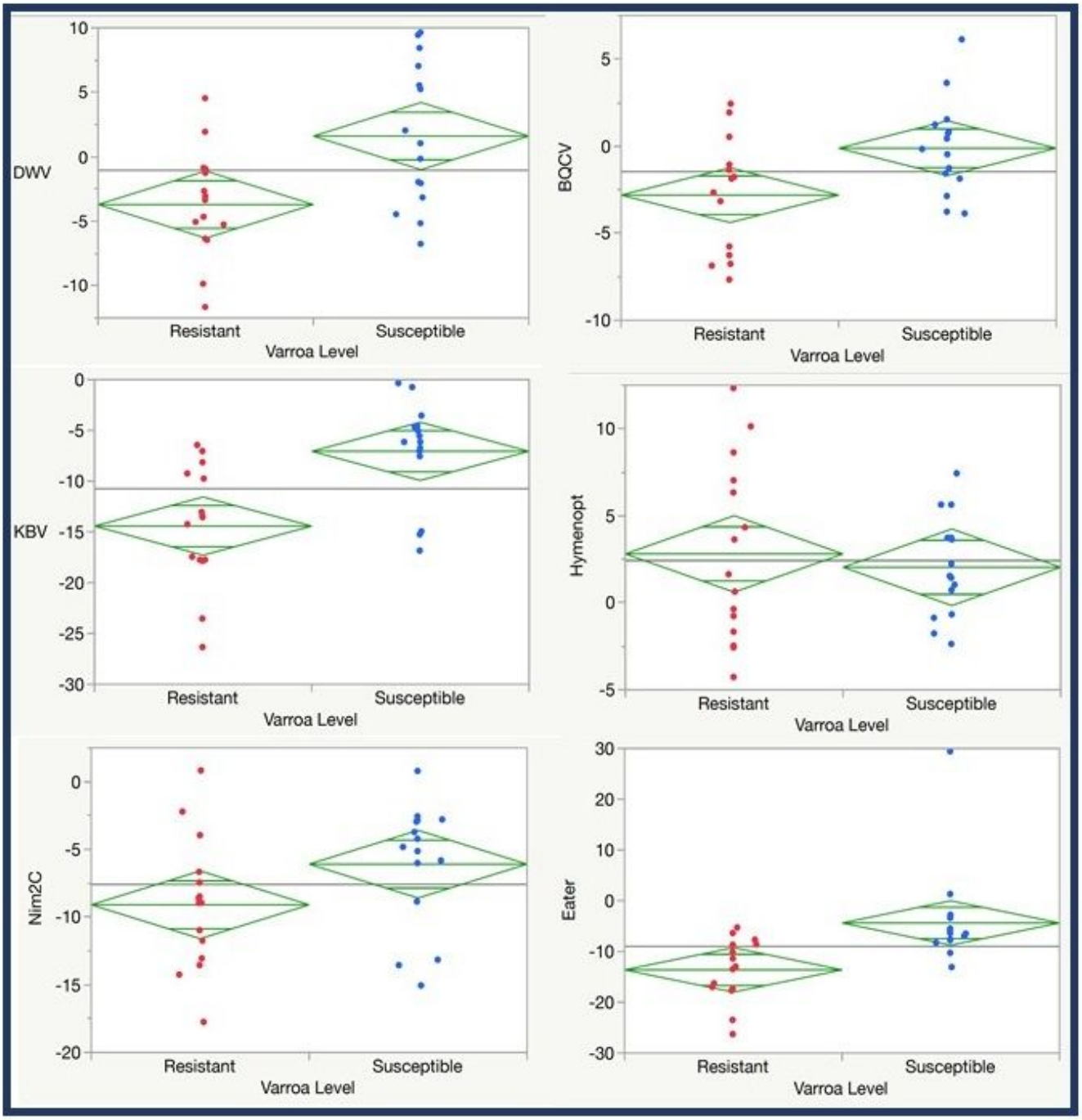

Figure 1

Figure 1

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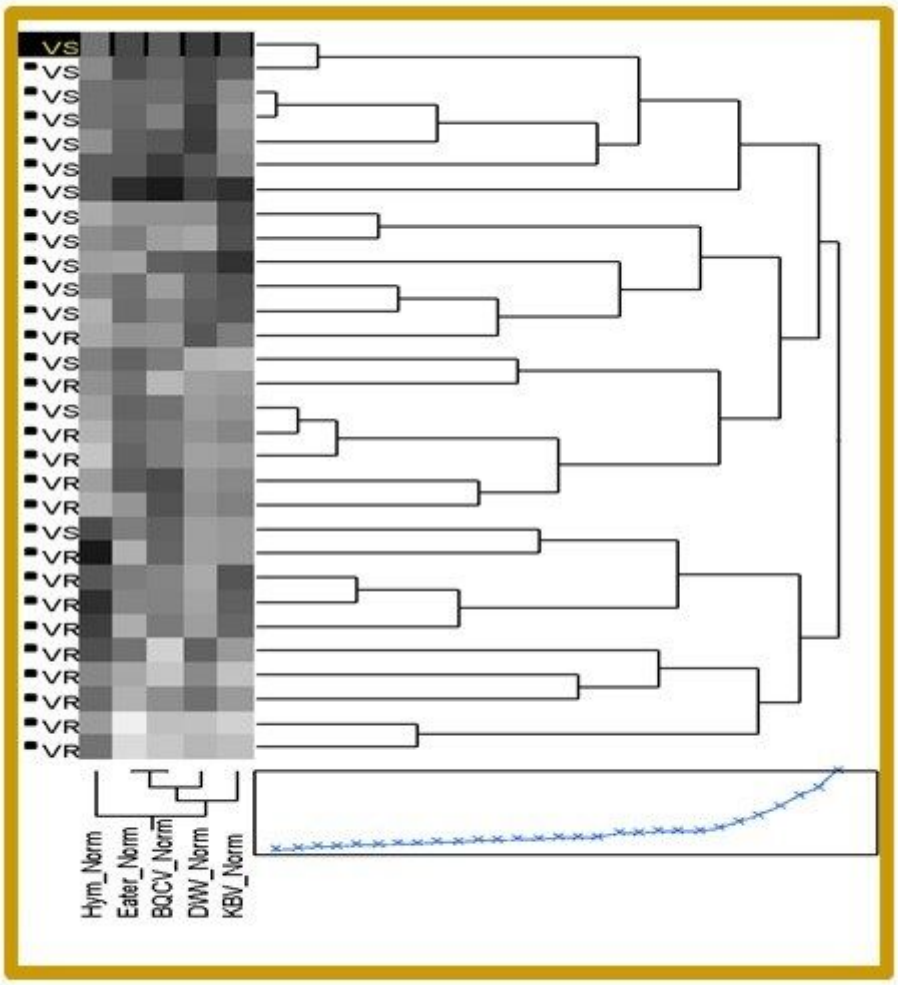

Figure 2

Figure 2

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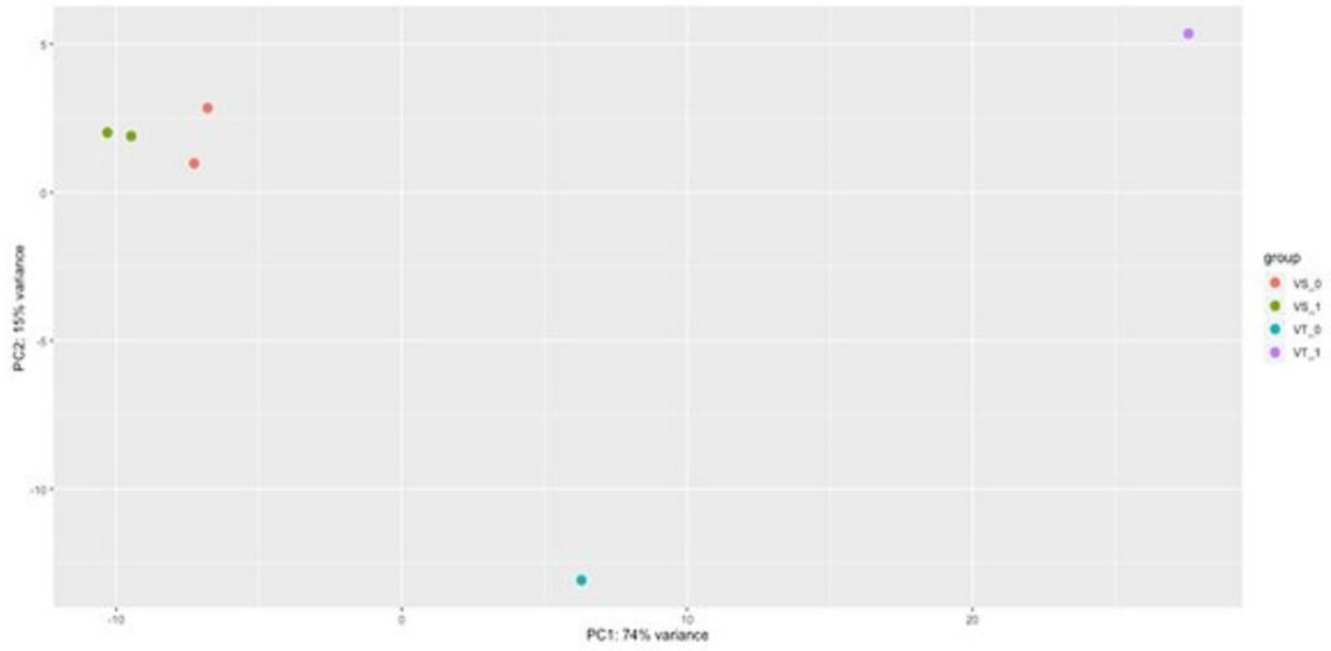
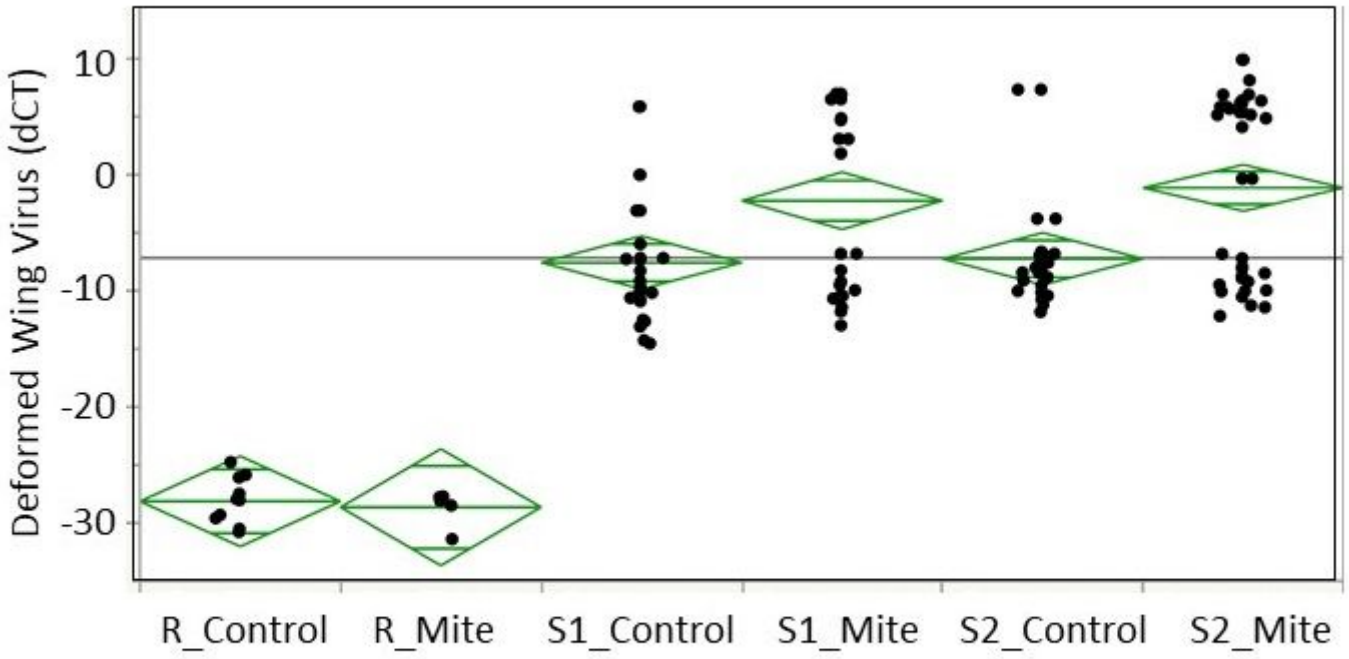


Figure 3

Figure 3

Principal Component Analysis of Gene Expression Data produced by DESeq 2 for VS1, VS0, VT1 and VT0 samples. VS1 = S_mite; VS0 = S_control; VT1 = R_mite; and, VT0 = R_control. Note that PC1 captures 74% of the total variance between the samples, and shows the extreme divergence of R_mite from S_mite and S_control. PC2 provides differentiation of R_control from R_mite, revealing much of the gene expression differences attributable to mite infestation of the R genetic background.



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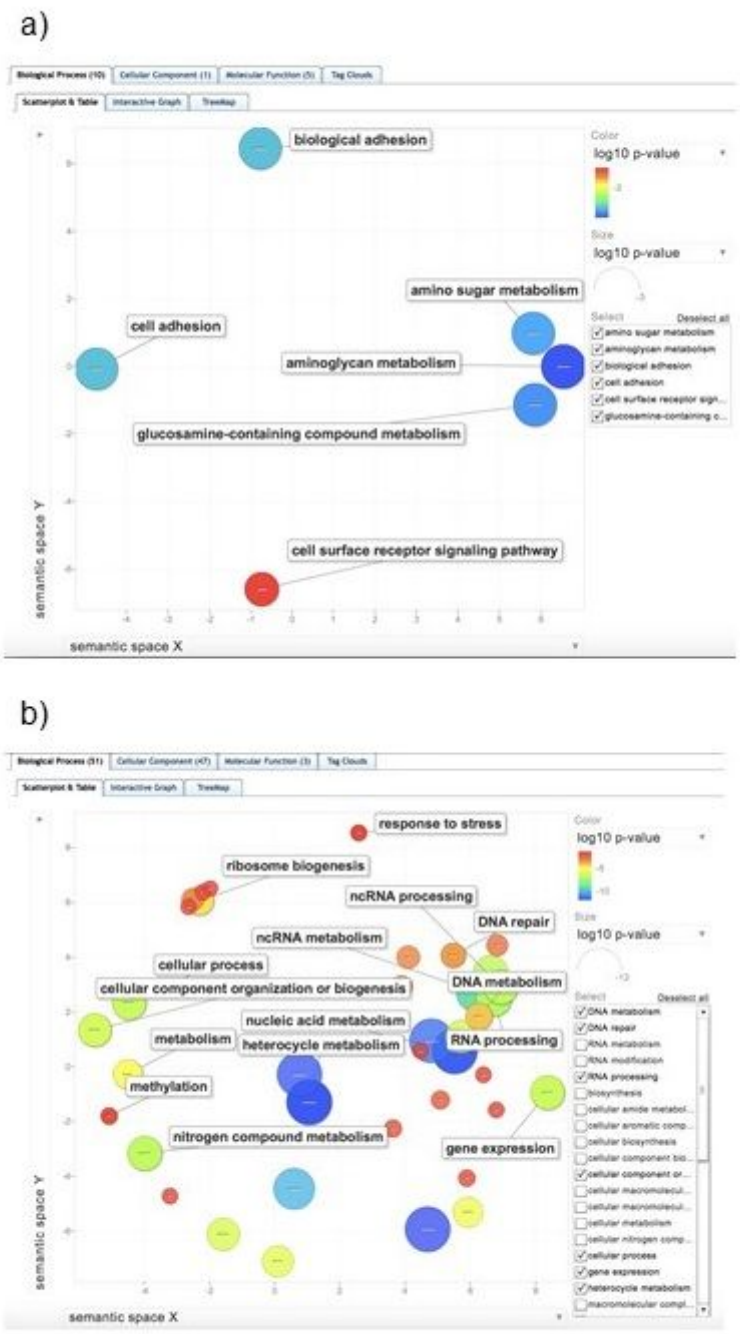

Figure 5

Figure 5

Scatterplot of enriched Biological Process Gene Ontology terms (GO BPs). a) Up-regulated in S bees with mites compared to R bees with mites (UP in S_mite v. R_mite), b) Down-regulated in S bees with mites compared to R bees with mites (DOWN in S_mite v. R_mite). GO enrichments and P-values were calculated using HymenopteraMine GO enrichment widgets after using HymenopteraMine database cross-references to convert gene identifiers to OGSv3.2, with the OGSv3.2 gene set as the background

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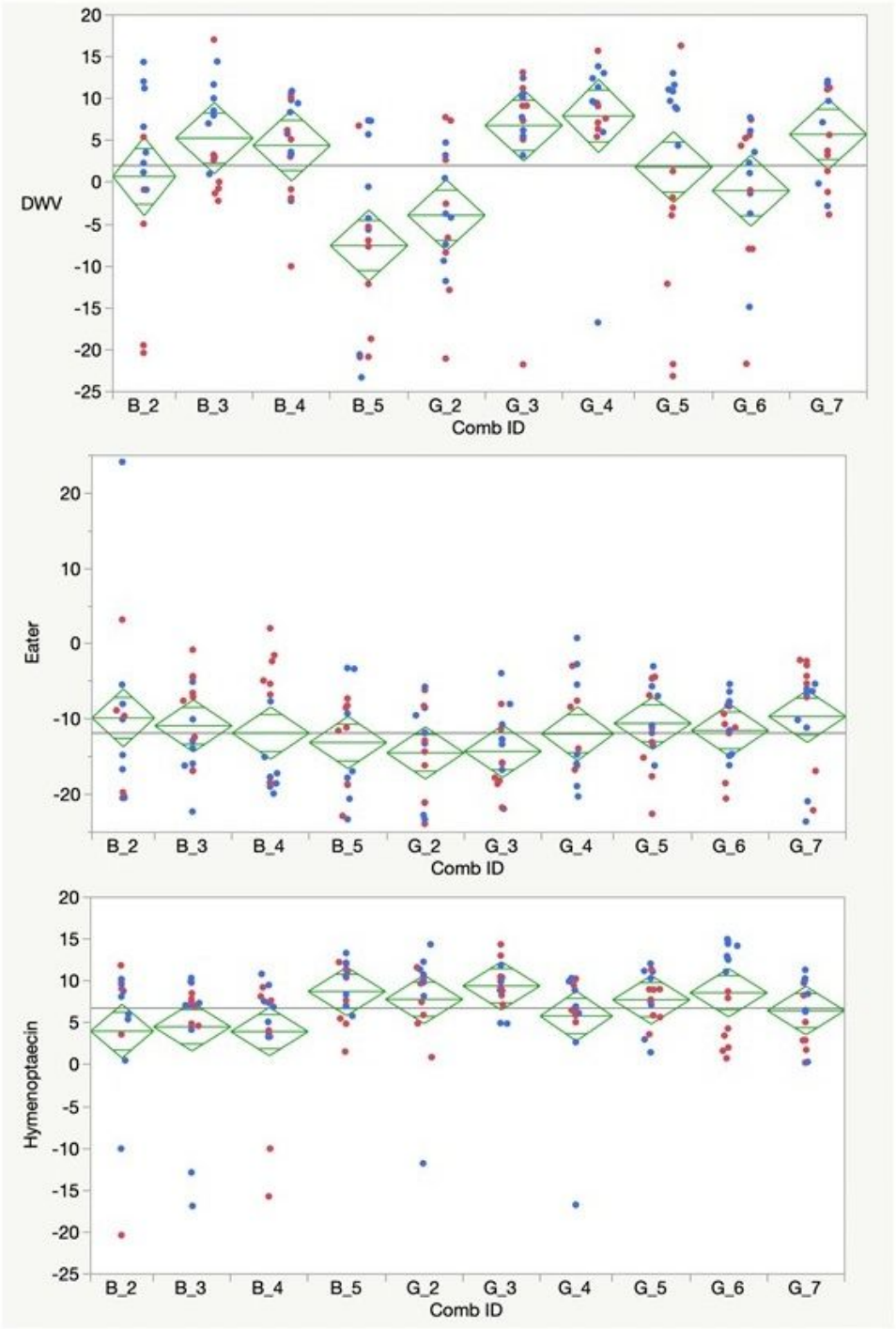

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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile1021921.xlsx
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