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Transcriptomic responses to diet quality and viral infection in *Apis mellifera*

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Abstract

Background: Parts of Europe and the United States have witnessed dramatic losses in commercially managed honey bees over the past decade to what is considered an unsustainable extent. The large-scale loss of bees has considerable implications for the agricultural economy because bees are one of the leading pollinators of numerous crops. Bee declines have been associated with several interactive factors. Recent studies suggest nutritional and pathogen stress can interactively contribute to bee physiological declines, but the molecular mechanisms underlying interactive effects remain unknown. In this study, we provide insight into this question by using RNA-sequencing to examine how monofloral diets and Israeli acute paralysis virus inoculation influence gene expression patterns in bees.

Results: We found a considerable nutritional response, with almost 2,000 transcripts changing with diet quality. The majority of these genes were over-represented for nutrient signaling (insulin resistance) and immune response (Notch signaling and JaK-STAT pathways). In our experimental conditions, the transcriptomic response to viral infection was fairly limited. We only found 43 transcripts to be differentially expressed, some with known immune functions (argonaute-2), transcriptional regulation, and muscle contraction. We created contrasts to explore whether protective mechanisms of good diet were due to direct effects on immune function (resistance) or indirect effects on energy availability (tolerance). A similar number of resistance and tolerance candidate differentially expressed genes were found, suggesting both processes may play significant roles in dietary buffering from pathogen infection.

Conclusions: Through transcriptional contrasts and functional enrichment analysis, we contribute to our understanding of the mechanisms underlying feedbacks between nutrition and disease in bees. We also show that comparing results derived from combined analyses across multiple RNA-seq studies may allow researchers to identify transcriptomic patterns in bees that are concurrently less artificial and less noisy. This work underlines the merits of using data visualization techniques and multiple datasets to interpret RNA-sequencing studies.

Keywords: Honey bee; RNA-sequencing; Israeli acute paralysis virus; Monofloral pollen; Visualization

1 Background

- 2 Commercially managed honey bees have undergone unusually large declines in the
- 3 United States and parts of Europe over the past decade [1, 2, 3], with annual

4 mortality rates exceeding what beekeepers consider sustainable [4, 5]. More than 70
5 percent of major global food crops (including fruits, vegetables, and nuts) at least
6 benefit from pollination, and yearly insect pollination services are valued worldwide
7 at \$175 billion [6]. As honey bees are largely considered to be the leading pollinator
8 of numerous crops, their marked loss has considerable implications for agricultural
9 sustainability [7].

10 Honey bee declines have been associated with several factors, including pesti-
11 cide use, parasites, pathogens, habitat loss, and poor nutrition [8, 9]. Researchers
12 generally agree that these stressors do not act in isolation; instead, they appear
13 to influence the large-scale loss of honey bees in an interactive fashion as the en-
14 vironment changes [10]. Nutrition and viral infection are two broad factors that
15 pose heightened dangers to honey bee health in response to recent environmental
16 changes.

17 Pollen is a main source of nutrition (including proteins, amino acids, lipids, sterols,
18 starch, vitamins, and minerals) in honey bees [11, 12]. At the individual level, pollen
19 supplies most of the nutrients necessary for physiological development [13] and is
20 believed to have considerable impact on longevity [14]. At the colony level, pollen
21 enables young workers to produce jelly, which then nourishes larvae, drones, older
22 workers, and the queen [15, 16]. Various environmental changes (including urban-
23 ization and monoculture crop production) have significantly altered the nutritional
24 profile available to honey bees. In particular, honey bees are confronted with a
25 less diverse selection of pollen, which is of concern because mixed-pollen (polyflo-
26 ral) diets are generally considered healthier than single-pollen (monofloral) diets
27 [17, 18, 19]. Indeed, reported colony mortality rates are higher in developed land
28 areas compared to undeveloped land areas [20], and beekeepers rank poor nutrition
29 as one of the main reasons for colony losses [21]. Understanding how low diversity

30 diets affect honey bee health will be crucial to resolve problems that may arise as
31 agriculture continues to intensify throughout the world [22, 23].

32 Viral infection was a comparatively minor problem in honey bees until the last
33 century when the ectoparasitic varroa mite (*Varroa destructor*) spread worldwide
34 [24]. This mite feeds on honey bee hemolymph [25], transmits multiple viruses,
35 and supports replication of some viruses [26, 27, 28, 29]. More than 20 honey bee
36 viruses have been identified [30]. One of these viruses that has been linked to honey
37 bee decline is Israeli acute paralysis virus (IAPV), a positive-sense RNA virus of
38 the family Dicistroviridae [31]. IAPV infection causes shivering wings, decreased
39 locomotion, muscle spasms, paralysis, and high premature death percentages in
40 caged infected adult honey bees [32]. IAPV has demonstrated higher infectious
41 capacities than other honey bee viruses under certain conditions [33] and is more
42 prevalent in colonies that do not survive the winter [34].

43 Although there is growing interest in how viruses and diet quality affect the health
44 and sustainability of honey bees, as well as a recognition that such factors might
45 operate interactively, there are only a small number of experimental studies thus
46 far directed toward elucidating the interactive effects of these two factors in honey
47 bees [35, 36, 37, 38, 39]. We recently used laboratory cages and nucleus hive experi-
48 ments to investigate the health effects of these two factors, and our results show the
49 importance of the combined effects of both diet quality and virus infection. Specifi-
50 cally, ingestion by honey bees of high quality pollen is able to mitigate virus-induced
51 mortality to the level of diverse, polyfloral pollen [40].

52 Following up on these findings, we now aim to understand the corresponding
53 underlying mechanisms by which high quality diets protect bees from virus-induced
54 mortality. For example, it is not known whether the protective effect of good diet
55 is due to direct, specific effects on immune function (resistance), or if it is due
56 to indirect effects of good nutrition on vigor (tolerance) [41]. Transcriptomics is

one means to better understand the mechanistic underpinnings of dietary and viral effects on honey bee health. Transcriptomic analysis can help us identify 1) the genomic scale of transcriptomic response to diet and virus infection, 2) whether these factors interact in an additive or synergistic way on transcriptome function, and 3) the types of pathways affected by diet quality and viral infection. This information, heretofore lacking in the literature, can help us better understand how good nutrition may be able to serve as a “buffer” against other stressors [42].

There are only a small number of published experiments examining gene expression patterns related to diet effects [43] and virus infection effects [44] in honey bees, but there have been several such studies in model organisms. Model insect studies can inform studies of honey bee transcriptomic responses, using functional inference of as-of-yet uncharacterized honey bee genes based on orthology to *Drosophila* and other model organisms. Previous *Drosophila* studies that examined various diet effects have found gene expression changes related to immunity, metabolism, cell cycle activity, DNA binding, transcription, and insulin signaling [45, 46, 47, 43]. While similar transcriptomic studies have been limited in honey bees, one study found that pollen nutrition upregulates genes involved in macromolecule metabolism, longevity, and the insulin/TOR pathway required for physiological development [43]. Numerous studies on the transcriptomic effects of virus infection in model organisms have shown that RNA silencing, transcriptional pausing, Toll pathways, IMD pathways, JAK/STAT pathways, and Toll-7 autophagy pathways play substantial roles in virus-host systems [48, 49]. Virus-bee systems have also revealed key factors of these antiviral conserved defense pathways, such as RNA silencing, Toll pathways, IMD pathways, JAK/STAT pathways, autophagy, and endocytosis [50].

As far as we know, there are few to no studies investigating honey bee gene expression patterns specifically related to monofloral diets, and few studies investigating honey bee gene expression patterns related to the combined effects of diet in any

84 broad sense and viral inoculation in any broad sense [38]. In this study, we examine
85 how monofloral diets and viral inoculation influence gene expression patterns in
86 honey bees by focusing on four treatment groups (low quality diet without IAPV
87 exposure, high quality diet without IAPV exposure, low quality diet with IAPV
88 exposure, and high quality diet with IAPV exposure). For our diet factor, we exam-
89 ined two monofloral pollen diets, rockrose (*Cistus* sp.) and chestnut (*Castanea* sp.).
90 Rockrose pollen is generally considered less nutritious than chestnut pollen because
91 it contains smaller amounts of protein, amino acids, antioxidants, calcium, and iron
92 [40, 51]. We conduct RNA-sequencing analysis on a randomly selected subset of the
93 honey bees we used in our previous study (as is further described in our methods
94 section). We then examine pairwise combinations of treatment groups, the main
95 effect of monofloral diet, the main effect of IAPV exposure, and the combined effect
96 of the two factors on gene expression patterns.

97 We also compare the main effect of IAPV exposure in our dataset to that obtained
98 in a previous study conducted by Galbraith and colleagues [44]. While our study
99 examines honey bees from naturally mated colonies, the Galbraith study examined
100 honey bees from single-drone colonies. As a consequence, the honey bees in our
101 study will be on average 25% genetically identical, whereas honey bees from the
102 Galbraith study will be on average 75% genetically identical [52]. We note that
103 the difference between these studies may be even greater than this as we used
104 naturally mated honey bees from 15 different colonies. We should therefore expect
105 that the Galbraith study may generate data with higher signal:to:noise ratios than
106 our data due to lower genetic variation between its replicates. At the same time, our
107 honey bees will be more likely to display the health benefits gained from increased
108 genotypic variance within colonies, including decreased parasitic load [53], increased
109 tolerance to environmental changes [54], and increased colony performance [55, 56].
110 Given that honey bees are naturally very polyandrous [57], our naturally mated

honey bees may also reflect more realistic environmental and genetic simulations. Taken together, each study provides a different point of value: Our study likely presents less artificial data while the Galbraith data likely presents less messy data. We wish to explore how the gene expression effects of IAPV inoculation compare between these two studies that used such different experimental designs. To achieve this objective, we use visualization techniques to assess the signal:to:noise ratio between these two datasets, and differential gene expression (DEG) analyses to determine any significantly overlapping genes of interest between these two datasets. As RNA-sequencing data can be biased [58, 59, 60], this comparison allowed us to characterize how repeatable and robust our RNA-sequencing results were in comparison to previous studies. It also allowed us to shine light on how experimental designs that control genetic variability to different extents might affect the resulting gene expression data in honey bees. We suggest that in-depth data visualization approaches can be useful for cross-study comparisons and validation of noisy RNA-sequencing data in the future.

Results

Mortality and virus titers

We reanalyzed our previously published dataset with a subset that focuses on diet quality and is more relevant to the current study. We show the data subset here to inform the RNA-sequencing comparison because we reduced the number of treatments from the original published data (from eight to four) [40] as a means to focus on diet quality effects.

As shown in Figure 1, mortality rates of honey bees 72 hours post-inoculation significantly differed among the treatment groups (mixed model ANOVA across all treatment groups, $df = 3, 54$; $F = 10.03$; $p < 2.34e-05$). The effect of virus treatment (mixed model ANOVA, $df = 1, 54$; $F = 24.73$; $p < 7.04e-06$) and diet treatment (mixed model ANOVA, $df = 1, 54$; $F = 5.32$; $p < 2.49e-02$) were significant, but

the interaction between the two factors (mixed model ANOVA, $df = 1, 54$; $F = 4.72e-02$, $p = 8.29e-01$) was not significant. We compared mortality levels based on pairwise comparisons: For a given diet, honey bees exposed to the virus showed significantly higher mortality rate than honey bees not exposed to the virus. Bees fed rockrose pollen had significantly elevated mortality with virus infection compared to uninfected controls (Benjamini-Hochberg, $p < 1.53e-03$), and bees fed chestnut pollen similarly had significantly elevated mortality with virus infection compared to controls (Benjamini-Hochberg, $p < 3.12e-03$) (Figure 1).

As shown in Figure 2, IAPV titers of honey bees 72 hours post-inoculation significantly differed among the treatment groups (mixed model ANOVA across all treatment groups, $df = 3, 33$; $F = 6.10$; $p < 2.03e-03$). The effect of virus treatment (mixed model ANOVA, $df = 1, 33$; $F = 15.04$; $p < 4.75e-04$) was significant, but the diet treatment (mixed model ANOVA, $df = 1, 33$; $F = 2.55$; $p = 1.20e-01$) and the interaction between the two factors (mixed model ANOVA, $df = 1, 33$; $F = 7.02e-01$, $p = 4.08e-01$) were not significant. We compared IAPV titers based on pairwise comparisons: Bees fed rockrose pollen had significantly elevated IAPV titers with virus infection compared to uninfected controls (Benjamini Hochberg, $p < 7.56e-03$). However, bees fed chestnut pollen did not have significantly elevated IAPV titers with virus infection compared to uninfected controls (Benjamini Hochberg, $p = 6.29e-02$). Overall, we interpreted these findings to mean that high-quality chestnut pollen could partially “rescue” high virus titers resulting from the inoculation treatment, whereas low-quality rockrose pollen could not (Figure 2).

Transcriptomic responses to virus infection and diet

We observed a substantially larger number of differentially expressed genes (DEGs) in our diet main effect ($n = 1,914$) than in our virus main effect ($n = 43$) (Supplementary table 1 A and B, Additional file 1). In the diet factor, more DEGs were upregulated in the more-nutritious chestnut group ($n = 1,033$) than in the

less-nutritious rockrose group ($n = 881$). In the virus factor, there were more virus-upregulated DEGs ($n = 38$) than control-upregulated DEGs ($n = 5$). While these reported DEG counts are from the DESeq2 package, we saw similar trends for the edgeR and limma package results (Supplementary table 1, Additional file 1 and Additional file 18).

GO analysis of the chestnut-upregulated DEGs revealed the following over-represented biological functions: Wnt signaling, hippo signaling, and dorso-ventral axis formation, as well as pathways related to circadian rhythm, mRNA surveillance, insulin resistance, inositol phosphate metabolism, FoxO signaling, ECM-receptor interaction, phototransduction, Notch signaling, JaK-STAT signaling, MAPK signaling, and carbon metabolism (Supplementary table 2, Additional file 1). GO analysis of the rockrose DEGs revealed pathways related to terpenoid backbone biosynthesis, homologous recombination, SNARE interactions in vesicular transport, aminoacyl-tRNA biosynthesis, Fanconi anemia, and pyrimidine metabolism (Supplementary table 3, Additional file 1).

With so few DEGs ($n = 43$) in our virus main effect comparison, we focused on individual genes and their known functionalities rather than GO over-representation (Table 1). Of the 43 virus-related DEGs, only 10 had GO assignments within the DAVID database. These genes had putative roles in the recognition of pathogen-related lipid products and the cleaving of transcripts from viruses, as well as involvement in ubiquitin and proteasome pathways, transcription pathways, apoptotic pathways, oxidoreductase processes, and several more functions (Table 1).

No interaction DEGs were observed between the diet and virus factors of the study, in any of the pipelines (DESeq2, edgeR, and limma).

The number of DEGs across the six treatment pairings between the diet and virus factor ranged from 0 to 955 (Supplementary table 8, Additional file 1). Again, diet level appeared to have greater influence on the number of DEGs than the virus

level. Across every pair comparing the chestnut and rockrose levels, regardless of the virus level, the number of chestnut-upregulated DEGs was higher than the number of rockrose-upregulated DEGs (Supplementary table 8 C, D, E, F, Additional file 1). Virus-treated bees showed equal to or more upregulated genes relative to controls, under both diet treatments (Supplementary table 8 A and B, Additional file 1). These trends were observed for all three pipelines used (DESeq2, edgeR, and limma).

Transcriptomic data visualization and comparison to a previous study

We wished to explore the signal:to:noise ratio between the Galbraith dataset and our dataset. Note that the Galbraith dataset contained three samples for each virus level, while our dataset contained twelve samples for each virus level. Basic PCA plots were constructed with the DESeq2 analysis pipeline and showed that the Galbraith dataset may separate the infected and uninfected honey bees better than our dataset (Additional file 2). We also noted that the first replicate of both treatment groups in the Galbraith data did not cluster as cleanly in the PCA plots. However, through this automatically-generated plot, we can only visualize information at the sample level. Wanting to learn more about the data at the gene level, we continued with new visualization techniques that are available online (<https://lrutter.github.io/bigPint>) and are in preparation for publication.

We used parallel coordinate lines superimposed onto side-by-side boxplots to visualize the DEGs associated with virus infection in the two studies. The background side-by-side boxplot represents the distribution of *all* genes in the data, and each parallel coordinate line represents one DEG. In a parallel coordinate line, connections between samples with positive correlations should be flat, while connections between samples with negative correlations should be crossed. We expect DEGs to show more variability between treatments than between replicates. This means the parallel coordinate lines should be flat between replicates but crossed between treatments. However, overplotting problems would obscure our visualization if we

were to plot all DEGs onto the same side-by-side boxplot. As a result, we used hierarchical clustering techniques to separate DEGs into common patterns as is described in the methods section.

We see that the 1,019 DEGs from the Galbraith dataset form relatively clean-looking visual displays, with consistent replicates and differences between treatments (Figure 3). We do see that the first replicate of the virus group (V.1) appears somewhat inconsistent with the other virus replicates in Cluster 1, confirming that the trend we saw in the PCA plot carried through into the DEG results. Cluster 4 reveals somewhat inconsistent replicates in the virus group, although most virus standardized read counts (group V) remain consistently larger than most control standardized read counts (group N). In contrast, we see that the 43 virus-related DEGs from our dataset do not look as clean in their visual displays (Figure 4). The replicates appear somewhat inconsistent in their estimated expression levels and there is not always such a large (or even consistent) difference between treatment groups. We see a similar finding when we also examine a larger subset of 1,914 diet-related DEGs from our study (Additional file 3).

We next used repLIcate TREatment (“litre”) plots, which we recently developed and published in our bigPint software package. Litre plots allow users to visualize one DEG onto the Cartesian coordinates of one scatterplot matrix. In the litre plot, each gene in the data is plotted once for every combination of replicates between treatment groups. For example, there are nine ways to pair a replicate from one treatment group with a replicate from the other treatment group in the Galbraith dataset (N.1 and V.1, N.1 and V.2, N.1 and V.3, N.2 and V.1, N.2. and V.2, N.2 and V.3, N.3 and V.1, N.3 and V.2, and N.3 and V.3). Hence, each gene in the Galbraith dataset is plotted as nine points in the litre plot. With 11,825 genes in the Galbriath data, 106,425 points would need to be plotted. Our dataset is even more dramatic: There are 144 ways to pair a replicate from one treatment group

246 with a replicate from the other treatment group, and with 15,314 genes in our data,
247 we would need to plot 2,205,216 points. For either dataset, plotting all these points
248 would reduce the speed of interactive functionality and cause overplotting problems.
249 As a result, we use hexagon bins to summarize this massive information. Once the
250 background of hexagons has been drawn to reveal the distribution of all between-
251 treatment sample pair combinations for *all* genes, the user can superimpose all
252 between-treatment sample pair combinations for one gene of interest.

253 Additional file 4 shows nine example litre plots for our dataset. The hexagon back-
254 ground is the same for all nine litre plots because it simply shows the distribution
255 of all between-treatment sample pair combinations for *all* genes in our dataset. In
256 each litre plot, there are 144 magenta points superimposed that show all between-
257 treatment sample pair combinations for one DEG of interest. Additional file 5 and 6
258 similarly each show nine example litre plots for the Galbraith dataset. We examined
259 individual DEGs from the first cluster (Additional file 5) and second cluster (Ad-
260 ditional file 6) of the Galbraith data because the first cluster had previously shown
261 less consistency in the first replicate of the treatment group (Figure 3). Notice that,
262 as previously explained, we now show each DEG as nine points for the Galbraith
263 dataset. We see that indeed the virus DEGs from our data (Additional file 4) show
264 less consistent replications and less differences between the treatment groups com-
265 pared to the virus DEGs from the Galbraith data (Additional files 5 and 6). We also
266 observe that, in the Galbraith dataset, the DEG points in the first cluster show less
267 tight cluster patterns than the DEG points in the second cluster (Additional files
268 5 and 6), an observation we saw previously in the parallel coordinate plots (Figure
269 3).

270 Finally, we used scatterplot matrices from the bigPint software to further assess
271 the DEGs. A scatterplot matrix is another effective multivariate visualization tool
272 that plots read count distributions across all genes and samples. Specifically, it rep-

resents every gene in the dataset as a black point in each scatterplot. DEGs can be superimposed as colored points to assess their patterns against the full dataset. We expect DEGs to mostly fall along the $x=y$ line in replicate scatterplots (denoting replicate consistency) but deviate from the $x=y$ line in treatment scatterplots (denoting significant treatment changes). The $x=y$ line is shown in red in our plots.

We created standardized scatterplot matrices for each of the four clusters (from Figure 3) of the Galbraith data (Additional files 7, 8, 9, and 10). We also created standardized scatterplot matrices for our data. However, as our dataset contained 24 samples, we would need to include 276 scatterplots in our matrix, which would be too numerous to allow for efficient visual assessment of the data. As a result, we created four scatterplot matrices of our data, each with subsets of 6 samples to be more comparable to the Galbraith data (Additional files 11, 12, 13, and 14). We can again confirm through these plots that the DEGs from the Galbraith data appeared more as expected: They deviated more from the $x=y$ line in the treatment scatterplots while staying close to the $x=y$ line in replicate scatterplots.

Despite the virus-related DEGs ($n = 1,019$) from the Galbraith dataset displaying the expected patterns more than those from our dataset ($n = 43$), there was significant overlap (p-value $< 2.2\text{e-}16$) in the DEGs between the two studies, with 26/38 (68%) of virus-upregulated DEGs from our study also showing virus-upregulated response in the Galbraith study (Figure 6).

Tolerance versus resistance

Using the contrasts specified in Table 2, we discovered 122 “tolerance” candidate DEGs and 125 “resistance” candidate DEGs. Within our 122 “tolerance” gene ontologies, we found functions related to metabolism (such as carbohydrate metabolism, fructose metabolism, and chitin metabolism). However, we also discovered gene ontologies related to RNA polymerase II transcription, immune response, and regulation of response to reactive oxygen species (Figure 5A). Within our 125

300 “resistance” gene ontologies, we found functions related to metabolism (such as car-
301 bohydrate metabolism, chitin metabolism, oligosaccharide biosynthesis, and general
302 metabolism) (Figure 5B).

303 To visually explore gene expression patterns related to tolerance and resistance,
304 we used hierarchical clustering to separate candidate DEGs into common patterns,
305 and then visualized these clusters using parallel coordinate lines superimposed onto
306 side-by-side boxplots. To reduce overplotting of parallel coordinate lines, we again
307 used hierarchical clustering techniques to separate DEGs into common patterns.
308 Perhaps unsurprisingly, we still see a substantial amount of noise (inconsistency
309 between replicates) in our resulting candidate DEGs (Additional files 15 and 16).
310 However, the broad patterns we expect to see still emerge: For example, based on
311 the contrasts we created to obtain the ‘tolerance’ candidate DEGs, we expect them
312 to display larger count values in the “NC” group compared to the “NR” group and
313 larger count values in the “VC” group compared to the “VR” group. Indeed, we see
314 this pattern in the associated parallel coordinate plots (Additional file 15). Likewise,
315 based on the contrasts we created to obtain the ‘resistance’ candidate DEGs, we
316 still expect them to display larger count values in the “VC” group compared to
317 the “VR” group, but we no longer expect to see a difference between the “NC”
318 and “NR” groups. We do generally see these expected patterns in the associated
319 parallel coordinate plots: While there are large outliers in the “NC” group, the “NR”
320 replicates are no longer typically below a standardized count of zero (Additional file
321 16). The genes in Cluster 3 may follow the expected pattern the most distinctively
322 (Additional file 16).

323 Post hoc analysis

324 To better understand sources of transcriptomic noise, we explored whether pathogen
325 response measurements (virus titers and mortality), which varied widely across
326 samples, were correlated with observed patterns in gene expression.

327 The R-squared values between gene read counts and pathogen response measure-
328 ments were generally low ($R\text{-squared} < 0.1$) across our dataset (Supplementary
329 table 9, Additional file 1). We further explored whether clusters of DEGs showed
330 higher correlations with pathogen response measurements than non-DEGs (the lat-
331 ter serving as a control, where we do not expect a correlation). A Kruskal–Wallis
332 test was used to determine if R-squared distributions of DEG clusters significantly
333 differed from those in the rest of the data. The p-values and Bonferroni correction
334 values for each of the 36 tests (as described in the methods section) is provided
335 in Supplementary table 9, Additional file 1. An overall trend emerges to suggest
336 that DEGs may have significantly larger correlation with the pathogen response
337 measurements compared to non-DEGs.

338 Discussion

339 Challenges to honey bee health are a growing concern, in particular the combined,
340 interactive effects of nutritional stress and pathogens [42]. In this study, we used
341 RNA-sequencing to probe mechanisms underlying honey bee responses to two ef-
342 fects, diet quality and infection with the prominent virus of concern, IAPV. In
343 general, we found a major nutritional transcriptomic response, with nearly 2,000
344 transcripts changing in response to diet quality (rockrose/poor diet versus chest-
345 nut/good diet). The majority of these genes were upregulated in response to high
346 quality diet, and these genes were over-represented for functions such as nutrient
347 signaling metabolism (insulin resistance), immune response (Notch signaling and
348 JaK-STAT pathways), and carbon metabolism (Supplementary table 2, Additional
349 file 1). These data suggest high quality nutrition may allow bees to alter their
350 metabolism, favoring investment of energy into innate immune responses.

351 One of the few studies that has investigated transcriptomic response to nutrition in
352 honey bees similarly found that pollen upregulates genes related to macromolecule
353 metabolism, insulin pathways, and TOR pathways [43]. Diet effects on transcrip-

354 tomics have been more extensively studied in the insect model *Drosophila*. One
355 recent transcriptomic study in *Drosophila melanogaster* reported an overexpression
356 of genes related to immunity, metabolism, and hemocyanin in a high-fat diet and
357 overexpression of genes related to cell cycle activity, DNA binding and transcription,
358 and CHK kinase-like protein activity in a high-sugar diet [45]. This same study also
359 discovered an upregulation of genes related to peptide and carbohydrate processing
360 in both high-fat and high-sugar diets, a finding the authors attributed to a general
361 increase in caloric intake. Another recent study investigated the transcriptomic ef-
362 fects of diets high in protein relative to sugar, diets high in sugar relative to protein,
363 and diets with equal amounts of protein and sugar [46]. *Drosophila mojavensis* and
364 *Drosophila arizonae* showed substantial differential expression between the dietary
365 conditions: genes involved in carbohydrate and lipid metabolism were upregulated
366 in response to high sugar low protein diets and genes involved in juvenile hormone
367 (JH) and ecdysone were upregulated in response to low sugar high protein diets. In-
368 terestingly, prior studies have suggested that JH regulates body size by controlling
369 ecdysone production, which modifies insulin signaling [47]. As we saw in our study,
370 these studies generally suggest that diet differences may relate to gene expression
371 changes in metabolism and immune responses in honey bees.

372 While some insect systems have shown relatively low transcriptional responses
373 to dicistrovirus infection [61, 62], previous work on honey bees has revealed many
374 hundreds of DEGs [44]. Discrepancies between datasets may be due to noise and
375 complexity of the honey bee microbiome. The transcriptomic response to virus infec-
376 tion in our experiment was fairly limited. We found only 43 differentially expressed
377 transcripts, some with known immune functions such as a gene with similarity to
378 MD-2 lipid recognition protein and argonaute-2, a protein that plays a central role
379 in RNA silencing (Table 1). We also found genes related to transcriptional regu-
380 lation and muscle contraction. The small number of DEGs in this study may be

381 partly explained by the large amount of noise in the data (Figure 4 and Additional
382 files 2B, 4, 11, 12, 13, and 14).

383 There have been numerous studies on the transcriptomic effects of virus infection
384 in model organisms like fruit flies and mosquitoes that can provide a useful frame-
385 work for interpreting virus responses in honey bees. These studies have shown that
386 RNA silencing is a major antiviral strategy, along with transcriptional pausing, Toll
387 pathways, IMD pathways, JAK/STAT pathways, and Toll-7-autophagy pathways
388 [48, 49]. Recent transcriptomic studies in honey bees have shown similar hallmarks
389 of these same antiviral defense mechanisms [50]. It is important to note that general
390 immune responses to viral infection in insects might be an indirect result of cellular
391 damage [49]. In fact, every virus-host interaction has its own particularities derived
392 from the diverse methods of replication and infection cycle evolved by different
393 viruses. An intricate set of pro- and anti-virus host factors such as ribosomal pro-
394 teins and autophagy pathways are involved, but the response depends on the virus
395 species, as has been elucidated in *Drosophila* [48, 49]. In addition, a non-sequence-
396 specific antiviral response mediated by unspecific dsRNA pathway was discovered
397 in honey bees [63, 64]. In the case of dicistroviruses, few works have studied the
398 impact of IAPV infection at transcriptional level. Chen et al. 2014 analyzed re-
399 sponses to IAPV infection in larvae and workers using microarrays [65]. Many of
400 the DEGs found were involved in immune response and energy-related metabolism,
401 particularly in adults but not in brood. The authors propose this observed differ-
402 ence could be connected to latent infections in larvae (where host immunity is not
403 perturbed) versus acute infections in adulthood (induced by stressors faced during
404 development) [65]. IAPV acute infection also alters the DNA methylation pattern
405 of numerous genes that do not overlap the genes that are up- or down-regulated
406 at the transcriptional level [44]. These works reiterate the conclusion that viruses
407 trigger particular antiviral mechanisms by different means and depending on several

408 factors. The honey bee antiviral pathways induced by specific viruses were recently
409 reviewed [50]; it is noteworthy that many honey bee factors discovered by tran-
410 scriptomics need further characterization to uncover their role in controlling (or
411 promoting) viral infection in honey bees.

412 Given the noisy nature of our data, and our desire to hone in on genes with real
413 expression differences, we compared our data to the Galbraith study [44], which
414 also examined bees response to IAPV infection. In contrast to our study, Galbraith
415 et al. identified a large number of virus responsive transcripts, and generally had
416 less noise in their data (Figure 3 and Additional files 2A, 5, 6, 7, 8, 9, and 10). To
417 identify the most consistent virus-responsive genes from our study, we looked for
418 overlap in the DEGs associated with virus infection on both experiments. We found
419 a large, statistically significant ($p\text{-value} < 2.2e\text{-}16$) overlap, with 26/38 (68%) of
420 virus-responsive DEGs from our study also showing response to virus infection in
421 Galbraith et al. (Figure 6). This result gives us confidence that, although noisy, we
422 were able to uncover reliable, replicable gene expression responses to virus infection
423 with our data.

424 Data visualization is a useful method to identify noise and robustness in RNA-
425 sequencing data [66]. In this study, we used extensive data visualization to improve
426 the interpretation of our RNA-sequencing results. For example, the DESeq2 pack-
427 age comes with certain visualization options that are popular in RNA-sequencing
428 analysis. One of these visualization is the principal component analysis (PCA) plot,
429 which allows users to visualize the similarity between samples within a dataset. We
430 could determine from this plot that indeed the Galbraith data may show more simi-
431 larity between its replicates and differences between its treatments compared to our
432 data (Additional file 2). However, the PCA plot only shows us information at the
433 sample level. We wanted to investigate how these differences in the signal:to:noise
434 ratios of the datasets would affect the structure of any resulting DEGs. As a result,

we also used three plotting techniques from the bigPint package: We investigated the 1,019 virus-related DEGs from the Galbraith dataset and the 43 virus-related DEGs from our dataset using parallel coordinate lines, scatterplot matrices, and litre plots. To prevent overplotting issues in our graphics, we used a hierarchical clustering technique for the parallel coordinate lines to separate the set of DEGs into smaller groups. We also needed to examine four subsets of samples from our dataset to make effective use of the scatterplot matrices. After these tailorizations, we determined that the same patterns we saw in the PCA plots regarding the entire dataset extended down the pipeline analysis into the DEG calls: Even the DEGs from the Galbraith dataset showed more similarity between their replicates and differences between their treatments compared to those from our data. However, the 365 DEGs from the Galbraith data in Cluster 1 of Figure 3 showed an inconsistent first replicate in the treatment group (“V.1”), which was something we observed in the PCA plot. This indicates that this feature also extended down the analysis pipeline into DEG calls. Despite the differences in signal between these two datasets, there was substantial overlap in the resulting DEGs. We believe these visualization applications can be useful for future researchers analyzing RNA-sequencing data to quickly and effectively ensure that the DEG calls look reliable or at least overlap with DEG calls from similar studies that look reliable. We also expect this type of visualization exploration can be especially crucial when studying wild populations with high levels of genetic and environmental variation between replicates and/or when using experiments that may lack rigid design control.

One of the goals of this study was to use our RNA-sequencing data to assess whether transcriptomic responses to diet quality and virus infection provide insight into whether high quality diet can buffer bees from pathogen stress via mechanisms of “resistance” or “tolerance”. Recent evidence has suggested that overall immunity is determined by more than just “resistance” (the reduction of pathogen fitness

within the host by mechanisms of avoidance and control) [67]. Instead, overall immunity is related to “resistance” in conjunction with “tolerance” (the reduction of adverse effects and disease resulting from pathogens by mechanisms of healing) [41, 67]. Immune-mediated resistance and diet-driven tolerance mechanisms are costly and may compete with each other [41, 68]. Data and models have suggested that selection can favor an optimum combination of both resistance and tolerance [69, 70, 71, 72]. We attempted to address this topic through specific gene expression contrasts (Table 2), accompanied by GO analysis of the associated gene lists. We found an approximately equal number of resistance ($n = 125$) and tolerance ($n = 122$) related candidate DEGs, suggesting both processes may be playing significant roles in dietary buffering from pathogen induced mortality. Resistance candidate DEGs had functions related to several forms of metabolism (chitin and carbohydrate), regulation of transcription, and cell adhesion (Figure 5B). Tolerance candidate DEGs had functions related to carbohydrate metabolism and chitin metabolism; however, they also showed functions related to immune response, including RNA polymerase II transcription (Figure 5A). Previous studies have shown that transcriptional pausing of RNA polymerase II may be an innate immune response in *D. melanogaster* that allows for a more rapid response by increasing the accessibility of promoter regions of virally induced genes [73]. These possible immunological defense mechanisms within our “tolerance” candidate DEGs and metabolic processes within our “resistance” candidate DEGs may provide additional evidence of feedbacks between diet and disease in honey bees [42].

There were several limitations in this study that could be improved upon in future studies. For instance, our comparison between the Galbraith data (single-drone colonies) and our data (naturally mated colonies) was limited by numerous extraneous variables between these studies. In addition to different molecular pipelines and bioinformatic preprocessing pipelines used between these studies, the Galbraith

study focused on one-day old worker honey bees that were fed sugar and artificial pollen diet, whereas our study focused on adult worker honey bees that were fed bee-collected monofloral diets. Furthermore, the Galbraith data used eviscerated abdomens with attached fat bodies and only considered symptomatic honey bees for their infected treatment group, whereas we used whole bodies and considered both asymptomatic and symptomatic honey bees for our infected treatment group. There are also differences in the hours post inoculation and possible differences in the inoculation amount between the studies. Further differences between the studies can be found in their corresponding published methods sections [40, 44]. The different factors between these two studies may be critical because particular antiviral factors in honey bees are linked to specific viruses, specific developmental stages, the analyzed tissue, the route of inoculation, and the time (post-inoculation) during which the study was performed. This was clearly demonstrated when comparing honey bee responses to two related iflaviruses with very different infection dynamics, sacbrood bee virus (SBV) vs. deformed wing virus (DWV) [74]. Authors observed differences in induction of defensin and hymenoptaecin immune-related genes, and suggested the results reflect adaptations to the different routes of transmission [74].

Moreover, our comparative visualization assessment between these two datasets was also somewhat limited because the virus effect in the Galbraith study used three replicates for each level, whereas the virus effect in our study used twelve replicates for each level that were actually further subdivided into six replicates for each diet level. Hence the apparent reduction in noise observed in the Galbraith data compared to our data in the PCA plots, parallel coordinate plots, scatterplot matrices, and litre plots may be an inadvertent product of the smaller number of replicates used and the lack of a secondary treatment group rather than solely the reduction in genetic variability through the single-drone colony design itself. With this in mind, while our current efforts may be a starting point, future studies can

shed more light on signal:to:noise and differential expression differences between naturally mated colony designs and single-drone colony designs by controlling for extraneous factors more strictly than what we were able to do in the current line of work.

In addition, this study used a whole body RNA-sequencing approach. In future related studies, it may be informative to use tissue-specific methods. Previous work has shown that even though IAPV replication occurs in all honey bee tissues, it localizes more in gut and nerve tissues and in the hypopharyngeal glands. Likewise, the highest IAPV titers have been observed in gut tissues [34]. Recent evidence has suggested that RNA-sequencing approaches toward composite structures in honey bees leads to false negatives, implying that genes strongly differentially expressed in particular structures may not reach significance within the composite structure [75]. These studies have also found that within a composite extraction, structures therein may contain opposite patterns of differential expression. We can provide more detailed answers to our original transcriptomic questions if we were to repeat this same experimental design only now at a more refined tissue level. Another future direction related to this work would be to integrate multiple omics datasets to investigate monofloral diet quality and IAPV infection in honey bees. Indeed, previous studies in honey bees have found that multiple omics datasets do not always align in a clear-cut manner, and hence may broaden our understanding of the molecular mechanisms being explored [44].

Conclusions

To the best of our knowledge, there are few to no studies investigating honey bee gene expression specifically related to monofloral diets, and few to no studies examining honey bee gene expression related to the combined effects of diet in any general sense and viral inoculation in any general sense. It also remains unknown whether the protective effects of good diet in honey bees is due to direct effects on

immune function (resistance) or indirect effects of energy availability on vigor and health (tolerance). We attempted to address these unresolved areas by conducting a two-factor RNA-sequencing study that examined how monofloral diets and IAPV inoculation influence gene expression patterns in honey bees. Overall, our data suggest complex transcriptomic responses to multiple stressors in honey bees. Diet has the capacity for large and profound effects on gene expression and may set up the potential for both resistance and tolerance to viral infection, adding to previous evidence of possible feedbacks between diet and disease in honey bees [42].

Moreover, this study also demonstrated the benefits of using data visualizations and multiple datasets to address inherently messy biological data. For instance, by verifying the substantial overlap in our DEG lists to those obtained in another study that addressed a similar question using specimens with less genetic variability, we were able to place much higher confidence in the differential gene expression results from our otherwise noisy data. We also suggested that comparing results derived from multiple studies varying in level of genetic and environmental variability may allow researchers to identify transcriptomic patterns that are concurrently more realistic and less noisy. Altogether, we hope our results underline the merits of using data visualization techniques and multiple datasets to understand and interpret RNA-sequencing datasets.

Methods

Mortality and virus titers

Details of the procedures we used to prepare virus inoculum, infect and feed caged honey bees, and quantify IAPV can be reviewed in our previous work [40, 33]. A linear mixed effects model was used to relate the mortality rates and IAPV titers to the main and interaction effects of the diet and virus factors. The model was fitted to the data by restricted maximum likelihood (REML) using the “lme” function in the R package “nlme”. A random (intercept) effect for experimental setup was

570 included in the model. Post-hoc pairwise comparisons of the four (diet and virus
571 combination) treatment groups were performed and Benjamini-Hochberg adjusted
572 p-values were calculated to limit familywise Type I error rates [76].

573 Design of two-factor experiment

574 For our nutrition factor, we examined two monofloral pollen diets, rockrose (*Cis-*
575 *tus* sp.) and chestnut (*Castanea* sp.). Rockrose pollen is generally considered less
576 nutritious than chestnut pollen due to its lower levels of protein, amino acids, antiox-
577 idants, calcium, and iron [40, 51]. For our virus factor, one level contained bees that
578 were infected with IAPV and another level contained bees that were not infected
579 with IAPV. This experimental design resulted in four treatment groups (rockrose
580 pollen without IAPV exposure, chestnut pollen without IAPV exposure, rockrose
581 pollen with IAPV exposure, and chestnut pollen with IAPV exposure) that allowed
582 us to assess main effects and interactive effects between diet quality and IAPV
583 infection in honey bees.

584 There are several reasons why our design focused only on diet quality (monofloral
585 diets) as opposed to diet diversity (monofloral diets versus polyfloral diets). First,
586 when assessing diet diversity, a sugar diet is often used as a control. However, such
587 an experimental design does not reflect real-world conditions for honey bees as
588 they rarely face a total lack of pollen [51]. Second, in studies that compared honey
589 bee health using monofloral and polyfloral diets at the same time, if the polyfloral
590 diet and one of the high-quality monofloral diets both exhibited similarly beneficial
591 effects, then it was difficult for the authors to assess if the polyfloral diet was better
592 than most of the monofloral diets because of its diversity or because it contained as
593 a subset the high-quality monofloral diet [51]. Third, as was previously mentioned,
594 honey bees are now confronted with less diverse sources of pollen. As a result, there
595 is a need to better understand how monofloral diets affect honey bee health.

596 RNA extraction

597 Fifteen cages per treatment were originally produced for monitoring of mortality.
598 From these, six live honey bees were randomly selected from each cage 36 hours
599 post inoculation and placed into tubes [33]. Tubes were kept on dry ice and then
600 transferred into a -80C freezer until processing. From the fifteen possible cages,
601 eight were randomly selected for RNA-sequencing. From these eight cages, two of
602 the honey bees per cage were randomly selected from the original six live honey
603 bees per cage. These two bees were combined to form a pooled sample representing
604 the cage. Whole body RNA from each pool was extracted using Qiagen RNeasy
605 MiniKit followed by Qiagen DNase treatment. Samples were suspended in water to
606 200-400 ng/ μ l. All samples were then tested on a Bioanalyzer at the Iowa State
607 University DNA Facility to ensure quality (RIN > 8).

608 Gene expression

609 Samples were sequenced starting on January 14, 2016 at the Iowa State University
610 DNA Facility (Platform: Illumina HiSeq Sequencing 2500 in rapid run mode; Cat-
611 egory: Single End 100 cycle sequencing). A standard Illumina mRNA library was
612 prepared by the DNA facility. Reads were aligned to the BeeBase Version 3.2 genome
613 [77] from the Hymenoptera Genome Database [78] using the programs GMAP and
614 GSNAP [79]. There were four lanes of sequencing with 24 samples per lane. Each
615 sample was run twice. Approximately 75-90% of reads were mapped to the honey
616 bee genome. Each lane produced around 13 million single-end 100 basepair reads.

617 We tested all six pairwise combinations of treatments for DEGs (pairwise DEGs).
618 We also tested the diet main effect (diet DEGs), virus main effect (virus DEGs), and
619 interaction term for DEGs (interaction DEGs). We then also tested for virus main
620 effect DEGs (virus DEGs) in public data derived from a previous study exploring
621 the gene expression of IAPV virus infection in honey bees [44]. We tested each
622 DEG analysis using recommended parameters with DESeq2 [80], edgeR [66], and

623 LimmaVoom [81]. In all cases, we used a false discovery rate (FDR) threshold of 0.05
624 [82]. Fisher's exact test was used to determine significant overlaps between DEG
625 sets (whether from the same dataset but across different analysis pipelines or from
626 different datasets across the same analysis pipelines). The eulerr shiny application
627 was used to construct Venn diagram overlap images [83]. In the end, we focused on
628 the DEG results from DESeq2 [80] as this pipeline was also used in the Galbraith
629 study [44]. We used the independent filtering process built into the DESeq2 software
630 that mitigates multiple comparison corrections on genes with no power rather than
631 defining one filtering threshold.

632 Comparison to prior studies on transcriptomic response to viral infection

633 We compare the main effect of IAPV exposure in our dataset to that obtained in a
634 previous study conducted by Galbraith and colleagues [44] who also addressed honey
635 bee transcriptomic responses to virus infection. We applied the same downstream
636 bioinformatics analyses between our count table and the count table provided in
637 the Galbraith study. When we applied our bioinformatics pipeline to the Galbraith
638 count table, we obtained different differential expression counts compared to the
639 results published in the Galbraith study. However, there was substantial overlap and
640 we considered this justification to use the differential expression list we obtained in
641 order to keep the downstream bioinformatics analyses as similar as possible between
642 the two datasets (Additional file 17).

643 We used honey bees from naturally mated colonies, whereas Galbraith et al. [44]
644 used honey bees from single-drone colonies. In light of this, we should expect the
645 Galbraith et al. dataset to contain lower genetic variation between its replicates
646 and higher signal:to:noise ratios than our dataset. We use visualization techniques
647 to assess the signal:to:noise ratio between these two datasets, and differential gene
648 expression (DEG) analyses to determine any significantly overlapping genes of in-
649 terest between these two datasets.

650 Visualization

651 We used an array of visualization tools as part of our analysis. We used the PCA plot
652 [84] from the DESeq2 package, a well-known and established tool. Along with that,
653 we used lesser-known multivariate visualization tools from our work-in-progress R
654 package called bigPint. Specifically, we used parallel coordinate plots [85], scatter-
655 plot matrices [86], and litre plots (which we recently developed based on “replicate
656 line plots” [87]) to assess the variability between the replicates and the treatments
657 in our data. We also used these plotting techniques to assess for normalization
658 problems and other common problems in RNA-sequencing analysis pipelines [87].

659 Furthermore, we used statistical graphics to better understand patterns in our
660 DEGs. However, in cases of large DEG lists, these visualization tools had overplot-
661 ting problems (where multiple objects are drawn on top of one another, making
662 it impossible to detect individual values). To remedy this problem, we first stan-
663 dardized each DEG to have a mean of zero and standard deviation of unity [88, 89].
664 Then, we performed hierarchical clustering on the standardized DEGs using Ward’s
665 linkage. This process divided large DEG lists into smaller clusters of similar pat-
666 terns, which allowed us to more efficiently visualize the different types of patterns
667 within large DEG lists (see Figures 3 and 4 for examples).

668 Gene ontology

669 DEGs were uploaded as a background list to DAVID Bioinformatics Resources 6.7
670 [90, 91]. The overrepresented gene ontology (GO) terms of DEGs were determined
671 using the BEEBASE_ID identifier option (honey bee gene model) in the DAVID
672 software. To fine-tune the GO term list, only terms correlating to Biological Pro-
673 cesses were considered. The refined GO term list was then imported into REVIGO
674 [92], which uses semantic similarity measures to cluster long lists of GO terms.

675 Probing tolerance versus resistance

676 To investigate whether the protective effect of good diet is due to direct, specific
677 effects on immune function (resistance), or if it is due to indirect effects of good nu-
678 trition on energy availability and vigor (tolerance), we created contrasts of interest
679 (Table 2). In particular, we assigned “resistance candidate DEGs” to be the ones
680 that were upregulated in the chestnut group within the virus infected bees but not
681 upregulated in the chestnut group within the non-infected bees. Our interpretation
682 of these genes is that they represent those that are only activated in infected bees
683 that are fed a high quality diet. We also assigned “tolerance candidate DEGs” to
684 be the ones that were upregulated in the chestnut group for both the virus infected
685 bees and non-infected bees. Our interpretation of these genes is that they represent
686 those that are constitutively activated in bees fed a high quality diet, regardless
687 of whether they are experiencing infection or not. We then determined how many
688 genes fell into these two categories and analyzed their GO terminologies.

689 Post hoc analysis

690 We found considerable noisiness in our data and saw, through gene-level visual-
691 izations, that our DEGs contained outliers and inconsistent replicates. Hence, we
692 wanted to explore whether our DEG read counts correlated with pathogen response
693 metrics, including IAPV titers, sacbrood bee virus (SBV) titers, and mortality rates.
694 For this process, we considered virus main effect DEGs (Figure 4), “tolerance can-
695 didate” DEGs (Additional file 15), and “resistance candidate” DEGs (Additional
696 file 16). For each DEG in each cluster, we calculated a coefficient of determination
697 (R-squared) value to estimate the correlation between its raw read counts and the
698 pathogen response metrics across its 24 samples. We then used the Kruskal–Wallis
699 test to determine if the distribution of the R-squared values in any of the DEG clus-
700 ters significantly differed from those in the non-DEG genes (the rest of the data).
701 As there were four clusters for each of the nine combinations of DEG lists (“tol-

erance” candidate DEGs, “resistance” candidate DEGs, and virus-related DEGs)
 and pathogen response measurements (IAPV titer, SBV titer, and mortality rate),
 this process resulted in 36 statistical tests.

Ethics approval and consent to participate

All honey bees used in this work were sampled in the United States, and no ethical use approval is required for this species in this country.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS]. Include our data, Galbraith data, scripts to reproduce tables and figures (on GitHub).

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

LR performed the bioinformatic and statistical analyses, produced the figures and tables, and drafted the manuscript. BB conceptualized the study and critically revised the manuscript. AD contributed to experimental design, carried out the laboratory experiments, and processed samples for virus titers and RNA-seq.

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928 **Figures**

Figure 1 Mortality rates for the four treatment groups, two virus groups, and two diet groups. Left to right: Mortality rates for the four treatment groups, two virus groups, and two diet groups. “N” represents non-inoculation, “V” represents viral inoculation, “C” represents chestnut pollen, and “R” represents rockrose pollen. The mortality rate data included 59 samples with 15 replicates per treatment group, except for the “NC” group having 14 replicates. ANOVA values and p-values for the statistical tests are listed in the text of the paper. The letters above the bars represent significant differences with a confidence level of 95%.

Figure 2 IAPV titers for the four treatment groups, two virus groups, and two diet groups. Left to right: IAPV titers for the four treatment groups, two virus groups, and two diet groups. “N” represents non-inoculation, “V” represents viral inoculation, “C” represents chestnut pollen, and “R” represents rockrose pollen. The IAPV titer data included 38 samples with 10 replicates per treatment group, except for the “NR” group having 8 replicates. ANOVA values and p-values for the statistical tests are listed in the text of the paper. The letters above the bars represent significant differences with a confidence level of 95%.

Figure 3 Parallel coordinate plots of the 1,019 DEGs after hierarchical clustering of size four between the virus-infected and control groups of the Galbraith data [44]. Parallel coordinate plots of the 1,019 DEGs after hierarchical clustering of size four between the virus-infected and control groups of the Galbraith study. “N” represents non-inoculation, “V” represents viral inoculation. Clusters 1, 2, and 4 seem to represent DEGs that were overexpressed in the virus inoculated group, and Cluster 3 seems to represent DEGs that were overexpressed in the non-inoculated control group. In general, the DEGs appeared as expected, but there is rather noticeable deviation of the first replicate from the virus-treated sample (“V.1”) from the other virus-treated replicates in Cluster 1.

Figure 4 Parallel coordinate plots of the 43 DEGs after hierarchical clustering of size four between the virus-infected and control groups of our study. Parallel coordinate plots of the 43 DEGs after hierarchical clustering of size four between the virus-infected and control groups of our study. “N” represents non-infected control group, and “V” represents treatment of virus. The vertical red line indicates the distinction between treatment groups. We see from this plot that the DEG designations for this dataset do not appear as clean compared to what we saw in the Galbraith dataset in Figure 3.

Figure 5 Gene ontology analysis results for the 122 DEGs related to our “tolerance” hypothesis and for the 125 DEGs related to our “resistance” hypothesis. GO analysis results for the 122 DEGs related to our “tolerance” hypothesis (A) and for the 125 DEGs related to our “resistance” hypothesis (B). The color and size of the circles both represent the number of genes in that ontology. The x-axis and y-axis are organized by SimRel, a semantic similarity metric [93].

Figure 6 Venn diagrams comparing the virus-related DEG overlaps between our dataset and the Galbraith dataset. Venn diagrams comparing the virus-related DEG overlaps between the Galbraith study (labeled as “G”) and our study (labeled as “R”). From left to right: Total virus-related DEGs (subplot A), virus-upregulated DEGs (subplot B), control-upregulated DEGs (subplot C). Both the total virus-related and virus-upregulated DEGs showed significant overlap between the studies ($p\text{-value} < 2.2\text{e-}16$) as per Fisher’s Exact Test for Count Data. There was one gene that was virus-upregulated in the Galbraith study but control-upregulated in our study.

929 **Tables**

BeeBase ID	Gene Name	Known functions	Us	Galbraith
GB41545	MD-2-related lipid-recognition protein-like	Implicated in lipid recognition, particularly in the recognition of pathogen related products	N	-
GB50955	Protein argonaute-2	Interacts with small interfering RNAs to form RNA-induced silencing complexes which target and cleave transcripts that are mostly from viruses and transposons	V	V
GB48755	UBA-like domain-containing protein 2	Found in diverse proteins involved in ubiquitin/proteasome pathways	V	V
GB47407	Histone H4	Capable of affecting transcription, DNA repair, and DNA replication when post-transcriptionally modified	V	V
GB42313	Leishmanolysin-like peptidase	Encodes a protein involved in cell migration and invasion; implicated in mitotic progression in <i>D. melanogaster</i>	V	V
GB50813	Rho guanine nucleotide exchange factor 11	Implicated in regulation of apoptotic processes, cell growth, signal transduction, and transcription	V	V
GB54503	Thioredoxin domain-containing protein	Serves as a general protein disulphide oxidoreductase	N	-
GB53500	Transcriptional regulator Myc-B	Regulator gene that codes for a transcription factor	V	V
GB51305	Tropomyosin-like	Related to protein involved in muscle contraction	N	N
GB50178	Cilia and flagella-associated protein 61-like	Induces components required for wild-type motility and stable assembly of motile cilia	V	V

Table 1 Known functions of the mapped subset of 43 DEGs in the virus main effect of our study. Whether the gene was overrepresented in the virus or non-virus group is also indicated for both our study and the Galbraith study. Functionalities were extracted from Flybase, National Center for Biotechnology Information and The European Bioinformatics Institute databases.

Contrast	DEGs	Interpretation	Results
V (all) vs N (all)	43	Genes that change expression due to virus effect regardless of diet status in bees	Table 1
NC vs NR	941	Genes that change expression due to diet effect in uninfected bees	Supplementary tables 4 and 5, Additional file 1
VC vs VR	376	Genes that change expression due to diet effect in infected bees	Supplementary tables 6 and 7, Additional file 1
VC upregulated in VC vs VR, and NC upregulated in NC vs NR	122	“Tolerance” genes that turn on by good diet regardless of virus infection status in bees	Figure 5A
VC upregulated in VC vs VR, but NC not upregulated in NC vs NR	125	“Resistance” genes that turn on by good diet only in infected bees	Figure 5B

Table 2 Contrasts in our study for assessing GO and pathways analysis.

Additional Files

Additional file 1 — Supplementary tables.

Table 1: Number of DEGs across three analysis pipelines for (A) the diet main effect in our study, (B) the virus main effect in our study, and (C) the virus main effect in the Galbraith study. For the diet effects, “C” represents chestnut diet and “R” represents rockrose diet. For the virus effects, “N” represents control non-inoculated and “V” represents virus-inoculated. **Table 2:** Pathways related to the 1,033 DEGs that were upregulated in the chestnut treatment from the diet main effect. **Table 3:** Pathways related to the 881 DEGs that were upregulated in the rockrose treatment from the diet main effect. **Table 4:** GO analysis results for the 601 DEGs that were upregulated in the NC treatment from the NC versus NR treatment pair analysis. These DEGs represent genes that are upregulated when non-infected honey bees are given high quality chestnut pollen compared to being given low quality rockrose pollen. **Table 5:** GO analysis results for the 340 DEGs that were upregulated in the NR treatment from the NC versus NR treatment pair analysis. These DEGs represent genes that are upregulated when non-infected honey bees are given low quality rockrose pollen compared to being given high quality chestnut pollen. **Table 6:** GO analysis results for the 247 DEGs that were upregulated in the VC treatment from the VC versus VR treatment pair analysis. These DEGs represent genes that are upregulated when infected honey bees are given high quality chestnut pollen compared to being given low quality rockrose pollen. **Table 7:** GO analysis results for the 129 DEGs that were upregulated in the VR treatment from the VC versus VR treatment pair analysis. These DEGs represent genes that are upregulated when infected honey bees are given low quality rockrose pollen compared to being given high quality chestnut pollen. **Table 8:** Number of DEGs across three analysis pipelines for all six treatment pair combinations between the diet and virus factor. “C” represents chestnut diet, “R” represents rockrose diet, “V” represents virus-inoculated, and “N” represents control non-inoculated. **Table 9:** Kruskal-Wallis p-value and Bonferroni corrections for the 36 combinations of DEG lists, pathogen response metrics, and cluster number. (XLS).

Additional file 2 — PCA plots for the Galbraith dataset and for our dataset.

PCA plots for the Galbraith dataset (A) and for our dataset (B). “V” represents virus-inoculated, and “N” represents control non-inoculated. The x-axis represents the principal component with the most variation and the y-axis represents the principal component with the second-most variation (PNG).

Additional file 3 — Parallel coordinate lines of the diet-related DEGs of our dataset.

Parallel coordinate plots of the 1,914 DEGs after hierarchical clustering of size six between the chestnut and rockrose groups of our study. Here “C” represents chestnut samples, and “R” represents rockrose samples. The vertical red line indicates the distinction between treatment groups. We see from this plot that the DEG designations for this dataset do not appear as clean compared to what we saw in the Galbraith dataset in Figure 3 (PNG).

Additional file 4 — Example litre plots from the virus-related DEGs of our dataset.

Example litre plots of the nine DEGs with the lowest FDR values from the 43 virus-related DEGs of our dataset. “N” represents non-infected control samples and “V” represents virus-treated samples. Most of the magenta points (representing the 144 combinations of samples between treatment groups for a given DEG) do not reflect the expected pattern as clearly compared to what we saw in the litre plots of the Galbraith data. They are not as clustered together (representing replicate inconsistency) and they sometimes cross the $x=y$ line (representing lack of difference between treatment groups). This finding reflects what we saw in the messy looking parallel coordinate lines of Figure 4 (PNG).

969 Additional file 5 — Example litre plots of DEGs from Cluster 1 of the Galbraith dataset.

970 Example litre plots of the nine DEGs with the lowest FDR values from the 365 DEGs in Cluster 1 (originally shown
971 in Figure 3) of the Galbraith dataset. "N" represents non-infected control samples and "V" represents virus-treated
972 samples. Most of the light orange points (representing the nine combinations of samples between treatment groups
973 for a given DEG) deviate from the $x=y$ line in a tight bundle as expected (PNG).

974 Additional file 6 — Example litre plots of DEGs from Cluster 2 of the Galbraith dataset.

975 Example litre plots of the nine DEGs with the lowest FDR values from the 327 DEGs in Cluster 2 (originally shown
976 in Figure 3) of the Galbraith dataset. "N" represents non-infected control samples and "V" represents virus-treated
977 samples. Most of the dark orange points (representing the nine combinations of samples between treatment groups
978 for a given DEG) deviate from the $x=y$ line in a compact clump as expected. However, they are not as tightly
979 bunched together compared to what we saw in the example litre plots of Cluster 1 (shown in Additional file 5). As a
980 result, what we see in these litre plots reflects what we saw in the parallel coordinate lines of Figure 3: The replicate
981 consistency in the Cluster 1 DEGs is not as clean as that in the Cluster 2 DEGs, but is still relatively clean (PNG).

982 Additional file 7 — Scatterplot matrix of DEGs from Cluster 1 of the Galbraith dataset.

983 The 365 DEGs from the first cluster of the Galbraith dataset (originally shown in Figure 3) superimposed as light
984 orange dots onto all genes as black dots in the form of a scatterplot matrix. The data has been standardized. "N"
985 represents non-infected control samples and "V" represents virus-treated samples. We confirm that the DEGs
986 mostly follow the expected structure, with their placement deviating from the $x=y$ line in the treatment
987 scatterplots, but adhering to the $x=y$ line in the replicate scatterplots. However, we do see that sample "V.1" may
988 be somewhat inconsistent in these DEGs, as its presence in the replicate scatterplots shows DEGs deviating from
989 the $x=y$ line more than expected and its presence in the treatment scatterplots shows DEGs adhering to the $x=y$
990 line more than expected. This inconsistent sample was something we observed in Figure 3 (PNG).

991 Additional file 8 — Scatterplot matrix of DEGs from Cluster 2 of the Galbraith dataset.

992 The 327 DEGs from the second cluster of the Galbraith dataset (originally shown in Figure 3) superimposed as dark
993 orange dots onto all genes as black dots in the form of a scatterplot matrix. The data has been standardized. "N"
994 represents non-infected control samples and "V" represents virus-treated samples. We confirm that the DEGs
995 mostly follow the expected structure, with their placement deviating from the $x=y$ line in the treatment
996 scatterplots, but adhering to the $x=y$ line in the replicate scatterplots (PNG).

997 Additional file 9 — Scatterplot matrix of DEGs from Cluster 3 of the Galbraith dataset.

998 The 224 DEGs from the third cluster of the Galbraith dataset (originally shown in Figure 3) superimposed as
999 turquoise dots onto all genes as black dots in the form of a scatterplot matrix. The data has been standardized. "N"
1000 represents non-infected control samples and "V" represents virus-treated samples. We confirm that the DEGs
1001 mostly follow the expected structure, with their placement deviating from the $x=y$ line in the treatment
1002 scatterplots, but adhering to the $x=y$ line in the replicate scatterplots (PNG).

1003 Additional file 10 — Scatterplot matrix of DEGs from Cluster 4 of the Galbraith dataset.

1004 The 103 DEGs from the fourth cluster of the Galbraith dataset (originally shown in Figure 3) superimposed as pink
1005 dots onto all genes as black dots in the form of a scatterplot matrix. The data has been standardized. "N"
1006 represents non-infected control samples and "V" represents virus-treated samples. We confirm that the DEGs
1007 mostly follow the expected structure, with their placement deviating from the $x=y$ line in the treatment
1008 scatterplots, but adhering to the $x=y$ line in the replicate scatterplots. We also see that the second replicate from
1009 the virus-treated sample ("V.2") may be somewhat inconsistent in these DEGs, as its presence in the replicate
1010 scatterplots results in the DEGs unexpectedly deviating from the $x=y$ line and its presence in the treatment
1011 scatterplots results in the DEGs unexpectedly adhering to the $x=y$ line (PNG).

1012 Additional file 11 — Scatterplot matrix of virus-related DEGs from our dataset, showing only replicates 1, 2, and 3.

1013 The 43 virus-related DEGs from our dataset superimposed as magenta dots onto all genes in the form of a
1014 scatterplot matrix. Only replicates 1, 2, and 3 are shown from both treatment groups. The data has been
1015 standardized. "N" represents non-infected control samples and "V" represents virus-treated samples. We see that,
1016 compared to the scatterplot matrices from certain clusters of the Galbraith data, the 43 DEGs from this subset of
1017 six samples from our data do not paint as clear of a picture, sometimes unexpectedly deviating from the $x=y$ line in
1018 the replicate plots and sometimes unexpectedly adhering to the $x=y$ line in the treatment plots (PNG).

1019 Additional file 12 — Scatterplot matrix of virus-related DEGs from our dataset, showing only replicates 4, 5, and 6.

1020 The 43 virus-related DEGs from our dataset superimposed as magenta dots onto all genes in the form of a
1021 scatterplot matrix. Only replicates 4, 5, and 6 are shown from both treatment groups. The data has been
1022 standardized. "N" represents non-infected control samples and "V" represents virus-treated samples. We see that,
1023 compared to the scatterplot matrices from certain clusters of the Galbraith data, the 43 DEGs from this subset of
1024 six samples from our data do not paint as clear of a picture, and most of them unexpectedly adhere to the $x=y$ line
1025 in the treatment plots (PNG).

1026 Additional file 13 — Scatterplot matrix of virus-related DEGs from our dataset, showing only replicates 7, 8, and 9.
 1027 The 43 virus-related DEGs from our dataset superimposed as magenta dots onto all genes in the form of a
 1028 scatterplot matrix. Only replicates 7, 8, and 9 are shown from both treatment groups. The data has been
 1029 standardized. "N" represents non-infected control samples and "V" represents virus-treated samples. We see that,
 1030 compared to the scatterplot matrices from certain clusters of the Galbraith data, the 43 DEGs from this subset of
 1031 six samples from our data do not paint as clear of a picture, sometimes unexpectedly deviating from the $x=y$ line in
 1032 the replicate plots and sometimes unexpectedly adhering to the $x=y$ line in the treatment plots (PNG).

1033 Additional file 14 — Scatterplot matrix of virus-related DEGs from our dataset, showing only replicates 10, 11, and
 1034 12.
 1035 The 43 virus-related DEGs from our dataset superimposed onto all genes in the form of a scatterplot matrix. Only
 1036 replicates 10, 11, and 12 are shown from both treatment groups. The data has been standardized. "N" represents
 1037 non-infected control samples and "V" represents virus-treated samples. We see that, compared to the scatterplot
 1038 matrices from certain clusters of the Galbraith data, the 43 DEGs from this subset of six samples from our data do
 1039 not paint as clear of a picture, and most of them unexpectedly deviate from the $x=y$ line in the virus-related
 1040 replicate plots (PNG).

1041 Additional file 15 — Parallel coordinate plots of the "tolerance" candidate DEGs.
 1042 Parallel coordinate plots of the 122 DEGs after hierarchical clustering of size four between the "tolerance" candidate
 1043 DEGs. Here "N" represents non-infected control group, "V" represents treatment of virus, "C" represents
 1044 high-quality chestnut diet, and "R" represents low-quality rockrose diet. The vertical red line indicates the
 1045 distinction between treatment groups. We see there is considerable noise in the data (non-consistent replicate
 1046 values), but that the general patterns of the DEGs follow what we expect based on our "tolerance" contrast (PNG).

1047 Additional file 16 — Parallel coordinate plots of the "resistance" candidate DEGs.
 1048 Parallel coordinate plots of the 125 DEGs after hierarchical clustering of size four between the "resistance"
 1049 candidate DEGs. Here "N" represents non-infected control group, "V" represents treatment of virus, "C" represents
 1050 high-quality chestnut diet, and "R" represents low-quality rockrose diet. The vertical red line indicates the distinction
 1051 between treatment groups. We see there is considerable noise in the data (non-consistent replicate values), but that
 1052 the general patterns of the DEGs follow what we expect based on our "resistance" contrasts (PNG).

1053 Additional file 17 — Venn diagrams comparing the virus-related DEG overlaps in the Galbraith data using our
 1054 pipeline and the pipeline used by Galbraith *et al.*
 1055 Venn diagrams comparing the virus-related DEG overlaps of the Galbraith data from the DESeq2 bioinformatics
 1056 pipelines used in the Galbraith study (labeled as "G.O.") and the DESeq2 bioinformatics pipelines used in our study
 1057 (labeled as "G.R."). While we were not able to fully replicate the DEG list published in the Galbraith study, our DEG
 1058 list maintained significant overlaps with their DEG list. From left to right: Total virus-related DEGs (subplot A),
 1059 virus-upregulated DEGs (subplot B), control-upregulated DEGs (subplot C) (PNG).

1060 Additional file 18 — Venn diagrams of main effect DEG overlaps across DESeq2, edgeR, and limma
 1061 Venn diagrams comparing DEG overlaps across DESeq2, edgeR, and limma for our diet main effect (top row), our
 1062 virus main effect (middle row), and the Galbraith virus main effect (bottom row). Within a given subplot, "D"
 1063 represents DESeq2, "E" represents edgeR, and "L" represents limma. From left to right on top row: Total
 1064 diet-related DEGs (subplot A), chestnut-upregulated DEGs (subplot B), rockrose-upregulated DEGs (subplot C).
 1065 From left to right on middle row: Total virus-related DEGs (subplot D), virus-upregulated DEGs (subplot E),
 1066 control-upregulated DEGs in our data (subplot F). From left to right on bottom row: Total virus-related DEGs
 1067 (subplot G), virus-upregulated DEGs (subplot H), control-upregulated DEGs in the Galbraith data (subplot I)
 1068 (PNG). With the exception of the limma pipeline resulting in zero DEGs in our virus main effect analysis, we found
 1069 significant overlaps between DEG lists across the different pipelines (DESeq2, edgeR, and limma). In general,
 1070 DESeq2 resulted in the largest number of DEGs and limma resulted in the least number of DEGs (PNG).

1071 Additional file 19 — Analysis of correlation between DEG read counts and pathogen response metrics
 1072 Distribution of R-squared values for DEG cluster read counts and pathogen response metrics. Columns left to right:
 1073 SBV titers, mortality rates, and IAPV titers. Rows top to bottom: Tolerance candidate DEGs, resistance candidate
 1074 DEGs, and virus-related DEGs. Each subplot includes five boxplots which represent the R-squared value distributions
 1075 for four DEG clusters and all remaining non-DEGs in the data. The top number above each boxplot represents the
 1076 number of genes included. The first four boxplots also include a bottom number, which represents the
 1077 Kruskal-Wallis p-value of the comparison of the R-squared distribution of the cluster and the R-squared distribution
 1078 of the non-DEG data (PNG).