

Gene expression responses to diet quality and viral infection in *Apis mellifera*

1.1 Introduction

Commerically managed honeybees have undergone unusually large declines in the United States and parts of Europe over the past decade ([van Engelsdorp et al. 2009](#), [Kulhanek et al. 2017](#), [Laurent et al. 2016](#)), with annual mortality rates exceeding what beekeepers consider sustainable ([Caron and Sagili 2011](#), [Bond et al. 2014](#)). More than 70 percent of major global food crops (including fruits, vegetables, and nuts) at least benefit from pollination, and yearly insect pollination services are valued worldwide at \$175 billion ([Gallai et al. 2009](#)). As honeybees are largely considered to be the leading pollinator of numerous crops, their marked loss has considerable implications regarding agricultural sustainability ([Klein et al. 2007](#)).

Honeybee declines have been associated with several factors, including pesticide use, parasites, pathogens, habitat loss, and poor nutrition ([Potts et al. 2010](#), [Spivak et al. 2011](#)). Researchers generally agree that these stressors do not act in isolation; instead, they appear to influence the large-scale loss of honeybees in interactive fashions as the environment changes ([Goulson et al. 2015](#)). Nutrition and viral infection are two broad factors that pose heightened dangers to honeybee health in response to recent environmental changes.

Pollen is the main source of nutrition (including proteins, amino acids, lipids, sterols, starch, vitamins, and minerals) in honeybees ([Roulston and Buchmann 2000](#), [Stanley and Linskens 1974](#)). At the individual level, pollen supplies most of the nutrients necessary for physiological development ([Brodschneider and Crailsheim 2010](#)) and is believed to have considerable impact on longevity ([Haydak 1970](#)). At the colony level, pollen enables

26 young workers to produce jelly, which then nourishes larvae, drones, older workers, and the
27 queen (Crailsheim et al. 1992, Crailsheim 1992). Various environmental changes (including
28 urbanization and monoculture crop production) have significantly altered the nutritional
29 profile available to honeybees. In particular, honeybees are confronted with less diverse
30 selections of pollen, which is of concern because mixed-pollen (polyfloral) diets are generally
31 considered healthier than single-pollen (monofloral) diets (Schmidt 1984, Schmidt et al. 1987,
32 Alaux et al. 2010). Indeed, reported colony mortality rates are higher in developed land
33 areas compared to undeveloped land areas (Naug 2009), and beekeepers rank poor nutrition
34 as one of the main reasons for colony losses (Engelsdorp et al. 2008). Understanding how
35 undiversified diets affect honeybee health will be crucial to resolve problems that may arise
36 as agriculture continues to intensify throughout the world (Neumann and Carreck 2010,
37 Engelsdorp and Meixner 2010).

38 Viral infection was a comparatively minor problem in honeybees until the last century when
39 Varroa destructor (an ectoparasitic mite) spread worldwide (Rosenkranz et al. 2010). This
40 mite feeds on honeybee hemolymph (Weinberg and Madel 1985), transmits cocktails of
41 viruses, and supports replication of certain viruses (Shen et al. 2005, Yang and Cox-Foster
42 2007, Yang and Cox-Foster 2005). More than 20 honeybee viruses have been identified (Chen
43 and Siede 2007). One of these viruses that has been linked to honeybee decline is Israeli
44 Acute Paralysis Virus (IAPV). A positive-sense RNA virus of the Dicistroviridae family
45 (Miranda et al. 2010), IAPV causes infected honeybees to display shivering wings, decreased
46 locomotion, muscle spasms, and paralysis, and 80% of caged infected adult honeybees die
47 prematurely (Maori et al. 2009). IAPV has demonstrated higher infectious capacities
48 than other honeybee viruses in certain conditions (Carrillo-Tripp et al. 2016) and is more
49 prevalent in colonies that do not survive the winter (Chen et al. 2014). Its role in the rising
50 phenomenon of “Colony Collapse Disorder” (in which the majority of worker bees disappear
51 from a hive) remains unclear: It has been implicated in some studies (Cox-Foster et al.
52 2007, Hou et al. 2014) but not in other studies (van Engelsdorp et al. 2009, Cornman et al.
53 2012, Miranda et al. 2010). Nonetheless, it seems likely that IAPV reduces colony strength
54 and survival.

55 Although there is growing interest in how viruses and diet quality affect the health and
56 sustainability of honeybees, as well as a recognition that such factors might operate
57 interactively, there are only a small number of experimental studies thus far directed toward
58 elucidating the interactive effects of these two factors in honeybees (DeGrandi-Hoffman and
59 Chen 2015, DeGrandi-Hoffman et al. 2010, Conte et al. 2011). We recently used laboratory
60 cages and nucleus hive experiments to investigate the health effects of these two factors,
61 and our results show a significant interaction between diet quality and virus infection.
62 Specifically, high quality pollen is able to mitigate virus-induced mortality to the level of
63 diverse, polyfloral pollen (Dolezal et al. 2018).

64 Following up on these phenotypic findings from our previous study, we now aim to understand
65 the corresponding underlying mechanisms by which high quality diets protect bees from
66 virus-induced mortality. For example, it is not known whether the protective effect of good
67 diet is due to direct, specific effects on immune function (resistance), or if it is due to indirect
68 effects of good nutrition on energy availability and vigor (resilience). Transcriptomics is
69 one means to achieve this goal. Transcriptomic analysis can help us identify 1) the genomic
70 scale of transcriptomic response to diet and virus infection, 2) whether these factors interact
71 in an additive or synergistic way on transcriptome function, and 3) the types of pathways
72 affected by diet quality and viral infection. This information, heretofore lacking in the
73 literature, can help us better understand how good nutrition may be able to serve as a
74 "buffer" against other stressors (Dolezal and Toth 2018). As it stands, there are only a small
75 number of published experiments examining gene expression patterns related to diet effects
76 (Alaux et al. 2011) and IAPV infection effects (Galbraith et al. 2015) in honeybees. As far
77 as we know, there are few to no studies investigating honeybee gene expression patterns
78 specifically related to monofloral diets, and few to no studies investigating honeybee gene
79 expression patterns related to the interaction effects of diet in any broad sense and viral
80 inoculation in any broad sense.

81 In this study, we examine how monofloral diets and viral inoculation influence gene
82 expression patterns in honeybees by focusing on four treatment groups (low quality diet
83 without IAPV exposure, high quality diet without IAPV exposure, low quality diet with
84 IAPV exposure, and high quality diet with IAPV exposure). We conduct RNA-sequencing
85 analysis on a randomly selected subset of the honeybees we used in our previous study (as
86 is further described in our methods section). We then examine pairwise combinations of
87 treatment groups, the main effect of monofloral diet, the main effect of IAPV exposure,
88 and the interactive effect of the two factors on gene expression patterns.

89 We also compare the main effect of IAPV exposure in our dataset to that obtained in
90 a previous study conducted by Galbraith and colleagues (Galbraith et al. 2015). As
91 RNA-sequencing data can be highly noisy, this comparison allowed us to characterize
92 how repeatable and robust our RNA-seq results were in comparison to previous studies.
93 Importantly, we use an in-depth data visualization approach to explore and validate our data,
94 and suggest such an approach can be useful for cross-study comparisons of RNA-sequencing
95 data in the future.

96 1.2 Methods

97 Details of the procedures we used to prepare virus inoculum, infect and feed caged honeybees,
98 and quantify IAPV can be reviewed in our previous work (Dolezal et al. 2018). The statistical
99 analysis we used to study the main and interaction effects of the two factors on mortality
100 and IAPV titers is also described in our earlier report (Dolezal et al. 2018).

101 1.2.1 Design of two-factor experiment

102 There are several reasons why we focused only on diet quality (monofloral diets) as opposed
103 to diet diversity (monofloral diets versus polyfloral diets). First, when assessing diet
104 diversity, a sugar diet is often used as a control. However, such an experimental design
105 does not reflect real-world conditions for honeybees as they rarely face a total lack of pollen
106 (Pasquale et al. 2013). Second, in studies that compared honeybee health using monofloral
107 and polyfloral diets at the same time, if the polyfloral diet and one of the high-quality
108 monofloral diets both exhibited similarly beneficial effects, then it was difficult for the
109 authors to assess if the polyfloral diet was better than most of the monofloral diets because
110 of its diversity or because it contained as a subset the high-quality monofloral diet (Pasquale
111 et al. 2013). Third, colonies used for pollination in agricultural areas (monoculture) face
112 less diversified pollens (according to Brodschneider, 2010). Pollinating areas are currently
113 undergoing landscape alteration and agriculture intensification, and bees are increasingly
114 faced with less diversified diets (monoculture) (Decourtye et al. 2010, Brodschneider and
115 Crailsheim 2010). As a result, there is a need to better understand how monofloral diets
116 affect honeybee health as a step toward mitigating the negative impact of human activity
117 on the honeybee population.

118 Consequently, for our nutrition factor, we examined two monofloral pollen diets, *Cistus*
119 (*Rockrose*) and *Castanea* (*Chestnut*). *Cistus* pollen is generally considered less nutritious
120 than *Castanea* pollen due to its lower levels of protein, amino acids, antioxidants, calcium,
121 and iron (Pasquale et al. 2013, Dolezal et al. 2018). For our virus factor, one level contained
122 bees that were infected with IAPV and another level contained bees that were not infected
123 with IAPV. This experimental design resulted in four treatment groups (*Cistus* pollen
124 without IAPV exposure, *Castanea* pollen without IAPV exposure, *Cistus* pollen with IAPV
125 exposure, and *Castanea* pollen with IAPV exposure) that allowed us to assess main effects
126 and interactive effects between diet quality and IAPV infection in honeybees.

127 1.2.2 RNA extraction

128 Fifteen cages per treatment were originally sampled. Six live honeybees from each cage
129 were randomly selected 36 hours post inoculation and placed into tubes. Tubes were kept
130 on dry ice and then transferred into a -80C freezer until processing. Eight cages were
131 randomly selected from the original 15 cages, and 2 honeybees per cage were randomly
132 selected from the original six live honeybees per cage. Whole body RNA from each pool of
133 two honeybees were extracted using Qiagen RNeasy MiniKit followed by Qiagen DNase
134 treatment. Samples were suspended in water to 200-400 ng/ μ l. All samples were then
135 tested on a Bioanalyzer at the DNA core facility to ensure quality (RIN>8).

1.2.3 Gene expression

Samples were sequenced starting on January 14, 2016 at the Iowa State University DNA Facility (Platform: Illumina HiSeq Sequencing; Category: Single End 100 cycle sequencing). A standard Illumina mRNA library was prepared by the DNA facility. Reads were aligned to the BeeBase Version 3.2 genome ([Consortium 2014](#)) from the Hymenoptera Genome Database ([Elsik et al. 2016](#)) using the programs GMAP and GSNAP ([Wu et al. 2016](#)). We tested all six pairwise combinations of treatments for DEGs (pairwise DEGs). We also tested the diet main effect (diet DEGs), virus main effect (virus DEGs), and interaction term for DEGs (interaction DEGs). We then also tested for virus main effect DEGs (virus DEGs) in public data derived from a previous study exploring the gene expression of IAPV virus infection in honeybees ([Galbraith et al. 2015](#)). We tested each DEG analysis using recommended parameters with DESeq2 ([Love et al. 2014](#)), edgeR ([Robinson et al. 2010](#)), and LimmaVoom ([Ritchie et al. 2015](#)). In all cases, we used a false discovery rate (FDR) threshold of 0.05 ([Benjamini and Hochberg 1995](#)). Fisher's exact test was used to determine significant overlaps between DEG sets (whether from the same dataset but across different analysis pipelines or from different datasets across the same analysis pipelines). The `eulerr` shiny application was used to construct Venn diagram overlap images ([Larsson 2018](#)).

@@@ What percent of reads mapped? @@@ Total number of raw reads @@@ How many lanes @@@ How many samples per lane

1.2.4 Comparison to previous studies on transcriptomic response to viral infection

We also compare the main effect of IAPV exposure in our dataset to that obtained in a previous study conducted by Galbraith and colleagues ([Galbraith et al. 2015](#)). While our study examines honeybees from polyandrous colonies, the Galbraith study examined honeybees from single-drone colonies. As a consequence, our honeybees will have an average of about 75% genetic variance, and the honeybees from the Galbraith study will have an average of about 25% genetic variance ([Page and Laidlaw 1988](#)). We should therefore expect that the Galbraith study may generate data with lower signal:to:noise ratios than our data due to the lower genetic variation between its replicates. At the same time, our honeybees will be more likely to display the health benefits gained from increased genotypic variance within colonies, including decreased parasitic load ([Sherman et al. 1988](#)), increased tolerance to environmental changes ([Crozier and Page 1985](#)), and increased colony performance ([Mattila and Seeley 2007](#), [Tarpy 2003](#)). Given that honeybees are naturally very polyandrous ([Brodschneider et al. 2012](#)), our honeybees 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. It is our hope that this aspect of our study may shine light on how experimental designs that control genetic variability to different extents might affect the resulting gene expression data in honeybees.

1.2.5 Visualization

We used @@@ visualization tools from @@@ and visual inference techniques to assess the signal:to:noise ratio in the datasets and to assess the suitability of the DEG calls.

1.2.6 Gene Ontology

DEGs were uploaded as a background list to DAVID Bioinformatics Resources 6.7 (Huang et al. 2009a, Huang et al. 2009b). The overrepresented gene ontology (GO) terms of DEGs were identified using the BEEBASE_ID identifier. To fine-tune the GO term list, only significant terms ($FDR < 0.05$) and those correlating to Biological Processes were considered. The refined GO term list was then imported into REVIGO (Supek et al. 2011), which uses semantic similarity measures to cluster long lists of GO terms.

1.3 Results

1.3.1 Phenotypic results

We reanalyzed our previously published dataset with a subset more relevant to our RNA-sequencing approaches in the current study that have a more focused question regarding diet quality. We briefly show it again here to inform the RNA-seq comparison because we reduced the number of treatments (from eight to four) from the original published data (Dolezal et al. 2018). When statistically analyzing the subset of this data that was used for RNA-sequencing analysis, we found that the mortality rates across diet quality and virus exposure at least numerically retained the same trends (Figure 1.1).

Mortality rates of honeybees 72 hour post-inoculation differed (or did not?) among the treatment groups (mixed model ANOVA across all treatment groups, $df=@@@$, $@@@$; $F=@@@$; $p<@@@$). The effect of virus treatment (mixed model ANOVA, $df=@@@$, $@@@$; $F=@@@$; $p<@@@$), diet treatment (mixed model ANOVA, $df=@@@,@@@$; $F=@@@$; $p<@@@$), and interaction between the two factors (mixed model ANOVA, $df=@@@,@@@$; $F=@@@$, $p=@@@$) did (or did not?) differ. The virus treatment was significant: For a given diet, honeybees exposed to the virus showed significantly higher mortality rate than honeybees not exposed to the virus (Tukey HSD, $p<0.05$). Without virus exposure, there

207 was only an intermediate reduction in mortality rate for bees fed Castanea pollen? (Tukey
208 HSD, $p>0.05$). However, with virus exposure, there was a significant reduction in mortality
209 rate for beeds fed Castanea pollen (Tukey HSD, $p<0.05$). Overall, we discovered that the
210 higher-quality Castanea diet had the ability to significantly reduce mortality in the presence
211 of IAPV infection compared to the lower-quality Cistus diet (Figure 1.1).

212 Comment on (Figure 1.1) @@@

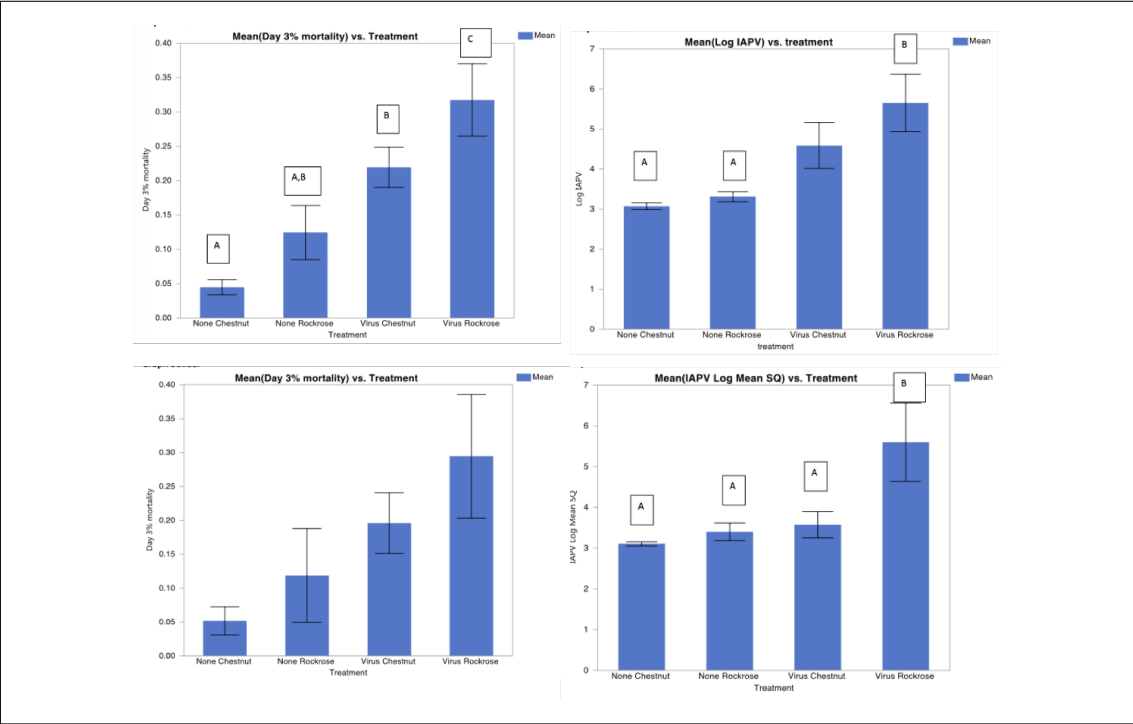


Figure 1.1: Mortality rates for all cages, mortality rates for subset of honeybees used for RNA-seq, IAPV titers for all cages, IAPV titers for subset of honeybees used for RNA-seq subset.

213 **1.4 Discussion**

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