HONEY BEE (*APIS MELLIFERA*) DISEASE ECOLOGY:

IN-HIVE PESTS AND NUTRITION AFFECT HOST-PATHOGEN INTERACTIONS

A Dissertation

by

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ABSTRACT

Honey bee-infecting pathogens are a leading cause of worldwide bee decline. However, it is oftentimes only in conjunction with other stressors that pathogens reach titers that begin to negatively impact the health of bees at both the individual and colony level. By studying the disease ecology of honey bees and their associated pathogens, we can better understand how these various stressors can impact transmission routes, host dynamics, and ecological drivers that contribute to pathogen spread. Two understudied biotic factors in relation to honey bee disease ecology include interspecies pathogen transmission and the effects of host nutrition on host-pathogen interactions. This dissertation adds to our current knowledge on these two subject areas by 1) identifying if in-hive ant pests act as reservoirs of honey bee-associated viruses, and 2) determining how macronutrient constituents in the honey bee diet affect the interactions between the bee host and its most ubiquitous fungal and viral pathogen. The overarching goal was to determine how these concurrent factors impact bee-pathogen interactions to develop solutions that can help beekeepers better mitigate colony losses.

The first chapter consists of a survey in which I determined the ant taxa (family Formicidae) that act as pests within managed apiaries in Texas and how these pests interact with honey bee colonies. I then examined whether any of six common honey bee-associated viruses were present and actively replicating in ants collected from within or outside of apiaries. In total, 14 genera of ants were found to be interacting with managed honey bee colonies in Texas, with the most common form of interaction being

the robbing of sugar resources from hives. I found that 12 of these 14 ant genera were reservoirs of honey bee-associated viruses. However, there was no evidence that active replication of these viruses was occurring within the ants. These results show for the first time that ants may be more than just nuisance pests of honey bees in United States, as they have the potential to aid in the transmission of honey bee-associated viruses, highlighting the need to better control for ants in managed honey bee colonies.

For the second and third chapters, I performed studies that involved a common honey bee-infecting fungal pathogen (*Nosema ceranae*) and viral pathogen (deformed wing virus, DWV) to determine how variation in the macronutrient content within the diet of honey bees impacts host-pathogen interactions. For both sets of experiments, I determined that bees infected with either one of these pathogens showed the highest consumption of a diet that was balanced in its macronutrient ratio of proteins (P) to lipids (L). This diet had a ratio of 30 parts protein and 20 parts lipid (30P:20L). However, the survivorship of bees differed across experiments, and it was significantly impacted by the type of infection and diet consumed. I observed significantly higher survivorship in *Nosema*-infected bees when they were fed a more protein-rich diet (40P:10L), while survivorship was higher in DWV-infected bees when they were fed a diet that was more balanced in its P:L ratio (30P:20L). These results, in conjunction with other analyses that measured bee physiology and gene expression, revealed that the protein and lipid content in the diet of honey bees influences host-pathogen systems, and bee hosts respond differently to infection based on pathogen type and the quality of diet consumed. This information can be used to create an optimized supplemental diet in

which we can manipulate the macronutrient content to address specific pathogen infections at different times of the year within honey bee colonies.

DEDICATION

I would like to dedicate my dissertation to my family for the incredible support they have always given me. They have always been my personal cheer squad, and I cannot thank them enough for it. I especially want to dedicate this accomplishment to my parents and grandparents, as they have always supported my career goals and were the ones who made me the person I am today.

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Advice and teaching of molecular techniques utilized in the research conducted as a part of Chapter 2 of this dissertation was provided by Dr. Tonya Shepherd. All other work for this chapter was independently conducted by the student and published in 2019. The work conducted in Chapters 3 and 4 was in part possible thanks to the assistance in cage assembly and data collection from Dr. Pierre Lau, Cora Garcia, and Jordan Gomez. The recipe for the artificial diets used for these chapters was created by Dr(s). Pierre Lau and Pierre Lesne for a previous study conducted in our laboratory. The use of honey bee injection assays utilized in Chapter 4 was done thanks to the teachings of Dr. Humberto Boncristiani within the laboratory of Dr. Jamie Ellis at the University of Florida.

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NOMENCLATURE

ABV	Acute bee paralysis virus
BQCV	Black queen cell virus
DNA	Deoxyribonucleic acid
DWV	Deformed wing virus
GFN	Geometric framework for nutrition
KBV	Kashmir bee virus
IAPV	Israeli acute paralysis virus
NI	non-infected
Nosema	*Nosema ceranae*
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
SBV	Sacbrood virus

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1. INTRODUCTION

1.1. The importance of honey bees and their decline

Honey bees (*Apis mellifera*) are critical pollinators of several agriculturally important crops worldwide (Gallai et al., 2009). In the U.S. alone, honey bees contribute an estimated $15 billion annually to the economy, primarily through the provision of pollination services (Calderone, 2012). Due in part to their large population sizes and the general ease of transporting hives, managed honey bees are preferred over other insect species for commercial crop pollination (Delaplane et al., 2000; Klein et al., 2007). However, beekeepers continuously struggle to keep their annual colony losses at a sustainable level (Kulhanek et al., 2017, Steinhauer et al., 2018). The most recent nationwide colony loss survey conducted in the U.S. reported that, on average, beekeepers lost over 40% of their colonies overwinter, the highest rate ever documented since the survey began over a decade ago (Bruckner et al., 2020). Extensive research has identified the primary stressors behind these unsustainable losses to include the "four Ps"- pesticides, parasites/pests, poor nutrition, and pathogens (Neumann & Carreck, 2010; Potts et al., 2010; Goulson et al., 2015; Hristov et al., 2020). Further research that leads to a better understanding of how these stressors individually and synergistically impact honey bee health is necessary in order to ensure the future of apiculture and maintain global food security.

1.2. Synergistic stress factors negatively impact honey bee health

The stressors that drive honey bee colony decline do not act in isolation. In fact, research has shown that they oftentimes interact with one another to cause large-scale colony loss (Smith et al., 2013; Goulson et al. 2015). These synergistic interactions between stressors is what has led to pathogen-associated colony losses within the past few decades (McMenamin et al., 2016). For example, approximately thirty years ago honey bee-infecting viruses were of little concern to beekeepers, primarily because they remained at low titers and caused covert, asymptomatic infections within individuals. It was not until the successful host switch of the ectoparasitic mite *Varroa destructor* from the Eastern honey bee (*Apis cerana*) to the Western honey bee (*A. mellifera*) that honey bee-infecting viruses became one of the major contributors of worldwide colony collapse (Gisder et al., 2009; Rosenkranz et al., 2010; Möckel et al., 2011). *Varroa* mites feed on immature and adult stages of honey bees, while also vectoring viruses between individuals within a colony and between colonies within managed apiaries (Rosenkranz et al., 2010; DeGrandi-Hoffman et al., 2017). Some of these viruses include deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), acute bee paralysis virus (ABPV), black queen cell virus (BQCV), Kashmir bee virus (KBV), and sacbrood virus (SBV) (Shen et al., 2005; Genersch & Aubert, 2010; Di Prisco et al., 2011; Posada-Florez et al., 2019). Many of these viruses accumulate within the mite and can reach extremely high titers prior to transmission. Incidentally, there is evidence that more virulent strains of some of these viruses have emerged over time and have been positively selected for due to *Varroa* parasitism (Gisder et al., 2009; Evans & Schwarz,

2011). Previous research has also shown a mutualistic symbiosis between *Varroa* and some of the viruses it vectors, particularly deformed wing virus (DWV). *Varroa* parasitism and DWV infection synergistically cause immunosuppression within the honey bee host, leading to enhanced reproduction of the mite and increased replication of the virus (Yang & Cox-Foster, 2005; Di Prisco et al., 2016; Zhao et al., 2019). The improved transmission of viral pathogens by *Varroa*, particularly DWV, highlights the critical need to better understand how synergetic stress factors interact within this host-pathogen system and can cause honey bee colony collapse.

Before the introduction of *Varroa*, honey bee-infecting viruses were primarily transmitted between individuals within a colony as covert, non-lethal infections. Viral transmission occurs naturally largely due to the eusocial lifestyle of honey bees. Factors that contribute to pathogen spread include a large number of genetically related individuals living in crowded conditions, warm hive temperatures, and periods of host confinement during the winter season (DeGrandi-Hoffman & Chen, 2015). In addition, pathogen transmission is aided by the multi-host nature of many honey bee-infecting pathogens that can infect and/or remain viable within other insect species, such as native bees, which can serve as alternative hosts or reservoirs of these viruses that can be reintroduced into naïve honey bee colonies through the sharing of floral resources between infected and non-infected individuals (Genersch et al., 2006; Meeus et al., 2010; Singh et al., 2010; Jilian et al., 2011; Peng et al., 2011; Zhang et al., 2012; Fürst et al., 2014). All these interactions have all been shown to be viable routes in which honey bee-infecting pathogens can be introduced into a colony (McMenamin et al., 2016).

1.3. Methods for dealing with pathogen infection of honey bee colonies

Although there are treatments available to address many of the fungal and bacterial pathogens that infect honey bees, there is not yet a commercially available option that can be used to directly treat or prevent viral infection (Brutscher et al., 2016). For this reason, beekeepers are only able to deal with viral infections within their colonies by reducing any concurrent and synergistic stress factors that can contribute to symptomatic viral infection and colony collapse. This strategy includes the prevention and control of parasites and pests that are capable of transmitting viruses to honey bee colonies. Since *Varroa* mites gained the ability to parasitize *A. mellifera* colonies, control efforts have predominantly focused on curtailing infestations through the use of pesticides. The types of pesticides used to treat for *Varroa* have changed over time, partly because the mites have developed resistance to several key chemical compounds (Spreafico et al., 2001; Pettis 2004; Rosenkranz et al., 2010) and also because we have gathered a better understanding of which miticides can negatively impact honey bee health (Mullin et al., 2010; Fisher II & Rangel, 2018; Walsh et al., 2020). As the mites continue to develop resistance to newer products (Rinkevich et al., 2020), there is a critical need to develop better integrated pest and pollinator management (IPPM) measures to address *Varroa* parasitism and the viruses they vector. One innovative IPPM approach is to ensure that honey bees have access to proper nutrition, as it has been shown that a good quality diet can break the cycle of immunosuppression between *Varroa*, DWV, and honey bees (DeGrandi-Hoffman & Chen, 2015; Dolezal & Toth, 2018).

Studying how concurrent factors impact host-pathogen interactions is imperative to better understanding the disease ecology of honey bees, especially because honey bees are constantly faced with multiple, concomitant stressors in natural settings. This includes better defining the modes of transmission through which honey bees can become infected with pathogens, as well as understanding which factors impact host physiology and thus, host-pathogen interactions. By better understanding the synergistic effects between the drivers of honey bee decline and what measures can be taken to address them, we can help develop management strategies that help beekeepers reduce yearly colony loss.

2. THE DETECTION OF HONEY BEE (*APIS MELLIFERA*)- ASSOCIATED VIRUSES IN ANTS

Payne, A. N., Shepherd, T. F. & Rangel, J. The detection of honey bee (*Apis mellifera*)-associated viruses in ants. *Sci. Rep.* 10(1), 1-8 (2020).

2.1. Overview

Interspecies virus transmission involving economically important pollinators, including honey bees (*Apis mellifera*), has recently sparked research interests regarding pollinator health. Given that ants (family Formicidae) are common pests within apiaries in the southern U.S., the goals of this study were to 1) survey ants found within or near managed honey bee colonies, 2) document what interactions are occurring between ant pests and managed honey bees, and 3) determine if any of six commonly occurring honey bee-associated viruses were present in ants collected from within or far from apiaries. Ants belonging to 14 genera were observed interacting with managed honey bee colonies in multiple ways, most commonly by robbing sugar resources from within hives. We detected at least one virus in 89% of the ant samples collected from apiary sites (n = 57) and in 15% of ant samples collected at non-apiary sites (n = 20). We found that none of these ant samples tested positive for the replication of Deformed wing virus, Black queen cell virus, or Israeli acute paralysis virus, however. Future studies looking at possible virus transmission between ants and bees could determine whether ants can be considered mechanical vectors of honey bee-associated viruses, making them a potential threat to pollinator health.

2.2. Introduction

More than 20 different viruses have been identified as able to infect honey bees with the majority belonging to the order Picornavirales (Chen and Siede 2007; McMenamin and Genersch 2015). Some of the most commonly detected viruses in honey bees within the

United States include Deformed wing virus (DWV), Black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), and Sacbrood virus (SBV) (DeGrandi-Hoffman and Chen 2015; McMenamin & Flenniken, 2018). Both DWV and SBV belong to the genus *Iflavirus* within the family Iflaviridae (de Miranda and Genersch 2010). The remaining three viruses belong to the family Dicistroviridae, with KBV and IAPV belonging to the genus *Aparavirus* and BQCV belonging to the genus *Cripavirus* (Bonning and Miller 2010; Chen et al. 2014).

However, despite these being commonly referred to as "honey bee" viruses, previous research has detected these viruses in a number of other arthropods including the ectoparasitic mite Varroa destructor (Di Prisco et al., 2011; Gisder et al., 2009), other insect pollinators such as hoverflies, bumblebees, and solitary bees (Genersch et al., 2006; Meeus et al., 2010; Singh et al., 2010; Jilian et al., 2011; Peng et al., 2011; Zhang et al., 2012; Fürst et al., 2014; Ravoet et al., 2014; McMahon et al., 2015; Parmentier et al., 2016; Tehel et al., 2016; Gisder & Genersch, 2017; Bailes et al., 2018; de Souza et al., 2019; Murray et al., 2019), and other Hymenopteran insects including some wasps and ants (Celle et al., 2008; Singh et al., 2010; Yañez et al., 2012; Levitt et al., 2013; Sébastien et al., 2015; Mordecai et al., 2016; Bigot et al., 2017; Gruber et al., 2017; Loope et al., 2019; Schläppi et al., 2019). Both direct and indirect interactions between honey bees and some of these arthropods (e.g., foraging at the same floral resource, parasitism, and predation) have been proposed as possible routes in which interspecies transmission of honey bee-associated viruses can occur (Durrer & Schmid-Hempel, 1994; Fries & Camazine, 2001; Graystock et al., 2015; Grozinger & Flenniken, 2019).

Many of these arthropods found to carry honey bee-associated viruses commonly interact with honey bees in some manner. For many of the native pollinators, this interaction most likely occurs through the overlapping use of floral resources as a source of nectar and pollen. Other forms of interaction can be in the form of pest species that interact with honey bees within their own hives.

In the southern United States, ants are ubiquitous within apiaries and are common pests of managed honey bees (Nagaraja & Rajagopal, 2000). However, despite their abundance, few studies have focused on identifying the ants that are common pests of honey bee colonies, or how ant pests interact with managed honey bees, especially in regards to interspecies virus transmission and the impact it might have on honey bee health. The first study to detect the replication of a honey bee-associated virus in an ant was conducted in France, where they found both the viral and replicative genome of Chronic bee paralysis virus (CBPV) in the carpenter ant, *Camponotus vagus* (Celle et al., 2008). A later study conducted in North America that screened for honey bee-associated viruses in arthropods found near apiaries (including the carpenter ant, *Camponotus* sp., and the pavement ant, *Tetramorium caespitum*) detected the presence of DWV, BQCV, IAPV, and SBV in *Camponotus* individuals. However, they did not detect any virus replication in the ants sampled (Levitt et al., 2013). Two studies conducted in New Zealand detected the presence of DWV, BQCV, and KBV, as well as the replicative form of DWV and KBV, in the Argentine ant, *Linepithema humile* (Sébastien et al., 2015; Gruber et al., 2017). In another study, Lake Sinai virus (LSV) and phylogenetically related viruses were detected in three species of harvester ants

including *Messor concolor*, *M. barbarus*, and *M. capitatus* (Bigot et al., 2017). Moreover, it was recently found that *Myrmica rubra* ants collected in Berlin, Germany can be infected with types A and B of DWV when fed infected honey bee pupae in caged environments (Schläppi et al., 2019).

In most of the above studies, ants were tested for the detection of honey bee-associated viruses without reporting how the ants naturally interacted with managed honey bees. To better understand which ants are pests within apiaries and how these pests interact with and potentially impact managed honey bees, we 1) surveyed and identified ants collected in or near apiaries in southern and central Texas, 2) documented the type of interactions observed between ants and managed honey bees, and 3) screened for the presence of DWV, BQCV, IAPV, ABPV, KBV, and SBV and the replication of DWV, BQCV, and IAPV in ants collected from apiary and non-apiary sites. Our study revealed a number of ant taxa that act as common pests of honey bees within apiaries and explored whether or not these ants may act as hosts of six honey bee-associated viruses, which could have important implications regarding honey bee health.

2.3. Methods

2.3.1. Sample collection

We began our study with a survey to identify the ants that act as pests of managed honey bee colonies. We collected a total of 57 ant samples from January 2017 to September 2018 from 21 apiaries across Texas where beekeepers had reported having issues with ants living within or around their hives. A sample consisted of individuals that belonged

to the same taxon and were collected at the same site, on the same day, and from the same honey bee hive or nearby ant colony. The number of individuals per sample ranged from one ant (e.g., species with solitary foraging habits), to a few hundred individuals (e.g., ant nests with high population densities). If a sample contained individuals at different life stages (i.e., immatures vs. adults), it was further divided into two distinct samples. Samples were collected with forceps and an aspirator either from within/on honey bee hives or from locations within 20 meters of a managed honey bee colony.

To better understand the extent to which honey bee-associated viruses are present in ants, 20 ant samples were collected from nine sites located at least 3.2 km away from any managed honey bee colonies (non-apiary sites). All samples were stored in 15 mL centrifuge tubes on dry ice upon collection in the field to maintain RNA integrity before being stored at -80°C in the laboratory. Ants were identified using printed keys and specimens from Texas A&M University's insect collection (Vinson et al., 2003; Cook et al., 2016).

2.3.2. RNA extraction

Each sample, consisting of whole-bodied ants, was homogenized in an Eppendorf tube using a pestle. Up to 20 mg of the homogenate was then used for total RNA extraction (Aurum Total RNA Mini Kit, Bio-Rad Laboratories, Hercules, CA), which included a DNase digestion step. The extracted RNA was eluted into a 40 µL solution and tested for its concentration and purity on a NanoPhotometer NP80 (Implen, Munich, Germany) before being stored at -80°C.

2.3.3. Diagnostic analysis for honey bee-associated viruses

The extracted total RNA underwent diagnostic analyses for common honey bee-associated viruses including DWV, BQCV, IAPV, ABPV, KBV, and SBV. To accomplish this, 250 ng of total RNA was first reverse transcribed with random primers (250 nM final concentration) in a 20 µL reaction (iScriptSelect cDNA Synthesis Kit, Bio Rad Laboratories, Hercules, CA). PCR amplification was performed with *Taq* DNA Polymerase (New England Biolabs, Ipswitch, MA). Virus-specific primers commonly used to screen honey bees for these viruses (Table 2.1), as well as cloned PCR products corresponding to each primer set that were used as positive controls, were acquired from the USDA-ARS Bee Research Laboratory in Beltsville, MD. The acquired PCR products that were used as positive controls can be visualized in Appendix A (Figure 1). PCR cycling conditions included an initial denaturation step at 94˚C for 2 min, followed by 30 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 1 min, without a final extension step. The resulting PCR products were visualized on a 3% agarose gel using gel electrophoresis, stained with ethidium bromide, and photographed under UV light. Samples that tested positive for each virus were confirmed via Sanger sequencing.

2.3.4. Detection of DWV, BQCV, and IAPV replication in ants

The three viruses that were initially screened for and detected in ant samples (DWV, BQCV, and IAPV) were further tested for replication within ants using tagged primers in a modified two-step RT-PCR (Yue & Genersch, 2005; Boncristiani et al., 2009). The use

of strand-specific RT-PCR (ssRT-PCR) to detect the replicated intermediate of viruses is described by de Miranda et al., 2013 (de Miranda et al., 2013). Briefly, 250 ng of total RNA was reverse transcribed with gene-specific primers (250 nM final concentration) in a 20 µL reaction (iScriptSelect cDNA Synthesis Kit, Bio Rad Laboratories, Hercules, CA). To target the negative-sense strand of the virus, only a forward primer complementary to the negative strand specific for either DWV (Genersch, 2005), BQCV (Peng et al., 2011), or IAPV (Di Prisco et al, 2011) was used in the reaction. Each forward primer contained a tag attached to its 5' end (Yue & Genersch, 2005) to increase the specificity of the primers and thus decrease the possibility of detecting false positives (de Miranda et al., 2013). The PCR reactions were subsequently carried out with a primer pair at a final primer concentration of 10 µM each. These two primers consisted solely of the tag sequence and a virus-specific reverse primer using the following PCR cycle conditions: an initial denaturation step at 94˚C for 2 min, followed by 35 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 1 min, with a final extension step of 72ºC for 10 min. PCR products were then visualized using gel electrophoresis on a 3% agarose gel. Primers used for the detection of viral replication are listed in Table 2.1. This method of ssRT-PCR can result in the attainment of false positives due to false-, self-, and mis-priming events that most likely occurs when residual primers from cDNA synthesis are carried over into the PCR reaction. To avoid these potential problems, samples that tested positive for replication underwent a second analysis to confirm the absence of false positives. Reverse transcription was carried out as previously described with additional controls including a template-free control, a RT-free control, and a

primer-free control for each tested sample. The resulting tagged cDNA was then treated with an Exonuclease-I (New England Biolabs, Ipswitch, MA) digestion step, which removes excess primers and has been shown to greatly reduce non-specific priming, and then diluted 10-fold prior to performing PCR (Craggs et al., 2001; de Miranda et al., 2013). PCR products were then visualized through gel electrophoresis using a 3% agarose gel.

Primer #	Primer name	Sequence (5'-3')	Amplicon size (bp)	Reference/Source
1	DWV.F.	GAGATTGAAGCGCATGAACA	130	USDA- ARS Honey Bee Laboratory, Beltsville, MD
2	DWV.R.	TGAATTCAGTGTCGCCCATA		
3	BQCV.F	TTTAGAGCGAATTCGGAAACA	140	
4	BQCV.R.	GGCGTACCGATAAAGATGGA		
5	IAPV.F.	GCGGAGAATATAAGGCTCAG	587	
6	IAPV.R.	CTTGCAAGATAAGAAAGGGGG		
7	ABPV.F.	ACCGACAAAGGGTATGATGC	124	
8	ABPV.R.	CTTGAGTTTGCGGTGTTCCT		
9	KBV.F.	TGAACGTCGACCTATTGAAAAA	127	
10	KBV.R.	TCGATTTTCCATCAAATGAGC		
11	SBV.F.	GGGTCGAGTGGTACTGGAAA	105	
12	SBV.R.	ACACAACACTCGTGGGTGAC		
13	tag only	agcctgcgcaccgtgg	not applicable	Yue et al., 2005
14	tag-DWV F15	agcctgcgcaccgtggTCCATCAGGTTCTCCAATAACGG	451	Yue et al., 2005
15	DWV B23	CCACCCAAATGCTAACTCTAACGC		Genersch, 2005
16	tag-BQCVsense	agcctgcgcaccgtggTCAGGTCGGAATAATCTCGA	419	Peng et al., 2011
17	BQCV-antisense	GCAACAAGAAGAAACGTAAACCAC		
18	tag-IAPVsense	agcctgcgcaccgtggGCGGAGAATATAAGGCTCAG	587	Di Prisco et al., 2011
19	IAPV-antisense	CTTGCAAGATAAGAAAGGGGG		

Table 2.1: List of primers used for RT-PCR and ssRT-PCR. Primer sets 1–12 were used in the diagnostic RT-PCR reactions for Deformed wing virus (DWV; primers 1 and 2), Black queen cell virus (BQCV; primers 3 and 4), Israeli acute paralysis virus (IAPV; primers 5 and 6), Acute bee paralysis virus (ABPV; primers 7 and 8), Kashmir bee virus (KBV; primers 9 and 10), and Sacbrood virus (SBV; primers 11 and 12). Primers 13–19 were used for the detection of the negative sense strand indicative of viral replication using strand-specific RT-PCR. Reverse transcription targeting the negative-sense strand

was conducted with primer numbers 14 for DWV, 16 for BQCV, and 18 for IAPV. The tag only primer (13) was the forward primer of all three viruses for PCR reactions, and primers 15, 17, and 19 were the reverse primers for DWV, BQCV, and IAPV respectively.

2.4. Results

2.4.1. Sample collection

We collected a total of 77 ant samples between 2017 and 2018 throughout Texas. A sample consisted of a group of individuals that belonged to the same taxon and were in the same life stage (i.e., all immatures or all adults) and were collected at the same site, on the same day, and from the same hive or nearby ant colony. Of the 57 samples collected at apiary sites, 26 were collected directly from within or on honey bee hives. The remaining 31 samples were collected within 20 meters of honey bee colonies, including structures or areas where beekeepers stored equipment such as unused hive bodies or frames. We identified a total of 14 ant genera, with the most common taxa being *Solenopsis invicta* (fire ants) and *Crematogaster* sp. (acrobat ants). In addition to ants collected within apiaries, 20 ant samples belonging to six different genera were collected from non-apiary sites to compare the presence of viruses between the two types of locations. A summary of the ant taxa collected from apiary and non-apiary sites is listed in Table 2.2 below.

Site	Ant taxa collected	Number of samples	Number (%) of samples that tested positive for a virus					
			DWV	BQCV	IAPV	ABPV	KBV	SBV
Apiary sites (n = 57)	*Aphaenogaster texana* (spine-waisted ant)	1	0	0	0	1 (100%)	0	0
	Brachymyrmex sp. (rover ant)	2	2 (100%)	1 (50%)	0	0	0	1 (50%)
	Camponotus sp. (carpenter ant)	4	3 (75%)	1 (25%)	1 (25%)	1 (25%)	0	0
	Crematogaster sp. (acrobat ant)	17	11 (64.7%)	9 (52.9%)	4 (23.5%)	11 (64.7%)	1 (5.9%)	3 (17.6%)
	Forelius sp. (cheese ant)	1	1 (100%)	0	0	0	0	0
	Formica sp. (field ant)	1	0	0	0	0	0	0
	Linepithema humile (Argentine ant)	2	1 (50%)	0	0	1 (50%)	1 (50%)	0
	Nylanderia sp. (crazy ant)	1	0	1 (100%)	1 (100%)	1 (100%)	0	0
	Pheidole sp. (big headed ant)	4	3 (75%)	2 (50%)	1 (25%)	0	0	0
	Pogonomyrmex sp. (harvester ant)	1	0	0	0	1 (100%)	0	0
	Pseudomyrmex gracilis (elongate twig ant)	4	3 (75%)	0	0	0	0	0
	Solenopsis invicta (fire ant)	18	14 (77.8%)	8 (44.4%)	5 (27.8%)	7 (38.9%)	4 (22.2%)	8 (44.4%)
	Tapinoma sp. (odorous house ant)	1	0	0	0	0	0	0
	Total number of samples	57	38 (67%)	22 (39%)	12 (21%)	22 (39%)	6 (11%)	12 (21%)
Non-apiary sites (n = 20)	*Brachymyrmex sp.* (rover ant)	1	0	0	0	0	0	0
	Crematogaster sp. (acrobat ant)	3	0	0	0	0	0	0
	Nylanderia sp. (crazy ant)	1	0	0	0	0	0	0
	Pheidole sp. (big headed ant)	3	0	0	0	0	0	0
	Pseudomyrmex gracilis (elongate twig ant)	1	0	0	0	0	0	0
	Solenopsis invicta (fire ant)	11	3 (27.3%)	0	0	0	2 (18.2%)	0
	Total number of samples	20	3 (15%)	0	0	0	2 (10%)	0

Table 2.2: Summary of ant samples collected from apiary and non-apiary locations throughout central Texas. The table includes information on the ant taxa collected, the

different interactions observed between ants and managed honey bees within/near hives (denoted as superscript letters alongside ant common names), and the prevalence of six honey bee-associated viruses in the sampled ants after performing diagnostic analysis using RT-PCR. A number of interactions were observed between ants and honey bees within managed apiaries including: (a) cohabitation of honey bees and ants (including brood and reproductives) within the same honey bee hive; (b) robbing of sugar resources (e.g., nectar, honey, and/or beekeeper-supplied sugar syrup) by ants from within the hive; (c) robbing of pollen; (d) foraging for honey/sugar from beekeeping equipment and/or supplies; (e) causing a honey bee colony to abscond due to an overwhelming level of robbing behavior by ants; (f) scavenging of dead adult bees; and (g) preying on honey bee brood or removing brood from the colony. Ants collected from apiary sites but without a letter indicating an interaction type were collected on or near a honey bee hive but were not observed interacting with the bee colony in any way. Viruses that were screened from collected ant samples included Deformed wing virus (DWV), Black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), Acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), and Sacbrood virus (SBV). Of the 57 ant samples that were collected from within apiaries and provided viable RNA, 51 (89%) tested positive for at least one virus of interest. For ants collected at non-apiary sites, only 3 of the 20 samples (15%) tested positive for at least one virus of interest. In many instances, a single ant sample tested positive for multiple viruses. The table does not include virus information for *Monomorium minimum*, as none of those samples provided viable RNA. Samples that tested positive for DWV, BQCV, and IAPV after the diagnostic RT-PCR

were then analyzed for the replication of these viruses by strand-specific RT-PCR. Data on the replication of viruses were not included, as they were negative for all tested samples.

2.4.2. Interactions observed between honey bees and ants

Ants at apiary sites were observed interacting with managed honey bees in multiple ways including robbing sugar or pollen resources from within the hive, scavenging dead honey bee adults, preying on honey bee brood, and cohabiting with bees within the hive (Figure 2.1). The most common interaction observed between ants and honey bees was the robbing of sugar resources including nectar, honey, and/or beekeeper-supplied sugar syrup (Figure 2.1a). Ants in the genera *Brachymyrmex*, *Forelius*, *Linepithema*, *Monomorium*, *Nylanderia* and *Solenopsis* were observed either foraging from beekeeper-supplied feeders or within wax cells where honey bees stored nectar. In some instances, entire bee colonies abandoned their hives (i.e., absconded) due to high rates of robbing, done mostly by ants with large populations such as *Nylanderia fulva* and *Linepithema humile*. Ants in the genera *Formica*, *Monomorium*, *Pheidole*, and *Solenopsis* were also observed robbing and transporting pollen out of hives.

Camponotus and *Solenopsis* ants were observed scavenging dead adult bees from within or near hives (Figure 2.1b), while *S. invicta* was the only species observed preying on bee brood (Figure 2.1c). This behavior was common for colonies that were weak or those that had absconded or collapsed and contained abandoned brood. Small colonies of *Brachymyrmex* ants were found living on top of hives, typically underneath

bricks that were placed to keep the hive lids from being blown off by the wind. Only ants in the genus *Crematogaster* were found living within honey bee hives (Figure 1d). This included whole ant colonies containing eggs, immatures, workers, and reproductives that lived either between the outer and inner covers of a hive or within tunnels that they had created through the wood of hive boxes.

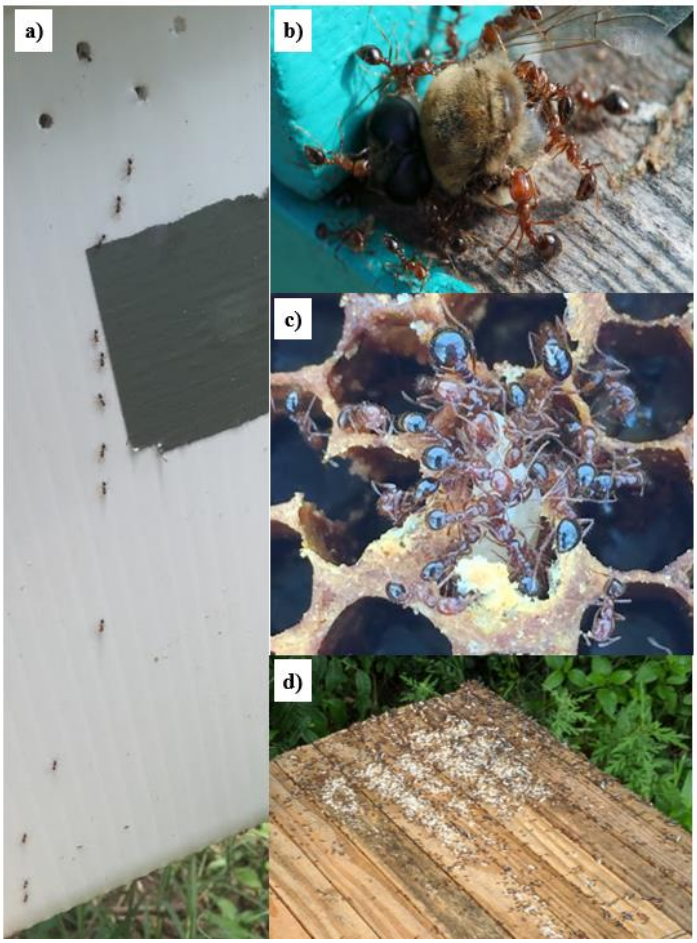

Figure 2.1: Interactions observed between ants and honey bees. Depiction of some of the different interaction types observed between honey bees and ants within apiaries including a) predation: *Solenopsis invicta* workers removing a bee larva from within a cell in a collapsed hive; b) scavenging: *S. invicta* transporting the head and thorax of a

dead adult drone out of a hive's entrance (photo credit: Pierre Lau); c) co-habitation: a *Crematogaster* sp. colony living within a honey bee top-bar hive (photo credit: Pierre Lau); and d) robbing of hive resources: *Crematogaster* sp. foraging trail leading out of a honey bee nucleus colony.

2.4.3. Diagnostic analysis of honey bee-associated viruses in ants

Of the 14 ant genera associated with honey bees that were collected within apiaries, 13 were screened for the presence of viruses. *Monomorium minimum* ants were excluded from the analysis due to their small body mass and the inadequate number of individuals collected per sample, which resulted in low RNA yield. Of the 57 ant samples collected from within apiaries, 51 (89%) tested positive for at least one virus. In many cases, we detected multiple viruses in a single ant sample. The most prevalent virus in ants collected from apiaries was DWV, with 38 of the 57 samples (66.7%) testing positive (Table 2.2). The least prevalent virus was KBV, with only six of the 57 samples (10.5%) testing positive. At non-apiary sites, only DWV and KBV were detected in *S. invicta* ants, with three of the 20 samples (15%) testing positive for DWV and two (10%) testing positive for KBV. A subset of samples that tested positive for each of our tested viruses are visualized in Appendix A (Figure 2).

2.4.4. No replication of DWV, BQCV, or IAPV detected in ants

When conducting the initial strand-specific RT-PCR to test for replication of DWV, BQCV, and IAPV, one sample had tested positive for replicating DWV, and four

samples tested positive for replicating IAPV. All samples that had tested positive for viral replication belonged to the genus *Crematogaster*, including one that consisted entirely of immature ants. However, when we tested these five samples again and performed a digest step using Exonuclease I and a 10-fold dilution following reverse transcription, we found that all of the previously positive samples tested negative for replication and were considered to originally have yielded false positive results, indicating a lack of viral replication in any of the ant samples collected from either apiary or non-apiary locations.

2.5. Discussion

Of the 500+ described ant species present in the United States, nearly 300 occur in the warm, subtropical climate of Texas, with around 20 species considered non-native pests (O'Keefe, 2000). Personal communications with commercial and backyard beekeepers (e.g., members of the Texas Beekeepers Association) made us aware of how common ants are within managed apiaries. Depending on the genus, ants have been observed robbing sugar resources and pollen from within hives, preying on bee brood, scavenging dead adult bees, or cohabiting with honey bees within their hives.

The ants most frequently found within apiaries were *S. invicta* and *Crematogaster* sp., both of which are common in Texas. Fire ants were often observed preying on bee brood and deceased adults, especially in hives that had collapsed or were weak and close to collapsing. For instance, it is common practice by beekeepers in Texas to place a frame of honeycomb infested with secondary pests (e.g., the greater and lesser

wax moths, *Galleria mellonella* and *Achroia grisella*) or diseased brood onto a *S. invicta* mound for the ants to "clean out" the comb for future use (Gene Ash, pers. comm.). In the case of acrobat ants, entire colonies, including immatures and reproductives, were found inhabiting hives between the inner and outer covers. In two different hives we observed that a colony of *Crematogaster* sp. had created tunnels and was dwelling within the wood of a hive box. This genus is typically arboreal but has been known to live within dead wood on the ground (Richard et al., 2001).

Other less commonly encountered ants within apiaries were two invasive species from South America that can reach large population densities in the United States: *Nylanderia fulva* (tawny crazy ants) and *Linepithema humile* (Argentine ants). In two of the 21 apiary sites we sampled from, honey bee colonies absconded due to overwhelming nectar robbing by invading tawny crazy ants or Argentine ants. Previous reports have documented the eventual absconding or collapse of honey bee colonies when they are invaded and overrun by these ants if they reach overwhelmingly large population densities (Fell, 1997).

The various associations we observed between ants and honey bees are potential routes for interspecies transmission of honey bee-associated viruses between these eusocial insects. As previously, growing body of research has recently focused on the detection of honey bee-associated viruses in other arthropod groups (Genersch et al., 2006; Celle et al., 2008; Gisder et al., 2009; Meeus et al., 2010; Singh et al., 2010; Di Prisco et al., 2011; Jilian et al., 2011; Peng et al., 2011; Yañez et al., 2012; Zhang et al., 2012; Levitt et al., 2013; Fürst et al., 2014; Ravoet et al., 2014; McMahon et al., 2015;

Sébastien et al., 2015; Mordecai et al., 2016; Parmentier et al., 2016; Tehel et al., 2016; Bigot et al., 2017; Gisder & Genersch, 2017; Gruber et al., 2017; Bailes et al., 2018; de Souza et al., 2019; Loope et al., 2019; Murray et al., 2019; Schläppi et al., 2019). This area of research is particularly important in the context of honey bee pathogen transmission given that viruses can rapidly mutate and adapt to novel hosts, particularly those that are genetically similar or whose biological niche overlaps with that of the original host. This rapid adaptation of viruses could have ecological consequences such as influencing changes in the structure of a community containing susceptible insects (Parrish, 2008).

It should be noted, however, that the identification of a virus in a novel host species is not indicative of a "spillover" event, the process through which a virus is transmitted from a reservoir population into a native or novel host. Instead, at least in the case of honey bee-associated viruses, it is more likely that spillover would have first occurred in susceptible hosts when honey bees were first introduced into the New World by European settlers during the seventeenth century (Sheppard, 1989). Any spillover event would have likely occurred in genetically similar species that had overlapping floral resources with honey bees (e.g., bumblebees and solitary bees), such that viruses could have been transmitted through the sharing of nectar or pollen (Singh et al., 2010; Mazzei et al., 2014). The growing number of newly-identified host species that have been found to foster honey bee-associated viruses speaks more about our advances in detecting these viruses then it does about the occurrence of a recent spillover event.

We discovered that 51 of 57 ant samples collected at apiary sites (89%) and three of 20 samples collected at non-apiary sites (15%) tested positive for the presence of one or more virus. Because the three samples collected from non-apiary sites were all *S. invicta*, we hypothesize that these omnivorous ants picked up the viruses by scavenging bee foragers that had died away from their hive. However, none of these samples tested positive for replication of DWV, BQCV, or IAPV. Overall, previous studies have tested only a relatively few number of ant species for the replication of DWV, BQCV, or IAPV (Levitt et al., 2013; Sébastien et al., 2015; Gruber et al., 2017; Schläppi et al., 2019). Argentine ants are one of the few ant species that have been tested for a honey bee-associated virus (DWV) in multiple areas worldwide including Argentina, New Zealand, Australia, and now the United States. Yet, only ants collected in New Zealand have been shown to have the replicative form of DWV (Sébastien et al., 2015; Gruber et al., 2017). This indicates that replication of honey bee-associated viruses in this species may be due to genetic variation of the virus, the ant, or both, as a result of geographical location. Argentine ants are not as common in Texas as in other areas of the United States, so it would be interesting to sample this species in a broader geographical range with an increased sample size in order to better answer this question.

Beyond being a pest for beekeeping operations, ants may be impacting bee health in more ways than previously thought. Despite lack of viral replication, ants that feed on infected honey bee brood or adults, or on infected sugar and pollen resources, may still act as mechanical vectors of honey bee-associated viruses. It is speculated that virus-containing ants disseminate viruses to honey bees by invading hives and transmitting the

viruses to nectar or honey cells while robbing, which can then enter bees that subsequently feed from these cells. This is especially likely of ants that are common pests within hives, such as *S. invicta* and *Crematogaster* sp. For instance, a previous study showed that ants can acquire honey bee-associated viruses through foodborne transmission (i.e., the ingestion of infected honey bee pupae) (Schläppi et al., 2019). However, further research looking at the possible transmission mechanisms of these viruses from ants to honey bees is needed to determine whether or not ants play a role in transmitting viruses to honey bees, which would contribute to the declining health of this important pollinator.

3. QUALITY OVER QUANTITY: HIGH-PROTEIN DIETS INCREASE PATHOGEN TOLERANCE OF HONEY BEES INFECTED WITH *NOSEMA CERANAE*

3.1. Overview

The ever-increasing demand for honey bee (*Apis mellifera*) pollination services of agricultural crops often leads colonies to be nutritionally stressed and at an increased risk of becoming infected with pathogens. Previous work has shown that the health of honey bees infected with pathogens can be improved by ensuring that they have access to diets that fulfill all their nutritional needs. One method of improving honey bee nutrition is supplementing colonies with commercially available pollen substitutes during times of pollen dearth. However, there are a myriad of pollen substitutes on the market, all of which vary in their makeup, including their macro- and micronutrient profiles. For example, the search for the ideal protein-to-lipid (P:L) macronutrient ratio that could help bees tolerate pathogen infection remains ongoing. The purpose of this study was to determine if there is an optimal P:L ratio in the honey bee diet that can ameliorate disease and positively impact the survivorship, physiology, and overall health of honey bees infected with the fungal pathogen *Nosema ceranae*. To do this, we conducted cage assays with cohorts of nurse-aged bees that were either uninfected or infected with *Nosema* and fed them one of four artificial diet treatments: a high P:low L diet (40P:10L), a low P:high L diet (20P:30L), an intermediate diet ratio at which non-infected honey bee colonies self-selected for in a separate study (30P:20L), or a

sucrose-only diet. We found that both non-infected and *Nosema*-infected bees had the largest consumption of the 30P:20L diet. However, bees that were fed the high-protein 40P:10L diet had the largest hypopharyngeal glands and a significantly higher survivorship over time when infected with *Nosema* compared to any of the other diet groups. Our results indicate that diet quality (i.e. protein content) provided a larger health benefit to *Nosema*-infected bees than the quantity of diet consumed. Overall, this chapter identified that higher protein content, in this case a macronutrient ratio of 40P:10L, in a honey bee's diet results in an increased tolerance to *Nosema* infection. This information can potentially be used in the future to better tailor commercially available pollen substitutes for managed colonies living on altered and changing landscapes.

3.2. Introduction

Pathogens can impose direct and indirect fitness costs on their host. Depending on the type and severity of infection, organisms can alter their diets as a mechanism to self-medicate against disease, implying that a dietary shift can improve an individual's fitness by increasing its resistance and/or tolerance to pathogen infection (Cotter et al., 2011; Shikano & Cory, 2015). However, host nutritional status can act as a double-edged sword regarding host-pathogen interactions. For example, while an increase in nutrient intake can lead to a boost in host immune function, it can also result in an increase of pathogen virulence due to the larger availability of resources utilized in replication (Bedhomme et al., 2004; Smith et al., 2005). Thus, there are two broad

strategies through which hosts can alter their nutritional status to mitigate the effects of infection: 1) illness-induced anorexia that reduces the availability of nutrients to the pathogen and/or decreases trade-offs between the immune system and digestion, or 2) an increase in nutrient intake to boost host immune function. The strategy that helps a host better deal with pathogen infection is generally dependent on the taxa involved in a particular host-pathogen system (Exton, 1997; Coop & Kyriazakis, 1999; Singer et al., 2014).

Examples of both strategies are found across different insect-pathogen systems. For example, the former strategy has been observed in fire ants (*Solenopsis invicta*). Workers have been shown to reduce their foraging activity, shift their diet to decrease lipid intake, and show a preference for carbohydrate-rich foods when infected with *Solenopsis invicta* virus-1 (Hsu et al., 2018). This dietary shift of macronutrients away from lipid-rich foods has also been observed in the Texas field cricket (*Gryllus texensis*). Individuals infected with the bacterial pathogen *Serratia marcescens* preferentially consume foods with less fat and exhibit a decrease in resistance to bacterial infection when fed a lipid-rich diet (Adamo et al., 2010). Illness-induced anorexia and the reduced intake of lipids is believed to be beneficial to some insect hosts because it decreases the trade-off between immune function and lipid transport. Other examples of illness-induced anorexia in insect hosts have shown hosts reducing their protein intake, as opposed to lipids, to better deal with infection (Rachimi et al., 2021). Conversely, hosts may use the other strategy and shift their diet to increase their nutritional intake, typically to consume a particular nutrient that may contribute to increased pathogen

tolerance (Lee et al., 2006; Povey et al., 2009). For example, caterpillars (*Spodoptera littoralis*) infected with nucleopolyhedrovirushave been shown to increase their relative consumption of protein and, in turn, have higher constitutive immune function which resulted in increased resistance to the virus (Lee et al., 2006).

In the case of honey bees (*Apis mellifera*), the consensus is that an increase in host nutritional status (which includes diet quantity, diversity, and quality), as opposed to illness-induced anorexia, improves host tolerance to pathogens (reviewed in: Dolezal et al., 2018; Alaux et al., 2010; Di Pasquale et al., 2013). For example, honey bees infected with the microsporidian gut pathogen *Nosema* spp. have been shown to increase the amount of diet consumed, preferring polyfloral over monofloral pollen, to combat infection (Alaux et al., 2010; Jack et al., 2016; Castelli et al., 2020). The microsporidian *Nosema ceranae* is an obligate, intracellular spore-forming parasite that infects ventricular epithelial cells in the honey bee midgut that are involved in digestion and nutrient intake. This can cause increased energetic stress and immunosuppression in its host (Antúnez et al., 2009; Fries, 2010; Chaimanee et al., 2012).

While diet quality has been linked to an increase in tolerance to pathogen infections, what constitutes “proper nutrition” for honey bees has not been clearly defined (Dolezal et al., 2018). In some cases, diet quality has been estimated based on the general nutrient composition of natural pollen and pollen substitutes (DeGrandi-Hoffman et al., 2016). This approach can be quite variable, however, as the nutritional composition of pollen or their substitutes as a proxy for diet quality can be referring to several constituents such as macronutrients, micronutrients, vitamins, antioxidant

capabilities, and other constituents (Di Pasquale et al., 2013; Glavinic et al., 2017). This highlights the need to better define what constitutes proper nutrition (i.e., diet quality), particularly in relation to the interactions between honey bees and their associated pathogens.

In this study, we used artificial diets in which we manipulated the macronutrient ratio of protein to lipids (P:L) to elucidate how diet quality impacts the host-pathogen interactions between honey bees and *Nosema ceranae*. We conducted a series of nutrition-based no-choice tests to determine whether honey bees infected with *N. ceranae* exhibited differences in host diet consumption, pathogen load, survivorship, and physiology when fed diets that varied in their P:L ratios. Overall, these results contribute to our understanding of how diet can be used to mitigate the effects of pathogen infection in honey bees.

3.3. Methods

3.3.1. Experiment setup

The experiment was carried out over a 30-day period from May to June 2021. The colonies used in this study were sourced from the Janice and John G. Thomas Honey Bee Facility located at the RELLIS campus of Texas A&M University in Bryan, TX, U.S.A. The colonies intended for use were preemptively treated with Apivar® Miticide Strips (Véto-pharma, Palaiseau, France) to control for the parasitic mite *Varroa destructor*(Rosenkranz et al., 2010). Upon inspection, only colonies that had zero mites per 100 bees (Macedo et al., 2002; Dietemann et al., 2013), were free of any visible

signs of brood diseases such as foulbrood and sacbrood, had a healthy capped brood pattern, were queenright, and were not infected with *Nosema* spp., were used in the study. Two frames with capped brood were pulled from each of five source colonies that met the above criteria (ten frames total) and placed within two five-frame nucleus hive boxes inside an incubator that was kept at 34ºC and ~80% relative humidity to mimic natural colony conditions (Winston, 1987). All adult bees were removed before the frames were placed in the nucleus boxes. Each box contained one frame from each colony to diminish the effect of the source colony as a random factor influencing the results. The frames were kept in the incubator for 24 h to allow for the emergence of age-matched, adult workers. Once bees emerged, they were collected and randomly placed within identical cages (n=25 bees/cage) made from modified deli containers that were outfitted with a wire mesh on top. Each cage was equipped with two gravity feeders, one containing a 50% (w:v) sucrose solution in dH_2O and the other containing water, both of which were filled *ad libitum*. The cages had two holes at the bottom that were used to place plastic queen-rearing cups (JZs-BZs, Santa Cruz, CA, U.S.A.) that contained the artificial diets. All components of the cage setup were sterilized with a 10% bleach and water solution prior to the start of the experiment.

3.3.2. Creation of artificial diets

We created three artificial honey bee diets in which the macronutrient ratio of proteins to lipids (P:L) could be manipulated while simultaneously keeping all other diet components constant. We chose isolated soy powder as the protein source due to its

balanced amino acid profile for bees (de Groot, 1953; Gorissen et al., 2018; Manning, 2016) and linseed oil as the lipid source because of its high proportion of relevant fatty acids, including a high omega 3-to-omega 6 fatty acid ratio (Kraus et al., 2019; Manning, 2016). The P:L ratios were selected based on previous work that used the Geometric Framework for Nutrition (GFN) to determine honey bee preferences for a particular P:L intake target. This included a 30P:20L diet, which had the P:L ratio towards which bees had previously been shown to regulate their nutrient intake (Lau et al., in prep). We also used a comparatively high P-to-low L ratio diet (40P:10L), and a low P-to-high L ratio diet (20P:30L). For all three diet types (40P:10L, 30P:20L, and 20P:30L), the combined weight of protein and lipid equaled 50% of the diet's total mass. The remaining mass had an identical proportion of Vanderzant vitamin mixture for insects (0.5%), cellulose (10%), and 50% w/v sucrose solution (39.5%).

3.3.3. Creation of the *Nosema ceranae* inoculum

We created an inoculum that contained a *N. ceranae* spore suspension with a concentration of 5.45×10^7 spores/μL. Due to the difficulty of culturing *N. ceranae* spores *in vitro* (Fries et al., 2013), spores were grown to a high concentration within generational cohorts of caged adult workers. This was accomplished by locating a colony of *Nosema*-infected workers in February 2021. In the following months leading up the experiment, cages of adult bees from the same colony were systemically infected with the spores cultivated from the previous cohort of infected individuals. This was

replicated for a total of four cohort cycles over a three-month period to acquire a spore count of 100,000 spores/bee.

To ensure spore viability, the *N. ceranae* inoculum was created on the same day that the experiment was started. To do this, bees were collected from the infection cages and used to create a pure spore suspension, as previously described (Cole, 1970; Fries et al., 2013). Briefly, the abdomens of 50 bees were separated, homogenized with a mortar and pestle, and suspended in 50 mL of autoclaved RO2 water. The homogenate was passed through a metal mesh to filter out debris, and the resulting filtrate was centrifuged at 4,000 rpm for 8 min to pellet the *N. ceranae* spores. The pellet was washed three times with autoclaved RO water. The pellet was centrifuged each time at 4,000 rpm for 8 min and the resulting supernatant was poured out. The pellet was then diluted with water and the final spore count was determined using a haemocytometer, as previously described (Cantwell, 1970). The spore count was performed in triplicate on three distinct aliquots of the diluted spore sample, and the three-count average was used to determine the pellet's spore concentration.

3.3.4. Infection of honey bees with *N. ceranae* using a group feeding method

Once each cage was assembled and filled with 25 newly emerged workers, we randomly split and assigned each cage to an experimental treatment that incorporated a specific infection status and diet group. Infection status refers to whether the caged bees were non-infected as our control group or experimentally infected with *N. ceranae* (*Nosema*). Diet group refers to cages being given either one of the three artificial diets (40P:10L,

30P:20L, and 20P:30L) or no diet as a negative control group in which bees were only fed sucrose solution and were devoid entirely of any protein or lipid food. In total, we had eight treatment groups (based on infection status and diet group) with eight biological cage replicates per treatment group (n = 64 cages total).

Cages were infected through a group feeding method using gravity feeders. The spore stock was mixed with 50:50 w:v sucrose solution made with autoclaved RO2 water to equal a dosage of 100,000 *N. ceranae* spores/bee. Each cage belonging to the *Nosema*-infected group was inoculated with 4 mL of the spore stock. The small volume of the inoculum introduced to each cage helped to ensure that the entirety of the inoculum was consumed by the bees, as previously described (Fries et al., 2013). Cages of bees belonging to the non-infected control group received 4 mL of just 50:50 w:v sucrose solution. The sucrose solutions were introduced into their respective cages at the same time to avoid a staggering of the infection timeline.

3.3.5. Experimental timeline and data collection

Artificial diets were first introduced into cages 24 h post-infection using plastic queen cups. These cups mimic the size of a honey bee comb cell and reduce the incidence of bees stepping into the receptacles and spilling the diets. Two queen cups were used per cage and were filled with that cohort's assigned diet (or remained empty, in the case of the no-diet control group). We weighed each diet cup at room temperature after adding the food to determine the initial diet weight, and then we placed both cups within their respective cage. All cups were exchanged daily and weighed approximately 24 h after

being removed from a cage to determine the post-consumed diet weight. This waiting period was necessary to allow for the diets to reach ambient humidity and temperature levels within the laboratory prior to being weighed. The daily exchange of diets was done over six consecutive days (n = 6 rounds of diet introduced into cages daily). Diet consumption was obtained by calculating the difference between the initial and final weights of each cup. We calculated the total amount of artificial diet consumed by nurse bees during the six days of the experiment, as well as the number of bees that were alive within each cage at each timepoint, to determine the cumulative per-capita consumption of diet (mg/bee) for each treatment group across all cages. Bee mortality was monitored throughout a 30-day period by counting and removing dead bees in each cage daily. On the final day of the experiment, all remaining live bees were removed from each cage and stored at -80°C until further analysis.

We used a subset of bees from each cage to measure average hypopharyngeal gland (HPG) acinus size and pathogen load. HPG acinus size is a common metric used to assess the nutritional status of honey bee nurses (Brodschneider & Crailsheim, 2010; Omar et al., 2017). To perform this analysis, we randomly removed two bees from each cage (n = 128 bees) seven days post-infection and stored them in -20°C for later HPG analysis. For each bee, both glands were dissected from the head and the size of five randomly selected acini was measured per gland (n = 10). Then, the average acinus size (μm^2) per treatment group was calculated. Detailed methods involving the dissection of the glands from the bee's brain and their visualization and measurement using microscopy is described in Corby-Harris & Snyder (2018). For pathogen load, two

randomly selected bees from each cage were removed 14 days post-infection and stored at -20°C for future analysis. The abdomens of each bee were used to conduct individual spore counts as previously described (Cantwell, 1970). Bees used to measure HPG size and pathogen load were excluded from the analysis of mortality across treatment groups.

3.3.6. Statistical analysis

The cumulative per-capita consumption of each artificial diet (mg/bee) by nurse bees was compared with a two-way ANOVA test, with infection status and diet type as the independent variables. This was followed by pairwise Tukey HSD tests. Daily bee survivorship over the 30-day experimental period was compared between treatment groups using multiple cox mixed effect models that used infection status and diet group as the fixed variables, and cage number as a random effect. Risk ratios were calculated based on likelihood of death by dividing the risk of death in one treatment group over the risk of death in another. HPG size was compared between infection status and diet group using an ANOVA test followed by Tukey HSD tests. The level of significance for all statistical tests was set at $\alpha = 0.05$ and performed using JMP software.

3.4. Results

3.4.1. Comparative consumption of artificial diets across all treatment groups

The mean cumulative per-capita consumption of our artificial diets by nurse bees was significantly affected by infection status ($F_{1,42} = 4.72$, $p = 0.036$), diet type ($F_{2,42} = 76.16$, $p < 0.0001$), and the interaction between infection status and diet type ($F_{2,42} = 8.03$, $p =$

0.0011; Figure 3.1). Of the three artificial diets we tested (40P:10L, 30P:20L, and 20P:30L), the median and more "balanced" diet of 30P:20L was significantly more consumed by bees in both the non-infected control groups and *Nosema*-infected (*Nosema*) treatment groups compared to bees fed the other two diets. A comparison of infection status only without factoring in the diet group showed that bees infected with *N. ceranae* consumed significantly more food (8.42 mg/bee) than their non-infected counterparts (7.06 mg/bee). When looking at the interactive effect of infection status and diet type on consumption, the treatment group with the greatest food consumption was that of *Nosema*-infected bees that were fed the 30P:20L diet, with a cumulative per-capita consumption of 15.65 mg/bee. The second highest consumption was observed in non-infected bees fed the 30P:20L diet (10.81 mg/bee). The high-protein (40P:10L) and high-lipid (20P:30L) diets had similar per-capita diet consumption for bees in both the non-infected group and *Nosema*-infected group, with these four groups having a mean cumulative per-capita consumption of < 6 mg/bee.

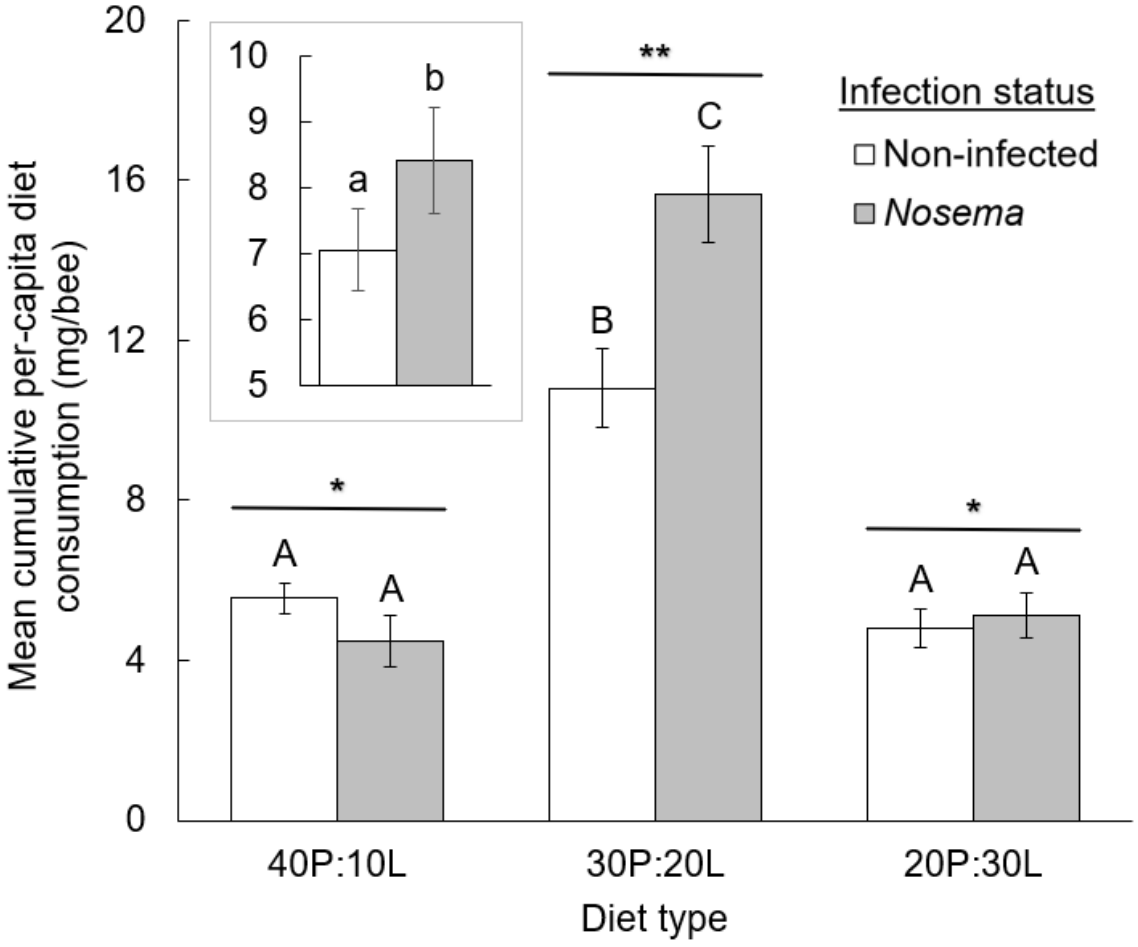

Figure 3.1: Effect of Nosema infection on the amount of artificial diets consumed by nurse bees. The mean amount of diet (mg/bee) cumulatively consumed by each nurse bee was measured over six days as a function of infection status and diet type. Diets varied in their protein-to-lipid (P:L) ratios, and infection status consisted of either non-infected control bees or bees infected with *Nosema ceranae* (*Nosema*). The panel in the upper left corner compares the mean cumulative per-capita consumption between control and *Nosema*-infected bees overall, irrespective of diet type. For the main figure, the bars are clustered by diet type, and bar color corresponds to infection group (Non-infected = white, *Nosema* = gray). The asterisks and letters above the bars denote significance (α =

0.05), and bars that do not share the same number of asterisks or the same letter are significantly different from one another. The asterisks denote significant differences between diet types, irrespective of infections status. Letters above each bar show significant differences between the interactive effect of infection status and diet type. Error bars represent the SEM.

3.4.2. Honey bee survivorship based on infection status and diet group

We measured daily bee mortality for each cage and compared the proportion of live bees across treatment groups remaining at the conclusion of the 30-day study. We found a significant effect of infection status (d.f. = 1, $\chi^2 = 57.61$, $p < 0.0001$), diet group (d.f. = 3, $\chi^2 = 218.06$, $p < 0.0001$), and the interaction between infection status and diet group (d.f. = 3, $\chi^2 = 22.86$, $p < 0.0001$) on bee survival (Figure 3.2). When considering only infection status and not diet group as a factor, honey bees infected with *Nosema* had significantly lower survivorship (26%) compared to non-infected bees (45%) by day 30 of the experiment (d.f. = 1, $\chi^2 = 50.82$, $p < 0.0001$; Figure 3.2). Bees infected with *Nosema* had a significantly higher risk of death than their non-infected counterparts (RR= 1.77, 95% CI 1.52-2.05, $p < 0.0001$; Table 4.1). When considering only diet group as a factor, bees had the highest survivorship when fed the high-protein 40P:10L diet (61%) compared to bees fed the 30P:20L diet (45%), 20P:30L diet (19%), and no diet negative control (15%). This corresponded to a significantly higher risk of death for bees that were fed any of the diet groups when compared to the 40P:10L diet (Appendix B, Table 1).

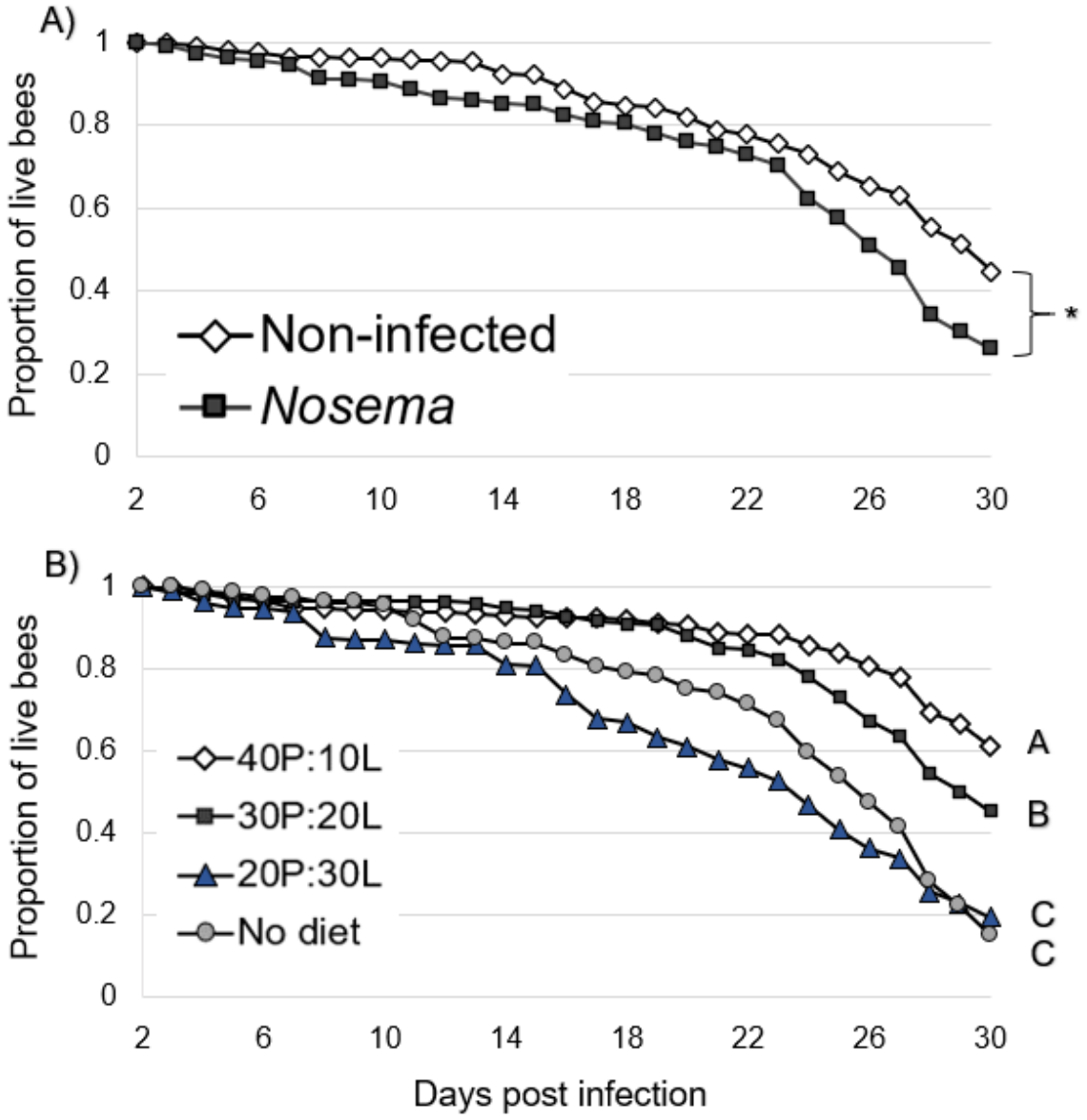

Figure 3.2: The individual effects of infection status and diet group on honey bee survivorship over time. The figure depicts the proportion of live, caged bees when A) grouped by infection status, and B) grouped by diet. Survivorship was measured as the average proportion of live individuals across all cages belonging to the same treatment group. Survivorship over time is represented as lines, with each marker showing the proportion of live bees on a particular day post-infection. In panel A, non-infected bees

are depicted by white diamonds, and *Nosema*-infected bees are depicted by black squares. The asterisk shows significance ($\alpha = 0.05$) between the NI and *Nosema* infection groups regarding the proportion of live bees remaining on Day 30 post-infection of the experiment. Panel B shows the proportion of live bees over time for each diet group including 40P:10L (white diamonds), 30P:20L (black squares), 20P:30L (blue triangles), and no diet (gray circles). The letters on the right of the figure denote significance ($\alpha = 0.05$) between treatment groups on day 30 post-infection, where lines ending in different letters are significantly different from one another. Survivorship for day 1 post-infection was not included in the analyses, as mortality on that day was linked to the injection/set-up process and not treatment. Live bees that were purposefully removed from cages on particular days of the experiment to measure hypopharyngeal gland sizes and pathogen load were not considered instances of mortality and were excluded from these survivorship analyses.

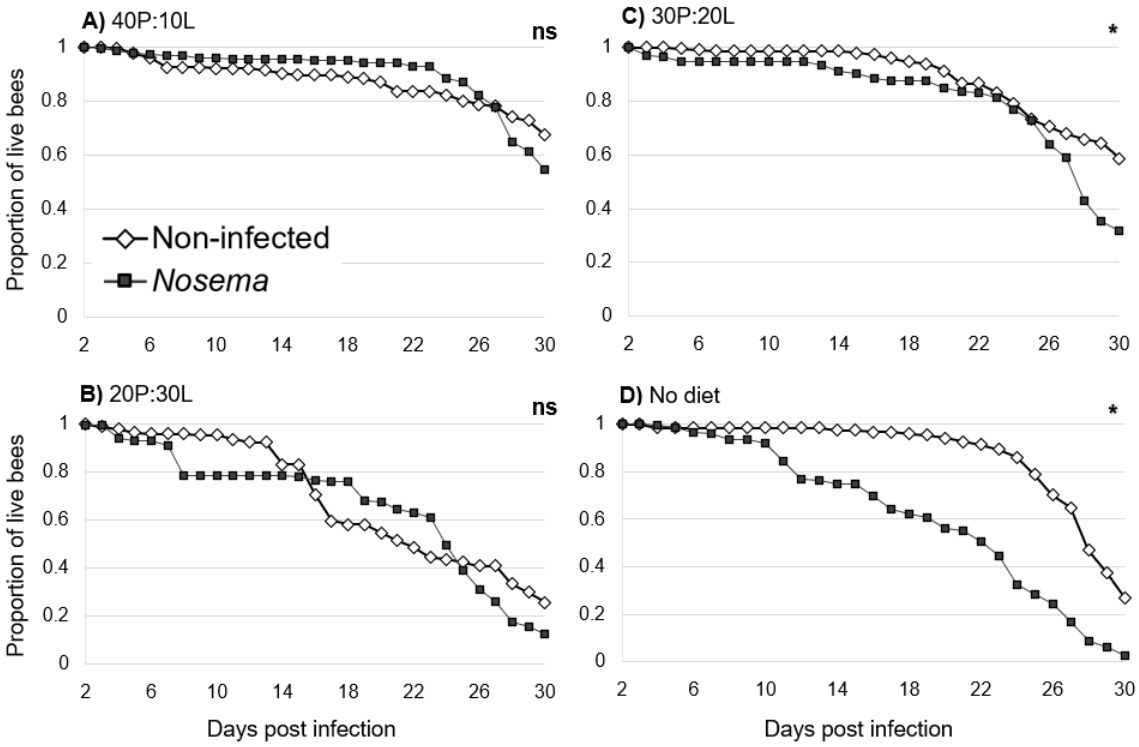

Figure 3.3: The interactive effect of infection status and diet group on honey bee survivorship over time. The four panels of line graphs illustrate the comparative survivorship of non-infected and *Nosema*-infected (*Nosema*) bees for each diet group (8 treatment groups total: ~200 bees/treatment group). The trial was conducted for 30 days post-infection. Each panel represents the interaction of infection status with a particular diet group: A) 40P:10L, B) 20P:30L, C) 30P:20L, and D) no diet. Survivorship was measured as the average proportion of live individuals across all cages belonging to the same treatment group. Survivorship over time is represented as lines, with each marker showing the proportion of bees that remained alive on a particular day post-infection. For all four panels, the survivorship of non-infected bees is depicted by white diamonds, and that of *Nosema*-infected bees by black squares. The interactive effect of infection status and diet group for each panel is marked above with an asterisk if significant ($p >$

0.05) or with the letters “ns” if not significant. Survivorship for day 1 post-infection was not included, and live bees that were purposefully removed from cages for other tests were excluded from the survivorship analysis.

3.4.3. Hypopharyngeal gland size analysis

Hypopharyngeal gland (HPG) size was measured as a physiological indicator of nutritional status in bees seven days post-infection (Figure 3.4). Average HPG acinus size was significantly correlated with diet group ($F_{3,120} = 7.17$, $p = 0.0002$), with bees in the two higher protein diets (40P:10L and 30P:20L) having significantly larger acini (9,323.35 μm^2 and 10,896.9 μm^2, respectively) compared to bees in the no diet group (6,331.68 μm^2). Infection status (irrespective of diet group) did not have a significant effect on HPG size ($F_{1,120} = 0.11$, $p = 0.75$). However, we saw a trend in which NI bees, regardless of diet group, had larger acini (8,957.87 μm^2) compared to *Nosema*-infected bees (8,727.29 μm^2). There was no significant interaction effect between infection status and diet group on HPG size ($F_{3,120} = 0.34$, $p = 0.80$). However, we observed that NI bees fed the 30P:20L diet had the largest HPG acini (11,444.89 μm^2), followed by the *Nosema*-infected bees fed the 30P:20L diet (10,348.91 μm^2). The smallest HPG sizes for the interaction between infection status and diet group belonged to bees in the NI no diet group (5,888.0 μm^2), followed by the *Nosema* no diet group (6,775.35 μm^2).

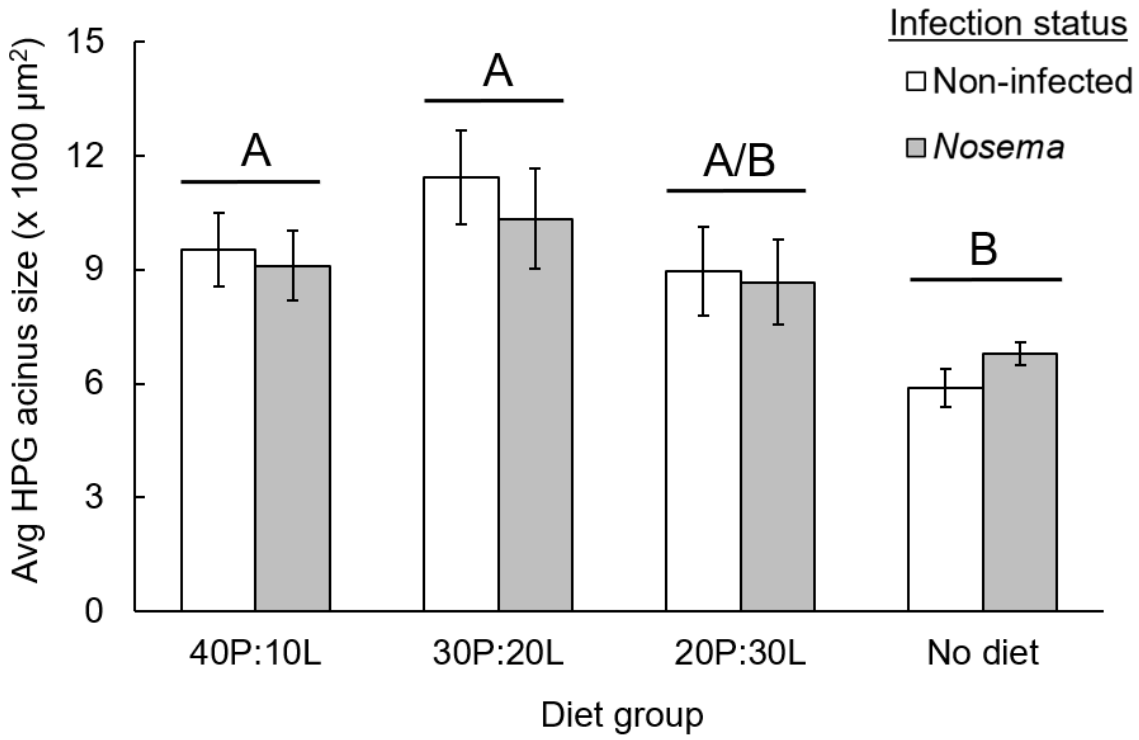

Figure 3.4: Average hypopharyngeal gland (HPG) acinus size of non-infected and Nosema-infected (Nosema) nurse bees fed artificial diets varying in their protein-to-lipid ratios. Bars represent the average HPG acinus size grouped by both infection status and diet group. The bars are clustered by diet group, which varied in protein-to-lipid (P:L) ratios. The no diet group was a negative control in which bees were not given a protein or lipid source and were only fed sucrose solution throughout the experiment. Bar color corresponds to infection status (Non-infected = white, *Nosema* = gray). Letters above the paired bars show the significant effect that diet had on the average HPG acinus size. Bars that do not share the same letter are significantly different from one another. There were no significant effects of either infection status alone or the interaction

between infection status and diet group on HPG size. Error bars represent the SEM and letters above the bars indicate significance at $p < 0.05$.

3.4.4. Pathogen load of *Nosema*-infected honey bees

Individual spore counts were performed for two randomly selected live bees per cage on day 14 post-infection in order to determine the average *Nosema* pathogen load per treatment group (Figure 3.5). We did not detect *Nosema* spores in any of the bees removed from a non-infected control cage (n = 64 bees total). For bees sampled from *Nosema*-infected cages, there was not a significant effect of diet group on pathogen load (d.f. = 3, $\chi^2 = 7.19$, $p = 0.0662$). When conducting mean comparisons using Student t tests, only the 30P:20L diet group that had the highest pathogen load (2.8×10^7 spores/bee) and the no diet control group that had the lowest pathogen load (5.03×10^6) were significantly different from one another ($p = 0.0131$).

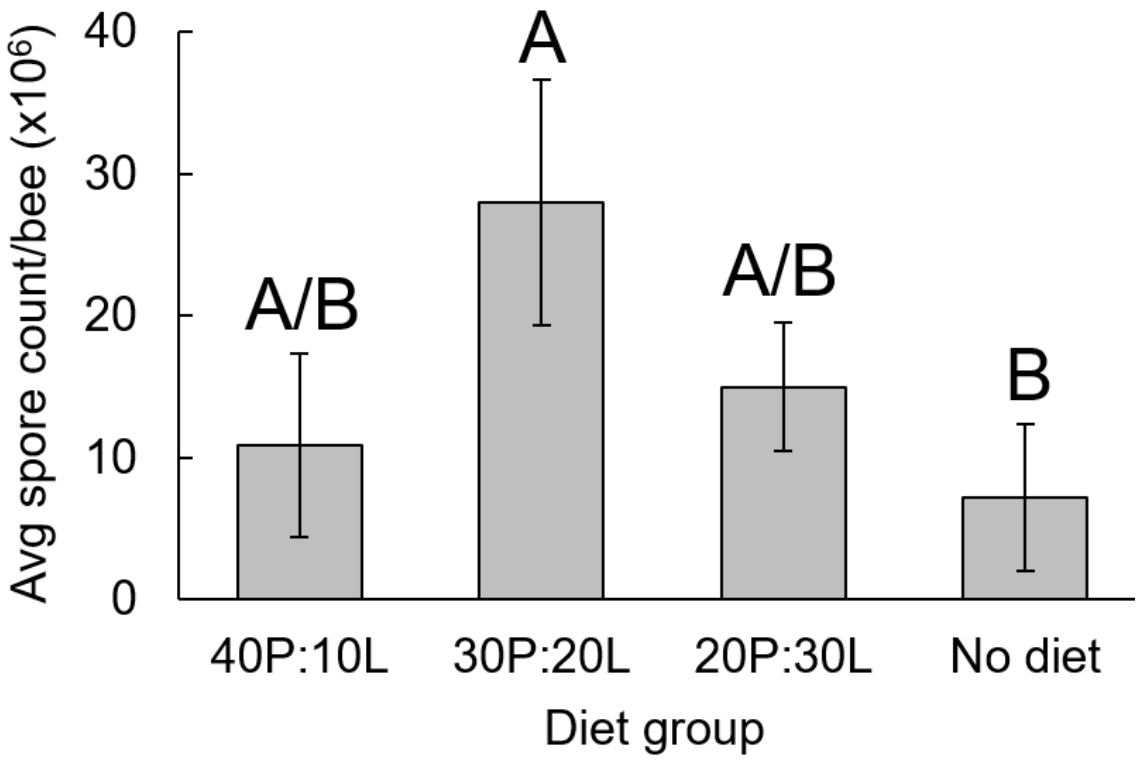

Figure 3.5: Effect of diet group on the pathogen load of *Nosema*- infected bees. The bars represent the average spore count/bee for each of the pairwise *Nosema*- infected, diet treatment groups (n= 16 bees/ treatment group). All bees sampled from non-infected cages and from all four diet groups were free of *Nosema* spores (not shown). Letters above the paired bars show the significant effect that diet had on the average spore count. Bars that do not share the same letter are significantly different from one another. Error bars represent the SEM and letters above the bars indicate significance at $p < 0.05$.

3.5. Discussion

The purpose of this study was to better define how diet quality (in regards to the ratio of proteins-to-lipids) impacts host-pathogen interactions between honey bees and the

microsporidian gut pathogen *Nosema ceranae*. We utilized artificial diets that varied in their protein-to-lipid (P:L) content in a no-choice test to determine how diets that varied in their ratio of these two macronutrients affected the tolerance of honey bee workers when infected with *N*osema. The diet groups consisted of a high P:low L diet (40P:10L), a low P:high L diet (20P:30L), an intermediate diet ratio at which non-infected honey bee colonies self-selected for in a separate study (30P:20L), and a sucrose-only diet that served as a negative control (Lau et al., in prep). The results of our study indicate that diet quality (i.e., P:L macronutrient composition) has a significant impact on honey bees workers infected with *Nosema*, more so than the quantity consumed of any one diet.

Previous studies that have looked at the interactive effect of host nutrition and *Nosema* infection on host-pathogen interactions have oftentimes either compared 1) different monofloral pollen types that varied in nutritional quality, 2) monofloral types vs polyfloral pollen blends, or 3) pollen that was supplemented with various protein/lipid sources to create diets that were broadly labelled as "high" or "low" quality. However, different pollen types can vary greatly on any number of nutritional components including a diverse composition of proteins, lipids, sugars, fibers, minerals, amino acids, waxes, etc. (Thakur & Nanda, 2020), which makes it difficult to determine what nutritional aspect of the pollen is having the largest impact on the host-pathogen interactions being observed. To better define how two particular diet components (proteins and lipids) impact host-pathogen interactions of *Nosema*-infected bees, we utilized honey bee-specific artificial diets in which all of the ingredients were

standardized and in which only the protein and lipid content were manipulated to create our different P:L diet groups.

We found that the intermediate 30P:20L diet was consumed significantly more than either the high-protein (40P:10L) or high-lipid (20P:30L) diets. Although we cannot make the claim that bees preferred consuming a particular P:L ratio, given that we did not perform choice tests using paired diets, our results do indicate that nurse bees exhibit nutrient regulation for a P:L intake target of 30 parts protein to 20 parts lipid. This result mirrors what was observed in a previous study by our research group, which used the same artificial diet formulation and found that nurse bees self-selected their consumption in choice-tests to reach a P:L intake ratio of 1.4:1, which, in no-choice experiments, was achieved by bees through consumption of the 30P:20L diet (Lau et al., in prep). While previous studies have demonstrated that forager bees will exhibit nutrient regulation in order to collect resources that complement a deficit within the colony (Hendriksma & Shafir, 2016; Zarchin et al., 2017), there is not a consensus on whether nurse bee regulate their diet based on preferential selection. Corby-Harris et al. (2018) concluded that honey bees do not preferentially select for and consume pollen based on its nutritional quality after performing choice-tests involving protein and lipid supplemented pollen types, while Stabler et al. (2021) found that nurse bees regulated their diet in choice tests to achieve a P:L intake target of 1.25:1 (a similar ratio to our 30P:20L diet ratio).

The significantly higher consumption of the 30P:20L diet compared to the other diet groups was true for both non-infected and *Nosema*-infected bees. This indicated that

nurse bees do not shift their consumption to be more protein or lipid-rich when infected with *Nosema*. In fact, we observed an effect of infection status on diet consumption where *Nosema*-infected bees consumed significantly larger quantities of the 30P:20L diet compared to the non-infected bees given the same diet type. This positive correlation between *Nosema*-infection and increased appetite is consistent with previous studies that have demonstrated that *Nosema ceranae* impacts the feeding behavior of honey bees, particularly by increasing hunger of infected individuals due to the energetic demands that this pathogen incurs on its host (Mayack & Naug, 2009; Naug & Gibbs; 2009). However, this increase in consumption by infected individuals was only observed for caged bees that were fed the 30P:20L diet. Overall, there was low consumption for the 40P:10L and 20P:30L diets, regardless of infection status. This may be a result of these more protein- and lipid-skewed diets not being as palatable to nurse bees compared to the more balanced 30P:20L ratio. However, further studies that utilize choice test with these diets would need to be conducted in order to better determine if this is the case.

A previous study done by Jack et al. (2016) found a positive correlation between pollen quantity consumed and survival in *Nosema*-infected bees. Based on these results, we predicted that survivorship of *Nosema*-infected individuals would be highest for the bees that consumed the largest quantity of food (in this case, bees fed the 30P:20L diet). However, bees fed the high-protein 40P:10L diet had a significantly higher tolerance to *Nosema* infection in regards to survivorship and overall risk of death when compared to bees given the 30P:20L, 20P:30L, and sucrose only diets. This was true when comparing survivorship based on diet group alone and when comparing the multivariate treatments

comprised of both infection status and diet group. In fact, consumption of the 40P:10L diet resulted in similarly high survivorship between non-infected and *Nosema*-infected bees, whereas consumption of the 30P:20L and 20P:30L diets resulted in significantly lower survivorship of bees infected with *Nosema* compared to their non-infected counterparts. Furthermore, *Nosema*-infected bees that belonged to the "no diet" group and were only fed sucrose had the lowest survivorship, with almost all bees across all cages belonging to this group having died by the conclusion of the study. This indicates that, at least in the extreme case of having to make the choice between being given no diet versus any diet at all, diet quantity did have a somewhat positive impact on host tolerance to infection. A study done by Tritschler et al. (2017) observed similar results where honey bees infected with *Nosema* only exhibited a reduction in lifespan when they were starved of protein. Our survivorship data supports the hypothesis that food consumption for workers infected with *Nosema* can overcome the energetic stress that this pathogen imposes (Naug & Gibbs, 2009; Porrini et al., 2011), leading to decreased mortality rates in highly infected individuals.

The size of hypopharyngeal glands (HPG) in nurse bees has been used in previous studies as a physiological measure of an individual's nutritional health and, as an extension, an indicator of a diet's quality (Di Pasquale et al., 2013; Corby-Harris & Snyder, 2018; Corby-Harris et al., 2018). For hypopharyngeal gland sizes, larger average acinus size was correlated with bees that were given access to one of our diet groups. Bees on average had significantly larger glands when fed one of the higher protein diets (40P:10L and 30P:20L) compared to bees fed the 20P:30L diet or no diet at all. This

correlation between increased protein consumption and larger HPG size in nurse bees is in agreement with previous studies that have observed the same result (Pernal & Currie, 2000; Renzi et al., 2016).

We found a correlation between amount of diet consumed and pathogen load, where bees that consumed larger quantities of diet had increased spore counts. Bees that belonged to the 30P:20L diet group had the largest amount of consumption and highest spore loads, followed by similar pathogen loads between bees fed the 40P:10L and 20P:30L diets. The only significant difference in pathogen load was between bees that only had access to sucrose and bees that fed on the highly consumed 30P:20L diet. Pollen consumption has been shown to be tied to *Nosema* spore load, with an increase in pollen consumption oftentimes equaling an increase of *Nosema* spores in honey bees (Porrini et al. 2011; Jack et al., 2016). However, a general increase in nutrient intake not only increases pathogen load, but it also appears to increase bee tolerance to *Nosema*-infection, leading to increased survivorship of the host (Zheng et al. 2014). In this study, this does not appear to be exactly the case, as DWV-infected bees that consumed the greatest amount of food (the 30P:20L diet), and that had the highest pathogen load, did not have the highest rate of survivorship. The DWV-infected bees fed the 40P:10L diet had a comparable pathogen load to the DWV 30P:20L bees, yet they had significantly higher survival. This is further evidence that bees are able to better tolerate *Nosema*-infection when fed a higher-protein diet vs the other tested P:L ratios.

4. EFFECTS OF MACRONUTRIENT CONTENT IN HONEY BEE DIETS ON DEFORMED WING VIRUS INFECTION

4.1. Overview

Across numerous host-pathogen systems, an organism's tolerance and/or resistance to a pathogen is impacted by its physiological state, which can be heavily influenced by host nutritional status. This type of immune response regulated by diet has not been thoroughly studied in social insects, however. For example, it is not known whether honey bees (*Apis mellifera*) exhibit dietary shifts in their macronutrient intake when infected with pathogens, or how different diets may impact a pathogen's ability to infect its honey bee host. Previous work done in our laboratory indicated that honey bee foragers actively regulate their macronutrient intake to reach a protein-to-lipid (P:L) ratio of ~1.4:1, preferring to consume a diet containing 30 parts protein (P) and 20 parts lipid (L). We also found that nurse bees prioritize the regulation of lipids and fatty acids over protein. Using this information, we infected cohorts of newly emerged workers with deformed wing virus (DWV), a commonly encountered honey bee-associated pathogen, and conducted cage assays in which cohorts of infected, mock-infected, and non-infected bees were fed one of four diet groups: a high P, low L diet (40P:10L), a low P, high L diet (20P:30L), an intermediate diet ratio (30P:20L), and a no-diet negative control. We compared diet consumption, survivorship, hypopharyngeal gland (HPG) size, and gene expression in bees across the different treatment groups. We found that the 30P:20L diet was the most consumed by infected and non-infected cohorts of caged

adult worker bees, confirming that bees preferentially regulate their diet to reach a 1.4P:1L intake ratio. Furthermore, bees infected with DWV that were fed the 30P:20L diet had the highest survivorship compared to bees in any of the other diet groups. HPG size was correlated with increasing protein content in the diet, but it was not impacted by infection status. RNA sequencing of uninfected and DWV-infected bees belonging to the four different diet groups revealed that infection status played a significant role in differential gene expression, while diet group did not. Gene ontology analysis revealed that many of the differentially expressed genes between infected and uninfected bees belonged to functional groups associated with immunity, including those involved in apoptosis. These results contribute to a better understanding of the dynamics between host-pathogen interactions and host nutritional status in honey bees, and they will ultimately guide our efforts to improve honey bee health.

4.2 Introduction

Positive-sense, single-stranded RNA viruses comprise the largest group of honey bee-infecting pathogens worldwide (Brutscher et al. 2016). To date, over 24 virus species, belonging primarily to the families Dicistroviridae and Iflaviridae, have been identified as being able to infect honey bees (McMenamin & Genersch, 2015; Brutscher et al., 2016; Gisder & Genersch 2017). The most ubiquitous and cosmopolitan of these viruses is deformed wing virus (DWV). This virus has been found to infect honey bees worldwide, as well as a wide range of other insect hosts (see Chapter 2 of this dissertation). Nationwide colony loss surveys performed every year by the Bee Informed

Partnership have consistently determined that DWV is the most prevalent virus infecting honey bee colonies in the United States. In fact, of the 739 samples taken from beekeeping operations across the country in 2020, 96% of them tested positive for the DWV-B viral strain and 76% tested positive for the DWV-A strain (Bee Informed Partnership, 2020).

Prior to the introduction of the ectoparasitic mite *Varroa destructor* into *A. mellifera* colonies in the 1980s, DWV primarily caused covert infections (Yue et al., 2007; Gisder et al., 2009; Möckel et al., 2011). Covert infections result in long-term persistence of the virus, along with typically asymptomatic levels of virulence within the host population (Sorrell et al., 2009). In the absence of *V. destructor,* prevalence of DWV is maintained covertly through several different vertical and horizontal transmission routes. Vertical transmission of DWV can occur venereally from drone sperm to virgin queens during mating and when honey bee queens transmit virions to their offspring (Chen et al., 2006; Yue et al, 2006; Yue et al., 2007). This form of transmission favors less virulent forms of the virus, as it relies on host survival and reproduction for viral replication and persistence within the host population (Fries & Camazine, 2001). Horizontal transmission of DWV occurs between individuals within a colony, primarily through food exchange between adults (trophallaxis), or from nurse bees to developing larvae during feeding bouts. Similar to vertical transmission, horizontal transmission through these routes have shown to typically result in covert, asymptomatic infections within developing larvae and adults (Chen et al., 2006; Möckel et al., 2011). However, the introduction of *V. destructor* into honey bee populations

resulted in an increased transmission of DWV, causing overt, symptomatic infections. This has been attributed to high viral accumulation within mites, greater DWV inoculation titers that are vectored from mite to bee, and the mite-influenced development of more virulent DWV strains (Gisder et al., 2009; Evans & Schwarz, 2011). Immature bees that are parasitized by *V. destructor* and infected with high titers of DWV typically develop crumpled wings that make flight as adults impossible. Furthermore, bees parasitized by *V. destructor* and infected with DWV at either the developmental or adult life stages commonly exhibit an overall reduction in adult lifespan, an increase in winter mortality, and a negative impact on learning and memory tasks that are crucial for foraging activities (Benaets et al., 2017; Dainat et al. 2012; Genersch & Aubert, 2010).

As is the case with most viral pathogens, there is currently no treatment option for infection with honey bee-associated viruses. Instead, beekeepers must minimize the concomitant effect of stressors that can contribute to viral replication in order to avoid overt, symptomatic infections within their colonies. Aside from controlling the *Varroa* mite, another prominent strategy that helps bees in mitigating viral infections is to ensure that colonies receive proper nutrition (Dolezal & Toth, 2018). However, what constitutes "proper nutrition" for honey bees is still poorly understood. Honey bees consume floral nectar for their carbohydrate needs and pollen as their source of protein, fats, and other nutritional components essential for brood-rearing (Wright et al., 2018). In terms of pollen consumption, proper nutrition typically refers to diets that are available in sufficient quantities (amount consumed), qualities (nutritional composition), and are

high in floral diversity (monofloral vs polyfloral sources) (Schmidt, 1984; Schmidt et al., 1987; Alaux et al., 2010; Brodschneider & Crailsheim, 2010; Hoover et al., 2022). Previous work has generally equated high-quality pollen diets to be those with a high protein content, as they are positively correlated with bee survivorship, glandular development, and brood rearing capacity (Schmidt et al., 1987; Pernal & Currie, 2000).

The three descriptors of proper pollen nutrition (quantity, quality, and diversity) have been shown to mitigate the effects of viral infections. For example, Dolezal et al. (2019) determined that honey bees simultaneously infected with IAPV and starved of pollen exhibited an increased exiting behavior that resulted in lower population sizes over time. This same study also found that bees infected with IAPV and fed a polyfloral pollen or high-quality, single-source pollen high (high in protein, amino acid content) had increased survivorship compared to when infected bees were fed a monofloral, low-quality diet. Another study compared infection levels in bees infected with BQCV that were fed either a pollen supplement or natural pollen. They found that bees consumed significantly more of the naturally foraged pollen, which contained higher levels of soluble protein. In turn, those colonies had decreased viral titers and had lower queen mortality than bees fed the pollen supplements (DeGrandi-Hoffman et al., 2016).

The purpose of this study was to determine whether the ratio of protein (P) to lipid (L) in bees' diet impacts their tolerance to Deformed wing virus (DWV) infection. To test this, we conducted a cage assay in which newly emerged bees were assigned to one of three infection treatments: a DWV-injected group, a mock-infected negative control group injected with phosphate buffered saline (PBS), or a non-injected negative

control group. Cages in the three infection groups were further divided into four diet treatment groups based on whether they were fed a high P:low L diet (40P:10L), a low P:high L diet (20P:30L), an intermediate diet ratio at which non-infected honey bee colonies self-selected for in a separate study (30P:20L), or a no diet negative control. We measured diet consumption, survivorship, hypopharyngeal gland size, and gene expression for bees in all treatment groups and compared our results across infection and diet groups over a 16-day period. Our results suggest that honey bees that consume a diet with a balanced P:L ratio (i.e., 30P:20L) can offset some of the negative effects of DWV infection compared to infected bees that feed on diets that are skewed towards being more protein or lipid rich. These findings add to our understanding of the impacts of host nutrition on honey bee-pathogen interactions and will ultimately help to produce a pollen substitute in which the macronutrient ratios can be manipulated to address nutrient deficiencies as a result of pathogen infection.

4.3 Methods

4.3.1 Experiment and cage setup

Honey bees used in this study were sourced from colonies at the Janice and John G. Thomas Honey Bee Facility located at the RELLIS campus of Texas A&M University in Bryan, TX, U.S.A. Source colonies were first evaluated for the level of infestation of *V. destructor* mites using the alcohol wash method (Dietemann et al., 2013; Macedo et al., 2002). Those that were identified as being free of *Varroa* were further screened for the absence of non-viral honey bee-infecting pathogens, including *Nosema spp.* and brood

diseases such as foulbrood and chalkbrood. Frames of capped brood from colonies that passed the screening process were removed from four colonies and placed within an incubator set at 34^{o}C and ~80% relative humidity, simulating the conditions inside field hives (Winston, 1987). The cages that housed the bees during the experiment were created from modified deli containers. Each cage consisted of a screened top, two gravity feeders providing water and sucrose *ad libitum*, and two holes in which plastic queen-rearing cups (JZs-BZs, Santa Cruz, CA, U.S.A.) containing the artificial diets could be easily introduced or removed (see Chapter 3 for more details).

Once all cages had been assembled and filled with 25 newly emerged honey bees each, we randomly split and assigned each cage to an experimental treatment that included both infection and diet group. Infection group referred to cages of bees that were either non-injected (NI), mock-infected with injection of phosphate buffered saline (PBS), or experimentally infected with deformed wing virus (DWV). Diet groups consisted of either three artificial diets (40P:10L, 30P:20L, and 20P:30L), or a no diet negative control in which bees were only fed sucrose solution. We had a total of twelve treatment groups based on both infection and diet group. The experiment consisted of two trial periods that each lasted 16 days: one took place from August to September 2021 and the second took place from September to October 2021. Each trial had three biological cage replicates per treatment. In total, there were six cages (three per trial) for each of the twelve experimental groups. The one exception was for treatment group DWV 20P:30L in which we included an extra cage (n=4) during the second trial period due to high mortality in the first trial and fear of low sample size for sequencing.

4.3.2 Creation of artificial diets

The artificial diets used in this experiment were made using the same ingredients and ratios as those outlined in Section 3.3.2 of this dissertation. In brief, we created diets in which we manipulated the protein-to-lipid ratios (P:L), while maintaining a standardized proportion of all other ingredients. Each diet consisted of isolated soy powder as the protein source, linseed oil as the lipid source, a 50% (w/v) sucrose solution, Vanderzant vitamin mixture for insects, and cellulose, with the percent mass of each component remaining the same as what was previously described in Chapter 3. The P:L ratios of the three experimental diets remained the same and consisted of a high P:low L diet (40P:10L), a low P:high L diet (20P:30L), and an intermediate diet ratio (30P:20L).

4.3.3 Creation of DWV inoculum and injection procedure

Adult honey bees with crumpled wings indicative of DWV infection were collected in August 2020 from colonies that were highly infested with *Varroa* mites in Franklin, Texas. A DWV inoculum was then created in the laboratory as previously described (de Miranda et al., 2013). Briefly, ten bees were ground up in PBS and then centrifuged for 10 min at 5,000 rpm. The resulting supernatant was passed through a 0.2 µM filter (Thermo Scientific Nalgene Syringe Filger, Catalogue No. 190-2520) using a B-D Luer-Lok 3-mL syringe (Thermo Scientific, Catalogue No. 309585). The presence of DWV and the absence of other common honey bee-associated viruses, including black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), acute bee paralysis virus

(ABPV), Kashmir bee virus (KBV), and sacbrood virus (SBV), was confirmed by real-time polymerase chain reactions (RT-PCR), as previously described (Payne et al., 2019). The absolute quantification of DWV genome equivalents within the inoculate was measured via quantitative RT-PCR (qRT-PCR) utilizing SYBR Green (Thermo Fisher Scientific, Waltham, MA) and the standard curve method (Gisder et al., 2009). To carry out both PCR steps, RNA was extracted from 50 µL of the filtered DWV inoculate using RiboZol™ (VWR International, Radnor, PA) following the manufacturer's protocol for biological fluids. The total RNA was then cleaned up prior to DNase treatment and cDNA synthesis using the E.Z.N.A. MicroElute RNA clean up kit following the manufacturer's desalting protocol (Omega Bio-tek, Inc., Norcross, GA). Reverse transcription was carried out using 100 ng of the cleaned-up total RNA using the iScript™ gDNA clear cDNA synthesis kit (Bio Rad Laboratories, Hercules, CA). The resulting cDNA was diluted tenfold before serving as the qPCR template for absolute quantification. The standard curve method was carried out by running the DWV inoculate against a triplicate dilution series using an externally obtained plasmid containing a DWV-specific insert (USDA's Honey Bee Laboratory in Beltsville, MD). The insert was amplified using PCR and diluted to cover a range of 10^{-1} to 10^{-7} molecules/µL to create our standard curve.

Newly emerged adult workers (approximately 24 h old) were placed in a container on ice to immobilize them prior to the injection treatment. Injections were performed with a Gilmont micrometer syringe (Cole-Parmer, Item # EW-07840-00) using a 30G x ½ in. needle (BD™, Item #305106). Bees in the experimental group were

injected with 1 µL of the DWV inoculate suspended in phosphate buffered saline (PBS), which contained approximately 1 x 10^6 viral RNA copies per µL, as determined through qPCR. Bees in the negative control PBS group were injected with 1 µL of the same PBS that was used in creating the DWV inoculum. Bees in the negative control non-injected (NI) group were not subjected to an injection process whatsoever. All injected bees were injected between the 4^{th} and 5^{th} segments on the left side of their abdomen (to mimic *Varroa* feeding). Only bees that were confirmed as having received the full injection internally were used in the experiment and placed within their respective cage (n = 25 bees/cage). Bees were separated by treatment group within the same incubator and remained in the same cage throughout the experiment.

4.3.4 Measurements of diet consumption, survivorship, and HPG size

Similar to the *Nosema* study described in Chapter 3 of this dissertation, caged bees received their assigned experimental diet during the first six days of the experiment. The diet was administered using plastic queen-rearing cups that were weighed and exchanged daily to determine the cumulative amount of diet consumed. The average per-capita diet consumption (mg/bee) was calculated by taking the total amount of diet consumed per cage each day and dividing it over the number of bees that were still alive at a given time point. The cumulative consumption/bee for each cage was then calculated by adding the daily values together. These cage-based cumulative per-capita consumption values were then averaged by treatment group. Individual mortality was measured daily, upon which dead bees were removed from each cage. Survivorship per cage and across treatment

groups was calculated daily, and all surviving bees on the final day of the experiment were collected and stored at -80ºC for future analyses. All cages (n=73), including the extra cage for DWV 20P:30L, were used for these analyses. We removed two live bees from each cage on day 8 post-infection to measure average hypopharyngeal gland (HPG) acinus size. The size of this gland is commonly used as a proxy to assess the nutritional status of nurse-aged bees (Omar et al., 2017), as described in Chapter 3 of this dissertation.

4.3.5 RNA extraction, library construction, and RNA-seq

A subset of DWV-infected and PBS-injected bees belonging to each of the four diet treatment groups (n = 8 treatment groups) were selected for RNA sequencing analysis: PBS-injected bees that were fed either the 40P:10L, 30P:20L, or 20P:30L diet, or no diet, and DWV-injected bees that were fed either the 40P:10L, 30P:20L, 20P:30L diet, or no diet. We had a total of five biological replicates per treatment group (n = 40 cages). The bees used for RNA extraction had been collected on the final day of the experiment and stored at -80ºC prior to sample preparation. Each of the 40 RNA samples consisted of pooled total RNA that had been extracted from ten whole-bodied bees belonging to their respective cage treatment.

Samples were prepared for sequencing by homogenizing ten randomly-selected bees per cage using liquid nitrogen, a mortar, and a pestle. RNA was extracted from each sample using RiboZol™ (ThermoFisher, Waltham, Massachusetts, U.S.A.) using the manufacturer's recommendations. Approximately 85 mg of homogenized tissue was

used in triplicate for each sample. The resulting extracted total RNA (final volume = 25 µL/sample) was combined amongst the triplicate extractions that was performed for each sample before undergoing RNA clean up using the E.Z.N.A. MicroElute RNA clean up kit following the manufacturer's protocol (Omega Bio-tek, Inc., Norcross, GA). Nucleic acid concentration and quality was measured on a NanoPhotometer NP80 (Implen, Munich, Germany) before being stored at −80°C and shipped out on dry ice to be sequenced by NovoGene CO (Beijing, China).

Each RNA sample passed standard quality control (QC) tests prior to sequencing. The mRNA within the total RNA samples was purified and fragmented using poly-T oligo-attached magnetic beads. Because we wanted to create a directional library, second strand cDNA synthesis was carried out using dUTP. The subsequent steps for directional library construction included end repair and A-tailing, adapter ligation, size selection, USER enzyme digestion to remove UTP-containing second strand cDNA, PCR amplification, and purification. The libraries were checked using a Qubit and RT-PCR for quantification and size distribution detection. The libraries were then pooled and sequenced on a Ilumina platform (NovaSeq 6000) using a 150 bp paired-end sequencing strategy.

4.3.6 Statistical analysis

The cumulative per-capita consumption of each artificial diet (mg/bee) by nurse bees was compared with a two-way ANOVA test, with the infection and diet group as independent variables, followed by pairwise Tukey HSD tests. Daily bee survivorship

over the 16-day experimental period was compared between treatment groups with multiple Cox mixed effect models that used the injection and diet groups as the fixed variable, with cage identification number as a random effect. Risk ratios were calculated using Cox-proportional hazard tests based on likelihood of bees in a given treatment group dying by dividing the risk of death in one treatment group over the risk of death in another. HPG size was compared between infection and diet group treatments using an ANOVA test followed by Tukey HSD tests. The level of significance for all tests was set at $\alpha = 0.05$.

For the RNA-seq data, the resulting raw reads were filtered by removing reads containing adapters, reads containing $n > 10\%$ (where n represented the number of bases that could not be determined), and low-quality reads. For QC, the base error rate of whole sequencing was = 0.03 for all samples. The clean reads were then mapped to the *Apis mellifera* genome (Amel_HAv3.1; Assembly accession number: GCF_003254395.2) using HISAT2 software. Differential gene expression was estimated using FPKM (expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced), which took into consideration the effects of both sequencing depth and gene length on the counting of fragments. Differentially expressed gene (DEG) analysis involved the normalization of the read counts, a model dependent *p*-value estimation, and a false discovery rate (FDR) value estimation. DEGs were then grouped based on function using gene ontology.

4.4 Results

4.4.1 Comparative cumulative, per-capita consumption for each artificial diet

When considering diet type alone irrespective of infection group, the protein-to-lipid (P:L) ratio within the artificial diets (i.e., 40P:10L, 30P:20L, and 20P:30L) had a significant effect on the amount of food consumed by nurse honey bees ($F_{2,46} = 47.75$, $p < 0.0001$; Figure 4.1). Bees consumed significantly larger quantities of the 30P:20L diet (30.69 mg/bee) over the six-day experimental period compared to the protein-rich 40P:10L diet (24.3 mg/bee) and the lipid-rich 20P:30L diet (9.84 mg/bee). When considering infection group alone, non-infected bees displayed a slightly higher mean diet consumption (22.64 mg/bee) compared to either PBS-injected (20.91 mg/bee) or DWV-infected bees (21.21 mg/bee) bees. However, there was no significant effect of infection status alone on diet consumption ($F_{2,46}= 0.35$, $p = 0.71$). Likewise, there was no significant interactive effect between infection group and diet type on the amount of food consumed by bees across treatment groups ($F_{4,46} = 1.41$, $p= 0.25$). Bees belonging to the NI 30P:20L diet had the highest diet consumption overall (35.66 mg/bee), followed by the PBS 30P:20L group (29.46 mg/bee). The infection, diet pairing with the lowest mean consumption were bees belonging to the NI 20P:30L group (8.48 mg/bee).

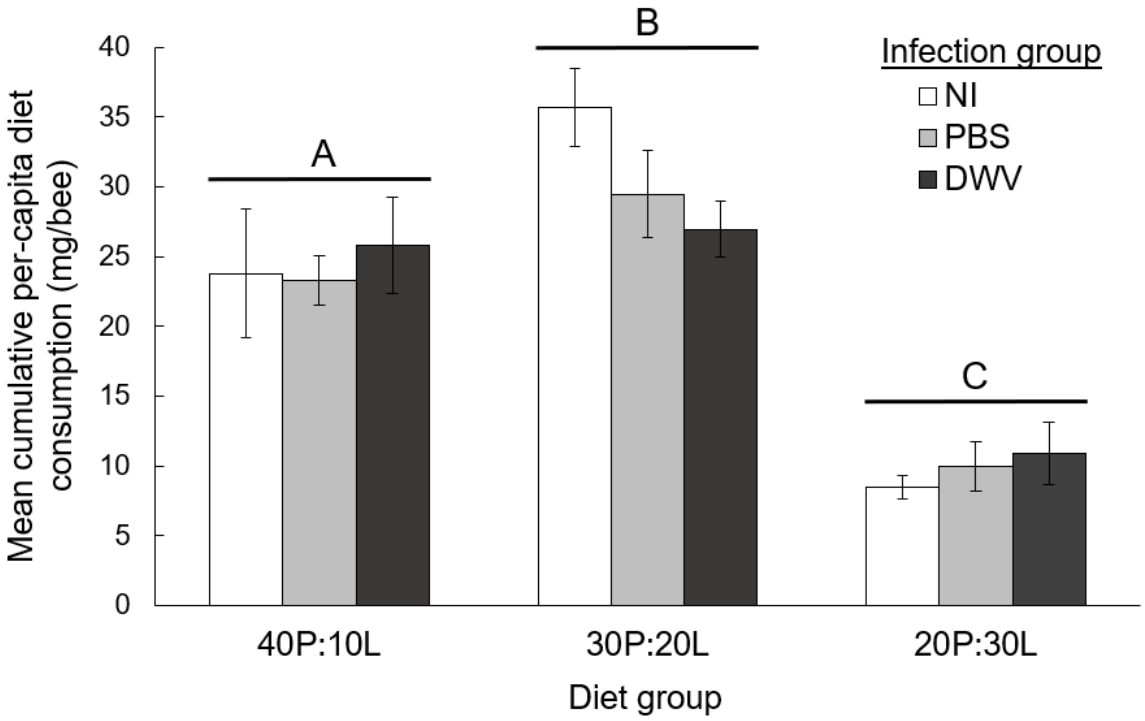

Figure 4.1: Effect of DWV-infection on the consumption of artificial diets that vary in their macronutrient ratio of protein to lipid. The mean amount of diet cumulatively consumed over a six-day period by each bee (mg/bee) was measured as a function of both diet type and infection group, with each caged bee belonging to a single diet and infection group over the entirety of the study. All bees within a respective cage (n = 25 bees/cage) were either non-injected (NI), mock-infected and injected with phosphate buffered saline (PBS) or injected with 1 x 10^6 viral RNA copies of deformed wing virus/µL (DWV). Diets varied in their macronutrient protein-to-lipid (P:L) ratio and included a protein-rich diet (40P:10L), a lipid-rich diet (20P:30L), and a more balanced diet of 30P:20L. The bars are clustered based on the diet group, shown on the x-axis, and each bar's color corresponds to the infection group (NI= white, PBS=gray, DWV=black). Letters above the clustered graphs depict statistically significant

differences between diet groups, where bars that do not share the same letter are significantly different from one another. There were no significant effects of solely infection group or the interactive effect between infection group and diet type on amount of diet consumed. Error bars represent the SEM and letters show statistical significance at $\alpha = 0.05$.

4.4.2 Survivorship based on infection and diet groups

We found a significant effect of infection group (d.f. = 2, $\chi^2 = 10.08$, $p = 0.0065$), diet group (d.f. = 3, $\chi^2 = 11.82$, $p = 0.008$), and the interaction between infection and diet group (d.f. = 6, $\chi^2 = 57.81$, $p < 0.0001$) on bee survival at the conclusion of the 16-day experimental period for both trials (Figures 4.2 and 4.3). Irrespective of the diet group, bees infected with DWV had a significantly higher risk of death than both NI bees (R.R. = 1.73, 95% C.I. = 1.21 - 2.46, $p = 0.0024$) and PBS-injected bees (RR= 1.54, 95% CI 1.10-2.14, $p= 0.011$; Appendix C, Figure 1). The average proportion of live bees at the conclusion of the experiment was 76% for all DWV-infected bees and 88% for both PBS-injected and non-injected (NI) bees (Figure 4.2, Panel A). We found no significant difference in risk of death between PBS-injected and NI bees (R.R. = 1.12, 95% C.I. = 0.78 – 1.61, $p = 0.53$).

Conversely, when only considering diet group as a factor, irrespective of infection status, there was a higher proportion of live bees by the end of the experiment (87%) when bees were fed the 30P:20L diet or the no diet negative control (90%) compared to bees that were fed the 40P:10L diet (79%) or the 20P:30L diet (80%)

(Figure 4.2, Panel B). Bees that were fed the 40P:10L diet had a significantly higher comparative risk of death when compared to bees that were either fed the 30P:20L diet (R.R. = 1.64, 95% C.I. = 1.12 - 2.40, $p = 0.012$) or fed no diet at all (R.R. = 1.87, 95% C.I. = 1.26 - 2.77, $p = 0.0019$). This resulted in a significantly lower proportion of live bees at the conclusion of the experiment when they were fed the 40P:10L diet compared to either bees fed the 30P:20L diet or given no diet (d.f. = 3, $\chi^2 = 19.16$, $p = 0.0003$). All other diet group comparisons, including 40P:10L vs 20P:30L (R.R. = 1.31, 95% C.I. = 0.91 - 1.89, $p = 0.14$), were not significant from one another when comparing risk of death and related survivorship.

When we calculated the interactive effect of infection and diet group on the proportion of live bees over time (Figure 4.3), along with the risk of death between all treatment group pairs (Appendix C, Figure 1), we found that DWV-infected bees had a significantly lower risk of death when fed the 30P:20L diet compared to DWV-infected bees assigned to any of the other three diet groups (d.f. = 3, $\chi^2 = 59.32$, $p < 0.0001$). Caged bees belonging to the DWV-infected group that were fed the 20P:30L diet had the overall highest risk of death and lowest proportion of live bees 16 days post infection (with a 7.48x higher likelihood of death and 61% survivorship) when compared against caged bees belonging to the DWV-injected group that was fed the 30P:20L diet, which had an average 94% survivorship rate (R.R. = 7.48, 95% C.I. = 3.57 – 15.64, $p < 0.0001$). Bees belonging to the DWV 40P:10L group had the second overall highest high risk of death and lowest proportion of live bees (a 6.96x higher likelihood of death and 65% survivorship) when also compared against the DWV 30P:20L group (R.R. = 6.96,

95% C.I. = 3.30 – 14.71, $p < 0.0001$). Similar to the DWV-infected bees, we found that survivorship and risk of death of non-infected bees was significantly affected by diet group (d.f. = 3, $\chi^2 = 19.31$, $p = 0.0002$). However, bees in the NI group had a significantly lower risk of death when fed the 20P:30L diet, or when they were fed no diet at all, compared to bees that were fed either the 40P:10L diet or the 30P:20L diet (Appendix C, Figure 1). In fact, the 30P:20L diet resulted in the lowest survivorship and highest risk of death for NI bees (79% survivorship, 3.83x likelihood) compared against bees in the no diet control group (95% survivorship, R.R. = 3.83, 95% C.I. = 1.75 – 8.38, $p = 0.0008$) and 20P:30L diet group (93% survivorship, R.R. = 3.03, 95% C.I. = 1.48 – 6.22, $p = 0.0025$). The NI infection group had non-significant risks of death for the comparisons of 30P:20L diet compared to the 40P:10L diet (R.R. = 1.32, 95% C.I. = 0.76 – 2.30, $p = 0.33$), or when they were fed the 20P:30L diet compared to when they were fed no diet at all (R.R. = 1.26, 95% C.I. = 0.50 – 3.20, $p = 0.62$). Finally, we found no interactive effect between any of the infection and diet group pairings for the PBS-injected bees (d.f. = 3, $\chi^2 = 0.49$, $p = 0.92$).

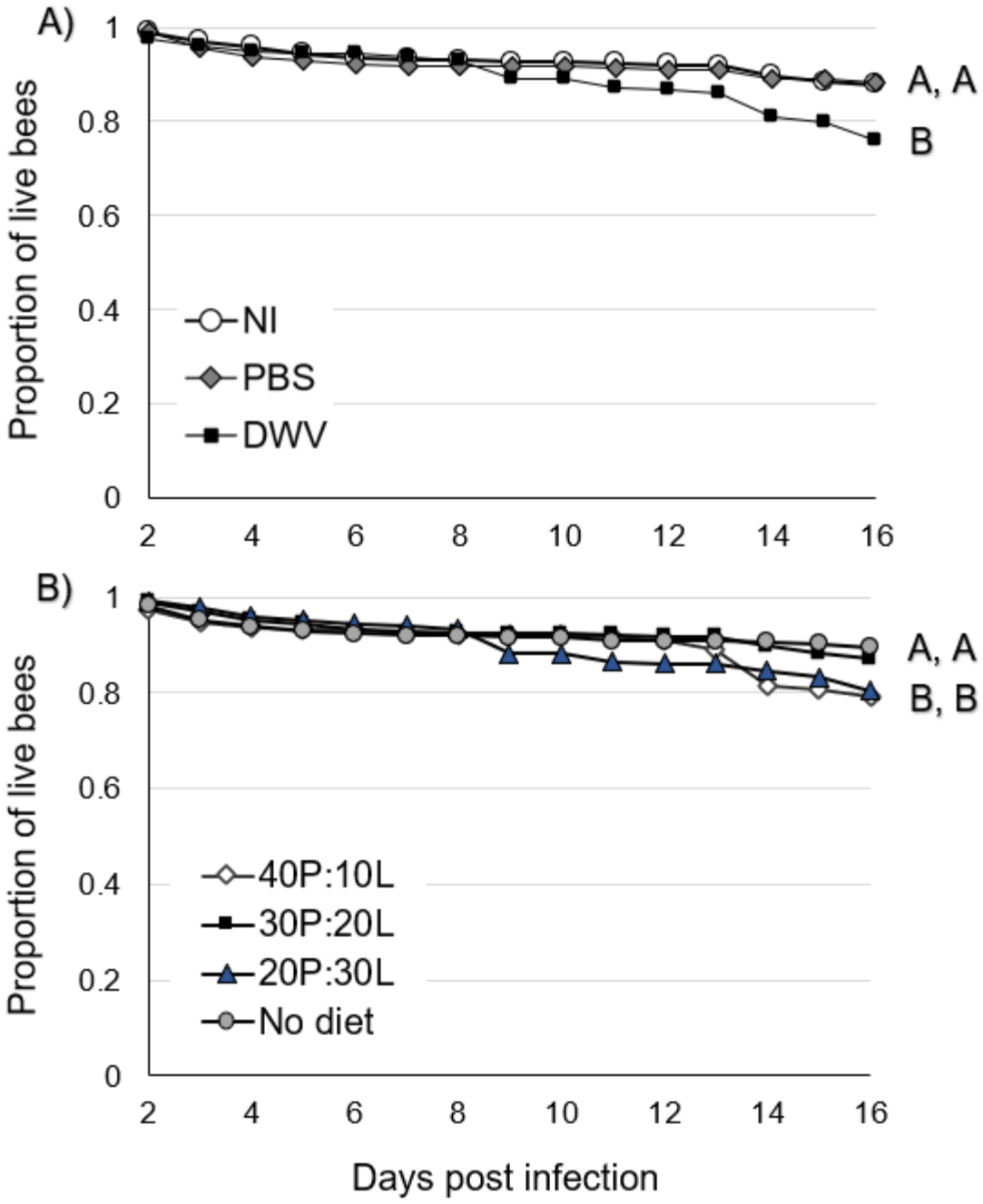

Figure 4.2: The individual effects of infection group and diet group on honey bee survivorship over time. Average percent survivorship of caged bees across two trials when segregated by A) infection group, and B) by diet group. Survivorship was measured as the average proportion of live individuals for all cages belonging to the

same treatment group. In panel A, non-injected (NI) honey bees are depicted by white circle markers, mock-infected honey bees injected with phosphate buffered saline (PBS) by gray diamonds, and DWV-infected bees by black squares. Panel B shows the proportion of live bees over time for each diet group including the 40P:10L diet (white diamonds), the 30P:20L diet (black squares), the 20P:30L diet (blue triangles), and a no diet negative control (gray circles). For both panels, the letters on the right of the figures denote significance between treatment groups ($p < 0.05$) on day 16 post-infection, and lines ending in different letters show significant differences between treatments. Bee survivorship for day 1 post-infection was not included in the analysis, as mortality on that day was linked to the injection/set-up process and not the treatment. Live bees that were purposefully removed from cages on day 7 post-infection to measure hypopharyngeal gland size were not considered in the survivorship analysis.

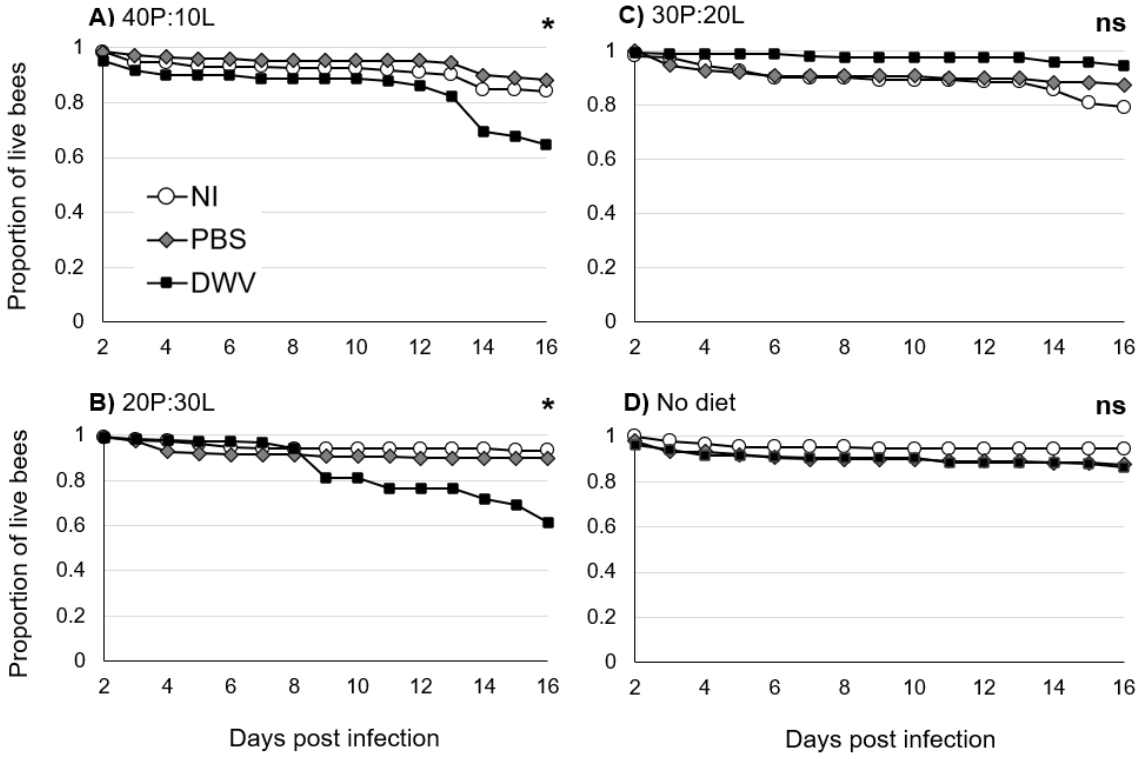

Figure 4.3: The interactive effect of infection and diet group on honey bee survivorship over time.. Comparative survivorship curves of non-injected bees (NI), mock-infected bees injected with phosphate buffered saline (PBS), and DWV-infected bees (DWV) for each diet group (12 treatments; ~150 bees/treatment group). Each panel represents the interaction effect of the infection group with a particular diet: A) 40P:10L, B) 20P:30L, C) 30P:20L, and D) no diet. Survivorship was measured as the average proportion of live bees per cage for all cages belonging to the same treatment group for both trials. For all four panels, NI bees are represented by white circle markers, PBS-injected bees by gray diamonds, and DWV-infected bees by black squares. The interactive effect of infection status and diet group for each panel is marked above with an asterisk if significant ($\alpha = 0.05$) or with the letters "ns" if not significant.

Survivorship for day 1 post-infection was not included, and live bees that were purposefully removed from cages for other tests were excluded from the survivorship analyses.

4.4.3 Hypopharyngeal gland size across treatment groups

The average HPG acinus size of nurse bees was significantly impacted by diet type ($F_{3,132} = 6.75$, $p = 0.0003$), with higher protein content within a diet generally correlating with larger average gland size. Bees that were fed the protein-rich 40P:10L diet had the largest average acinus size (10,978.5 μm^2), while bees that were fed the intermediate 30P:20L and the lipid-biased 20P:30L diet had an average acinus size of 9,112.2 μm^2 and 6,973.5 μm^2, respectively. Bees that only received sucrose as a food source had an average acinus size of 7,207.7 μm^2, which was numerically larger (but not significantly so) than the acinus size of bees that were fed the high-lipid, low-protein 20P:30L diet (6,973.5 μm^2). We did not find a significant effect of either infection status alone ($F_{2,132} = 1.97$, $p = 0.1441$) or an interactive effect between infection status and diet group ($F_{6,132} = 0.99$, $p = 0.44$) on average HPG size.

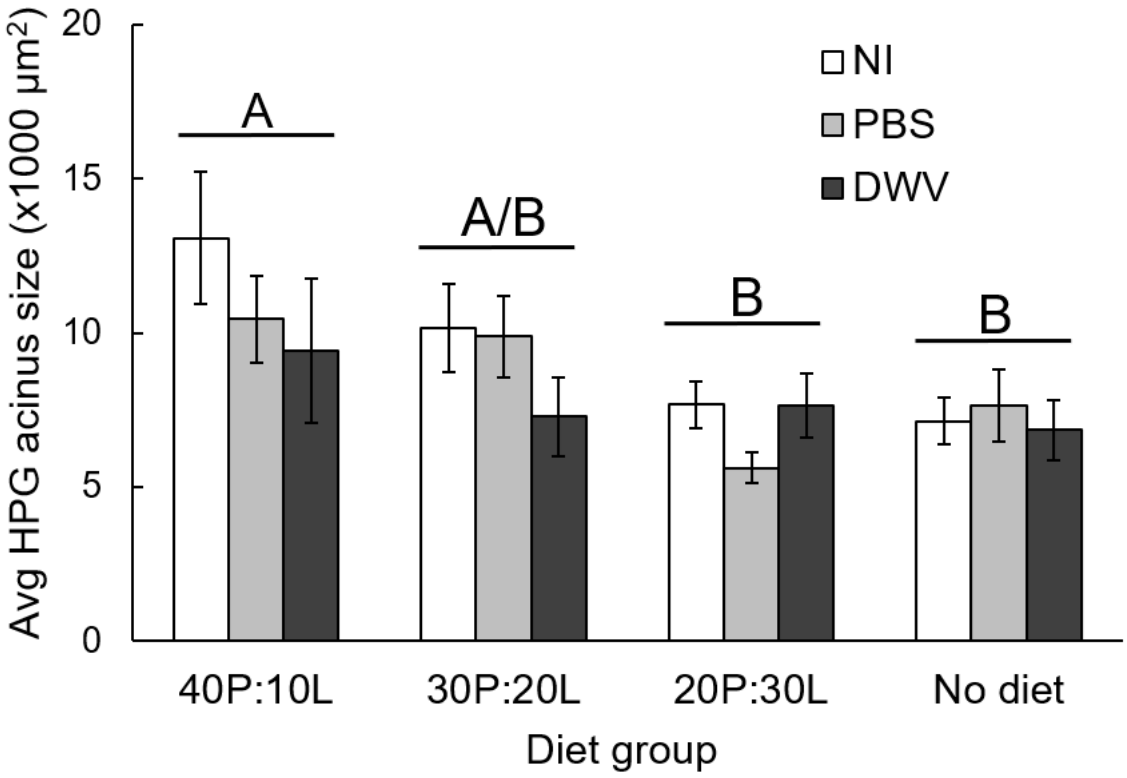

Figure 4.4: Average hypopharyngeal gland (HPG) acinus size of non-infected and DWV-infected honey bee nurses fed artificial diets varying in their macronutrient ratios. Two bees per cage (n = 146) were removed eight days post-infection to measure HPG size. Both HPGs from each bee were dissected, the size of five randomly selected acini per gland (n = 10) was measured, and the average acinus size per treatment group was calculated (μm^2). The bars represent the average HPG acinus size for each infection and diet group pairing. Bars are clustered based on the diet group (shown on the x axis), which varied in their ratio of protein-to-lipids (P:L). The no diet group was a negative control in which bees were not given a protein or lipid source and only fed sucrose. Bar colors correspond to the infection group: non-injected (NI) bees in white, mock-infected bees injected with PBS (PBS) in gray, and the DWV-infected bees (DWV) in black.

Letters above the clustered graphs show the significant effect that diet group had on the average HPG acinus size (mean + SEM). Bars that do not share the same letter are significantly different from one another. There were neither a significant effects of infection group alone nor an interactive effect between infection and diet groups on HPG size. Error bars represent SEM and letters show significance at $\alpha = 0.05$.

4.4.4 RNA sequencing of DWV-infected and PBS-injected bees

Purified samples of total RNA from pooled extractions had an average concentration of 1,020.6 ± 264.7 ng/μL. For sequencing purposes, aliquots of these samples were diluted into RNase-free water at a final volume of 25 μL, with average yields of 326.6 ± 84.7 ng/μL. cDNA libraries were generated for each of eight treatment groups: PBS-injected bees that were given either the 40P:10L diet, 30P:20L diet, 20P:30L diet, or no diet at all, and DWV-infected bees that were given either the 40P:10L diet, 30P:20L diet, 20P:30L diet, or no diet at all. A total of 40 cDNA libraries were created, consisting of five biological replicates per treatment group. After sequencing, each of the 40 libraries had over 40 million clean reads (over 98% of the total reads for all samples) and over 6 Gb of clean bases. The base error rate was 0.03 for all samples, and the Q20 percentage of clean reads was >95%.

After mapping the filtered reads to the *A. mellifera* genome to evaluate differential gene expression, we found that 10,260 genes were expressed across all libraries. The highest differentially expressed gene (DEG) count was for the comparison between all DWV-infected bees against all PBS-injected bees, regardless of the diet

group. A total of 1,981 genes were differentially expressed in DWV-injected bees compared to PBS-injected bees, with an almost identical number of genes being up-regulated (989) and down-regulated (992) (Figure 3.5). Four additional DEG comparisons were made between the DWV and PBS groups based on the four different diet groups. The DWV 40P:10L group had 648 DEGs compared to the PBS 40P:10L group. The same DEG analysis of DWV vs PBS was performed for the other three diet groups: 30P:20L (847 genes), 20P:30L (879), and no diet (767). These comparisons revealed that all the DWV-infected groups had a higher number of DEGs compared to their PBS-injected control, regardless of diet group. For the above comparisons involving the 40P:10L and no diet groups, a larger number of genes were upregulated vs down. The opposite was true for the 30P:20L and 20P:30L groups which had a higher number of genes that were downregulated. The impact of infection status on gene expression is further emphasized by the hierarchical clustering map shown in Figure 3.6.

Compared to infection status, the effect of diet group on gene expression differences between treatments was less pronounced (Appendix C, Table 1). We performed six comparisons that measured the gene count for DEGs based on diet group. For example, the second overall highest DEG count (1,388 genes) was for the comparison between PBS-injected bees that were fed the 40P:10L diet and PBS-injected bees that were fed no diet at all. Comparatively, DWV-infected bees that were fed the 40P:10L diet compared to those that were fed no diet only had 672 DEGs. The lowest number of DEGs overall occurred when PBS 20P:30L was compared against PBS no diet (425 genes), followed by DWV 20P:30L vs DWV no diet (235 genes). Of these six

comparisons that were based on diet group, five resulted in a higher number of up-regulated genes compared to down-regulated genes. The only comparison that resulted in a higher number of down-regulated genes was that for DWV-injected bees that were fed the 20P:30L diet compared to those that were fed no diet.

We also performed mapped read counts for specific honey bee viruses of interest to determine their prevalence using the raw read sequences for each library (Appendix C, Table 3). We used previously published, virus-specific primers to determine the mapped read count for eight honey bee-associated viruses (Appendix C, Table 2). This included three variants of DWV (DWV-A, B, C) and five other relatively common honey bee-associated viruses (including black queen cell virus-BQCV, acute bee paralysis virus-ABPV, Israeli acute paralysis virus-IAPV, Kashmir bee virus- KBV, chronic bee paralysis virus-CBPV, sacbrood virus-SBV, and Lake Sanai virus-LSV). We found that all 20 of the DWV-infected libraries contained sequences that were specific for both the DWV-A strain and the DWV-B strain. We did not detect the DWV-C strain in any of the libraries. Likewise, we did not detect any of the non-DWV viruses in any library. Aside from one outlier sample (PBS 30P:20L cage 1), none of the PBS-injected libraries had any mapped reads that corresponded to any of the viruses tested. The PBS sample that tested positive for a virus did so for the DWV-B strain. We further performed a mapped read count using a primer that is specific to the negative-sense strand of DWV, which would indicate that viral replication of the virus occurred at one point within our biological samples. We confirmed relatively high mapped read counts of the stand- specific DWV sequence within all twenty DWV sample. There was no

instance of this sequence in any of the PBS-injected samples, indicating that the single PBS-injected sample that had mapped reads for DWV-B may have been an artifact.

Enrichment analyses using gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to determine which biological functions and pathways were significantly associated with the differentially expressed genes for each group comparison. The significantly up- and downregulated GO groups for each of the eleven comparisons are listed in Appendix C, Table 4. Based on the GO enrichment analysis results for the comparison of all the DWV groups vs all the PBS control groups, electron transfer activity (GO: 0009055) and oxidoreductase activity acting on NAD(P)H (GO:0016651), both in the molecular function category, were the only groups found to be significantly upregulated ($p$adj < 0.05). There weren't any GO groups that were significantly downregulated for the comparison of DWV to PBS, irrespective of diet group. For DWV-infected bees fed either the 40P:10L, 30P:20L, or 20P:30L diets, oxidoreductase activity (GO:0016491) was significantly upregulated when compared to DWV-infected bees in the no diet/sucrose only control group. There were GO terms that were significantly downregulated for both the comparisons of DWV 40P:10L vs DWV no diet and DWV 20P:30L vs DWV no diet, including the biological processes of cell communication (GO:0007154), signaling (GO:0023052), signal transduction (GO:0007165), and response to stimulus (GO:0050896). None of these GO terms were significantly downregulated in the control comparison groups of PBS 40P:10L, 30P:20L, and 20P:30L vs PBS no diet. For the comparison of DWV 30P:20L vs DWV no diet, there were not any significantly downregulated GO terms.

For the KEGG enrichment analysis (Appendix C, Table 5), we found that for all DWV-infected bees vs PBS-control bees, the oxidative phosphorylation (KEGGID: ame00190), spliceosome (KEGGID: ame03040) , and apoptosis- fly (KEGGID: ame04214) pathways were all significantly upregulated, while peroxisome (KEGGID: ame04146) was significantly downregulated. When comparing DWV bees vs PBS bees by diet group (i.e. DWV 40P:10L vs PBS 40P:10L), there were not any pathways that were significantly downregulated except for the comparison made between DWV and PBS bees that were given no diet. For DWV no diet vs PBS no diet, there were multiple pathways involving various lipid and fatty acid metabolism that were significantly downregulated. For DWV-infected bees fed either the 40P:10L, 30P:20L, or 20P:30L diets vs the DWV no diet control, phototransduction- fly (KEGGID: ame04745) was significantly downregulated for all three of these comparison groups. For the equivalent PBS-control comparisons, only PBS 40P:10L vs PBS no diet had significant downregulation of phototransduction- fly (KEGGID: ame04745). Of all the comparisons made, DWV 40P:10L vs DWV no diet had the highest number of significantly upregulated pathways (n=27), which included peroxisome (KEGGID: ame04146) and tyrosine metabolism (KEGGID: ame00350).

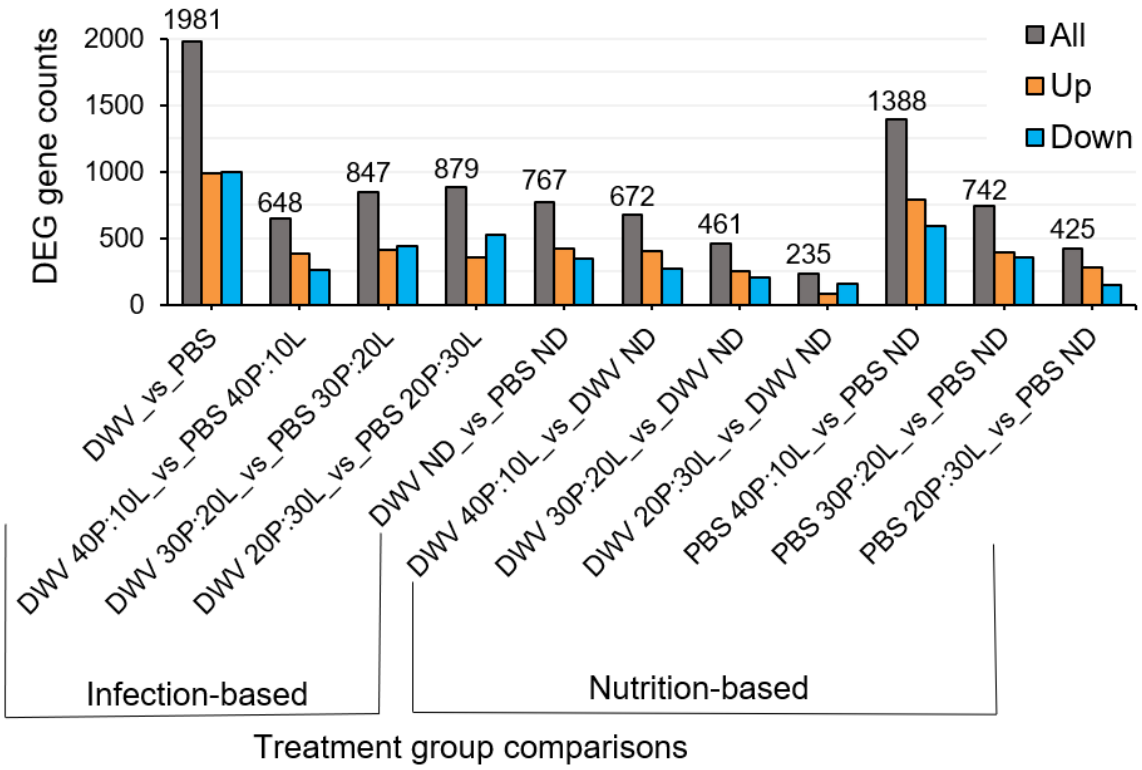

Figure 4.5: Gene counts of differentially expressed genes (DEGs) between the different infection status and diet group pairings. We had a total of eleven comparisons that were either based on infection status or diet group. Five comparisons were based on infection status and compared the number of DEGs between DWV-infected and PBS-injected bees that were fed the different diet groups. The other six comparisons were nutrition-based and compared how many genes were differentially expressed between different diet groups while belonging to the same infection group. The bars correlate to the number of DEGs for each comparison group. The gene count values correlate to the number of DEGs that were present for the first group when it was compared against the second group in the pairing (ex: DWV-infected bees vs. PBS-injected bees). The total number of DEGs for each comparison is depicted in gray and

has the total number displayed above each bar. The number of up-regulated (Up) genes is shown in orange, and the number of down-regulated genes (Down) is shown in blue.

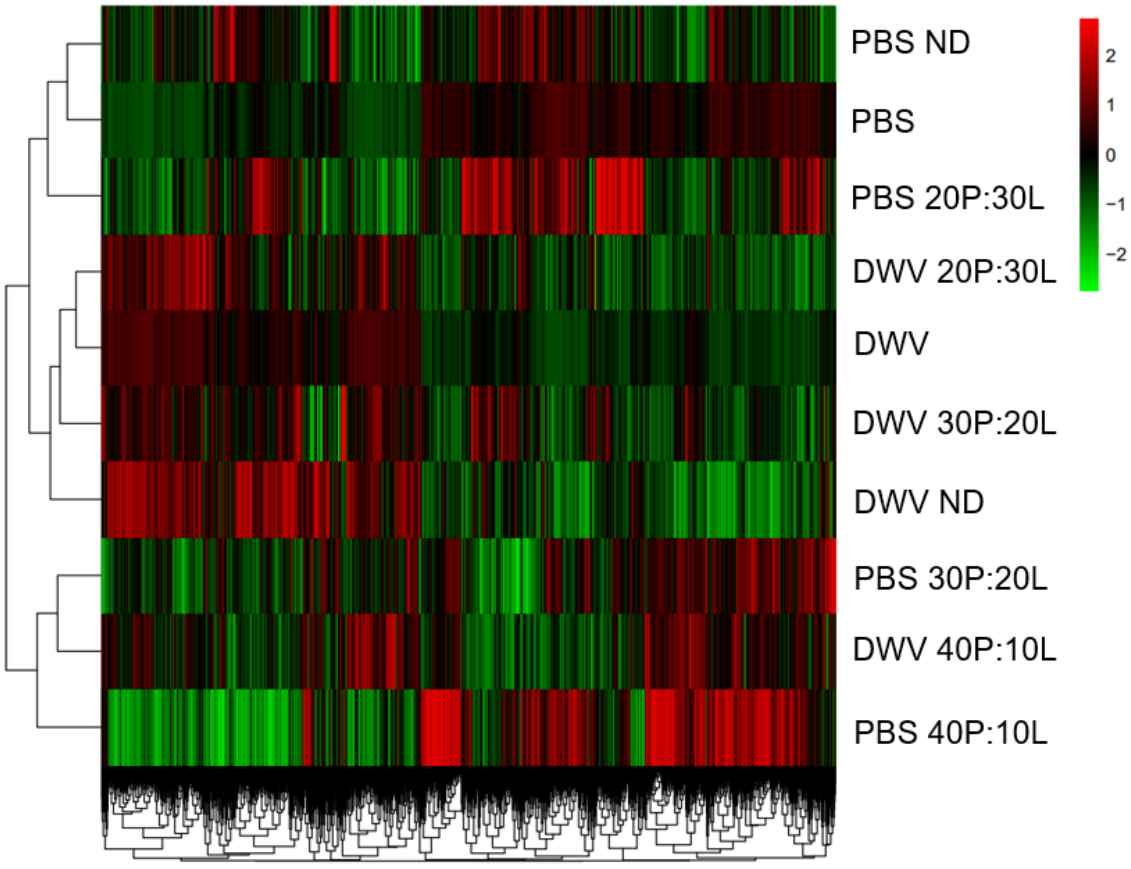

Figure 4.6: Hierarchical clustering heatmap of differential gene expression. The results of the FPKM cluster analysis were clustered using the $\log_{10}$ (FPKM+1) value. Red cells denote genes with low expression levels, while green cells denote genes with high expression levels. The color ranging from red to green indicates $\log_{10}$ (FPKM+1) values that range from large to small. Biological replicates were grouped based on infection status alone (PBS-injected bees vs. DWV-infected bees; n = 20 each), or by the multivariate treatment groups involving infection status and diet group (PBS-injected

bees that were fed either the 40P:10L, 30P:20L, or 20:30L diet, or no diet at all, as well as DWV-infected bees that were fed either the 40P:10L, 30P:20L, or 20P:30L diet, or no diet at all; n = 5).

4.5 Discussion

In this study, we determined how the protein-to-lipid (P:L) macronutrient composition of a honey bee's diet impacts its tolerance to infection with deformed wing virus (DWV). To our knowledge, this is the first study to single out how P:L ratios within diets impact viral infection within honey bees. Previous studies that have looked at the impacts of nutrition on host-pathogen interactions of honey bees and associated viruses have oftentimes used pollen as their nutritional source. However, flowering plants vary greatly in the nutritional quality of their pollen relative to honey bee fitness (Schmidt et al., 1987). Depending on plant taxon, a pollen grain's total macronutrient composition can range from having as little as 2.5% to as much as 61% protein, and as low as 2% to as high as 20% lipid, (Roulston et al. 2000) and can vary greatly on any number of other nutritional components including a diverse composition of sugars, fibers, minerals, amino acids, waxes, etc. (Thakur & Nanda, 2020). Oftentimes, this leads to comparisons being made between pollen types or pollen substitutes that are broadly classified as either "high" or "low" quality, in which quality can refer to any number of nutritional components (Schmidt, 1984; Schmidt 1987; Alaux et al., 2010). For instance, a study done by Dolezal et al. (2019) found evidence that honey bees fed a high quality, single-source pollen are able to mitigate IAPV-induced mortality to the same degree as when

fed a diverse, polyfloral pollen diet. While they postulated that this may be attributed to micronutrient availability, the authors pointed out that the different pollen types used in their study varied in regard in other aspects of nutritional quality besides micronutrient content such as protein levels and amino acid content, making it difficult to pinpoint the exact mechanism by which high quality diets increases viral tolerance in honey bees.

To better answer by which mechanism nutrition impacts viral infection in honey bees and what nutritional component(s) determines a diet's quality in relation to pathogen tolerance, we chose to use artificial diets, as opposed to natural pollen, in which we could manipulate one select aspect of the nutrient formulation while maintaining equal amounts of all other diet components. We created diets in which we manipulated the ratio of proteins-to-lipids (P:L) to more precisely define how variation of select nutritional components in a honey bee's diet impact their tolerance to DWV infection. We conducted a cage study that included non-injected (NI), mock-infected (PBS), and DWV-infected honey bees fed one of four diet groups: a high protein, low lipid diet (40P:10L), a low protein, high lipid diet (20P:30L), an intermediate diet ratio at which non-infected honey bee colonies self-selected for in a separate study (30P:20L), and a sucrose-only (no diet) negative control (Lau et al., in prep).

Our results determined that the intermediate diet (30P:20L) had the highest cumulative consumption per bee. This is the same result as what was observed in a previous study by our research group and in the study described in Chapter 3 of this dissertation. In all three of these studies, nurse bees were found to regulate their consumption to reach an P:L intake ratio of 30P:20L (Lau et al., in prep). Consumption

of this diet was higher than the 40P:10L and 20P:30L diets, regardless of infection status or the interactive effect of infection and diet group. These results indicate that nurse bees will consistently regulate their diet to reach a P:L intake target of 30 parts protein to 20 parts lipid, and they do not shift their intake towards a more protein- or lipid-rich diet when infected with DWV. However, based on our results, it is not definitively clear if 30P:20L is in fact a "golden" ratio at which nurse bees will regulate their diet to regardless of pathogen-induced stress, or if the 40P:10L and 20P:30L diets were simply unpalatable for bees due to their imbalance of these macronutrients. Overall, there were very low levels of consumption for the 20P:30L diet for all three infection groups. However, although the 30P:20L diet was consumed significantly more than the 40P:10L diet when all infection treatments were grouped together and for both the non-injected and PBS control groups individually, for DWV-infected bees, the amount of 30P:20L and 40P:10L diet consumed was comparable and not significantly different. To better determine if 30P:20L is in fact a "golden" ratio at which nurse bee consistently regulate to or if palatability of the more skewed diets was a significant factor for observed differences in consumption, future studies should implement nutrition-based choice tests to better define honey bee diet preference for a particular P:L ratio when under pathogen-induced stress. Although previous studies have consistently found forager bees to regulate their collection of pollen based on the nutritional needs of the colony (Hendriksma & Shafir, 2016; Zarchin et al., 2017), the literature is not in agreement on whether nurse bees exhibit regulation based on a diet's nutritional quality. Corby-Harris et al. (2018) determined that nurse bees do not exhibit diet regulation based on

nutritional quality, while Stabler et al., 2021 found that nurse bees regulate their intake to achieve a P:L ratio of 1.25:1 (similar to our 30P:20L ratio). Given that all three of our infection groups had the highest consumption of the 30P:20L diet, our study supports the idea that nurse bees regulate their diet to reach a particular P:L intake target.

We compared survivorship at the conclusion of the 16-day study and observed that the highest proportion of live bees occurred in cages that were 1) either non-injected or injected with PBS compared to being infected with DWV, and 2) when fed either the 40P:10L or 30P:20L diet groups compared to the 20P:30L diet and sucrose- only (no diet) group. For the DWV-infected groups specifically, bees had the highest percent survivorship when fed the 30P:20L diet or the sucrose only diet. In comparison, bees infected with DWV and fed either the high-protein 40P:10L or high-lipid 20P:30L diets had significantly lower survivorship compared to non-infected controls fed these same diets. As previously mentioned, DWV-infected bees consumed comparable amounts of the 40P:10L and 30P:20L diets, indicating that diet nutritional quality, and not quantity of diet consumed, more so determined observed differences in survivorship. In the study performed by Dolezal et al. (2019), they found that bees starved of a source of protein, lipid, and nutrients had the highest mortality when infected with IAPV compared to bees that were given any form of pollen diet. This is contradictory to our results, as bees devoid of a diet and infected with DWV had comparably high survivorship to their non-infected and PBS controls, and this DWV no diet group had higher survivorship than bees belonging to either the DWV 40P:10L and DWV 20P:30L groups. These results indicate that pathogen species can have differential interactions with host nutritional

status and that consuming a diet with a balanced P:L ratio such as 30P:20L can offset some of the negative effects of DWV infection, particularly when compared to DWV-infected bees that are fed imbalanced diets that are skewed towards being more protein- or lipid-rich.

Within a honey bee colony, young nurse bees are the primary consumers of pollen as they are the ones responsible for producing the proteinaceous glandular secretions necessary for brood feeding and development (Haydak, 1970; Herbert et al., 1977). Previous studies have used the size of hypopharyngeal glands (HPG) in nurse bees as a physiological measure of an individual's nutritional health and, as an extension, an indicator of a diet's quality for nurse bee development (Di Pasquale et al., 2013; Corby-Harris & Snyder, 2018; Corby-Harris et al., 2018). When comparing the average acinus size of hypopharyngeal glands dissected out of bees 7 d.p.i. amongst all treatment groups, we found that protein quantity was positively correlated with HPG size, which has been demonstrated in past studies (Pernal & Currie, 2000; Renzi et al., 2016). Overall, bees fed the high-protein 40P:10L diet had significantly larger HPG size than both the 20P:30L (of which bees consumed very little of) and no diet groups. The relatively small amount of the low-protein 20P:30L diet consumed by nurse bees resulted in comparably small HPG acini size to the bees fed a sucrose-only diet, most likely due to insufficient protein intake necessary for glandular development for both groups. DWV has been previously detected in the HPGs of honey bees (Li et al., 2019), and infection with DWV has been shown to cause hypoplasia of the HPGs in highly infected, symptomatic individuals parasitized by varroa (Koziy et al., 2019). However,

we did not find HPG acinus size to be significantly impacted by infection status or the interactive effect of infection and diet group. This may be attributed to the fact that our study experimentally infected adults as opposed to sampling bees that were infected during pupal development, or it may be a result of differences in host phenotype between overt and covert DWV infections (DeGrandi-Hoffman et al., 2010). Based on the results here and those of previous studies, it appears that the HPG size of worker bees infected with covert levels of DWV as adults are not negatively impacted by infection. Instead, it appears that factors such as diet quantity, diet quality, age of bee, and presence/absence of brood play a more significant role on HPG size in nurse bees (Pernal & Currie, 2000; Renzi et al., 2016).

We conducted RNA-seq and analyzed differential expression of genes (DEGs) of DWV-infected bees fed our different diet groups, using the PBS-injected group as a comparative control for infection status and the no diet/sucrose only group as a comparative control for diet type. The PBS group was chosen as opposed to the non-injected group in order to control for differential gene expression caused by the injection process itself. Of the eleven different comparisons between treatments made based on infection status and diet group, we found that the overall comparison of the DWV vs PBS infection groups, irrespective of diet, had the largest number of DEGs (1981 DEGS) that were conversely expressed, with a near equal number of genes that were up- (989) and down-regulated (992). This equates to approximately 20% of genes being differentially expressed between the DWV-infected and PBS control groups, as honey bees have 10,157 known genes (Honeybee Genome Sequencing Consortium, 2006).

When analyzing the DEGs using GO enrichment to determine significance based on functional groups, we found that for the comparison of all DWV groups vs all PBS groups, electron transfer activity (GO: 0009055) and oxidoreductase activity acting on NAD(P)H (GO:0016651), both in the molecular function category, were the only groups found to be significantly upregulated ($p$adj < 0.05), and there were not any GO terms that were significantly downregulated for this comparison. Oxidoreductase activity appeared to be linked to consuming diet, as this GO term was significantly upregulated in DWV-infected bees fed any one of the three artificial diets (40P:10L, 30P:20L, and 20P:30L) when compared to DWV-infected bees fed sucrose only. Using KEGG enrichment to determine significance of pathways, we determined that this same comparison of DWV vs PBS resulted in significant upregulation of the oxidative phosphorylation (KEGGID: ame00190), spliceosome (KEGGID: ame03040), and apoptosis- fly (KEGGID: ame04214) pathways and significant downregulation of the peroxisome (KEGGID: ame04146) pathway. From the results of GO and KEGG enrichment, it appears that DWV-infection has significant impacts on oxidation-related processes with bees, which may be tied to acceleration in temporal polyethism of sampled workers (Cervoni et al., 2017). Various pathogens have been shown to attribute to the acceleration of temporal polyethism (i.e. age-related biases in task performance) in honey bees and result in nurse-aged bees taking on forager-related tasks earlier in life than normal (Woyciechowski & Kozłowski 1998; Goblirsch et al., 2013; Natsopoulou et al., 2016). The bees in this study were sampled for RNA-seq analysis on the final day experiment, making them approximately 16 days old. Bees at this age typically perform

in-hive tasks such as comb building and food packing, and it is not until they reach approximately 23 days old that they begin to take on foraging activities (Seeley, 1982; Johnson, 2008). In honey bees, increased levels of oxidative phosphorylation and antioxidant-related structures/enzymes (such as peroxisomes) are typically found in older, forager bees compared to nurses as foragers require larger quantities of ATP moieties for flight muscle function and a positively correlated, increased need for removal of reactive oxygen species (ROX) (Corona & Robinson, 2006; Cervoni et al., 2017). However, bees infected with DWV in this study had a significantly upregulated oxidative phosphorylation pathway, more so associated with forager-aged bees, when compared to the PBS control bees. Also, although there was a significant upregulation of oxidative phosphorylation, there was a significant downregulation in the peroxisome pathway, contrary to what is typically observed (Cervoni et al., 2017). Bees with symptomatic DWV-infection have been shown to undergo precocious foraging, exhibit an overall reduction in adult lifespan, and have reduced learning and memory capabilities that are crucial for foraging activities (Benaets et al., 2017; Dainat et al. 2012; Genersch & Aubert, 2010). These symptoms, along with the deformed wings from which DWV gets its name, are largely neurological in nature. The cause of these symptoms may be attributed to the significant downregulation of the peroxisome pathway in DWV-infected bees, as seen here. Peroxisomes are oxidative organelles found in the cytoplasm of eukaryotic cells that are involved in breaking down long chain fatty acids, aid in the synthesis of phospholipids such as plasmalogens, and contribute to the reduction of reactive oxygen species (ROX) that are the main cause of aging in

organisms. The downregulation of peroxisomes can lead to impairment in the biosynthesis of phospholipids such as plasmalogens (which in part protect internodal myelin from oxidative damage) and can result in neurological damage or disease (Luoma et al., 2015). The DWV-infected bees did show evidence of an immune response with the significant upregulation of the apoptosis pathway. Apoptosis has been shown to be a primary defense mechanism in another social insect, the fire ant *Solenopsis invicta*, in response to infection with a viral pathogen (Hsu et al., 2019).

There were GO terms that were significantly downregulated for both the comparisons of DWV 40P:10L vs DWV no diet and DWV 20P:30L vs DWV no diet, including the biological processes of cell communication (GO:0007154), signaling (GO:0023052), signal transduction (GO:0007165), and response to stimulus (GO:0050896). However, none of these GO terms were significantly downregulated in the control comparison groups of PBS 40P:10L, 30P:20L, and 20P:30L vs PBS no diet, implying that downregulation of these GO terms was a result of DWV-infection. However, downregulation of these terms (or any form of downregulation for that matter) did not occur in DWV-infected bees fed the 30P:20L. This information, tied with our survivorship results, provides evidence that the 30P:20L ratio was able to ameliorate the effects of DWV-infection and help bees better tolerate the effects of pathogen infection.

5. CONCLUSIONS

The culmination of this research is evidence that host-pathogen interactions must account for more than just the direct effect of pathogens on their honey bee host. To better understand honey bee disease ecology and how to address pathogen infection, the interacting factors and associated stressors that can contribute to pathogen transmission and host immunity must also be considered. In these studies, we looked at how ants that act as in-hive pests and how host nutrition impact host-pathogen interaction of honey bees. In the first chapter of this dissertation, we determined that many taxa of ants were observed interacting with managed honey bee colonies and were commonly found to be carrying honey bee-associated viruses. However, we did not find evidence that any of these honey bee-associated viruses were replicating within the sampled ants. Follow up studies to this research would be determining if transmission is occurring between ants and honey bees and by what mode of transmission.

It is well known that a honey bee's tolerance and/or resistance to pathogen infection is greatly impacted by its physiological state, including its nutritional health. However, many gaps exist in our understanding of the effects of host nutrition on honey bee-pathogen interactions, including what nutrient composition of a honey bee's diet is best suited to help bees mitigate the effects of infection. Chapters 2 and 3 of this dissertation looked at how diet quality impacted adult honey bees infected with either a fungal pathogen (*Nosema ceranae*) or a viral pathogen (deformed wing virus, DWV). For both chapters, we discovered that nurse bees have the highest amount of

consumption when given the more balanced 30P:20L diet, regardless of infection status or pathogen type. For both studies, the protein-biased 40P:10L diet was the next highest consumed diet, followed by the lipid-biased 20P:30L diet which was consumed the least by a significant margin for both pathogen types. This 30P:20L ratio was the same intake target as what our lab had previously determined honey bees preferentially forage for and consume without the added implications due to difference in infection status. While infection with DWV did not appear to impact appetite, infection with *Nosema* did cause bees to consume greater quantities of this balanced diet compared to non-infected controls. Based on the results of this previous study and the results this dissertation's two pathogen-nutrition chapters, it appears that honey bee nurses do not shift their nutritional intake to be more protein- or lipid-rich when infected with either the tested fungal or viral pathogen and instead seem to continuously select for a diet that is 30 parts protein and 20 parts lipid, regardless of infection status. The survivorship of bees was significantly impacted by both diet and infection status, with significantly higher survivorship occurring in *Nosema*-infected bees when fed a more protein-rich diet (40P:10L) and in DWV-infected bees when fed a diet that was balanced in its P:L ratio (30P:20L). The size of hypopharyngeal glands in nurse bees was significantly impacted by protein content, but not infection status, for both experiments. Pathogen load was significantly impacted by consumption of a diet in *Nosema*-infected bees. Transcriptomic analysis of DWV-infected bees revealed a higher level of differential gene expression than non-infected controls. However, diet type did not appear to significantly impact the differential expression of genes. The results of both experiments

reveal that the protein and lipid composition within honey bee diets does affect the host-pathogen system involving honey bees, with differences in host response based on pathogen type. Further studies, in conjunction with those described here, can lead to the creation of an optimized supplemental diet that can be manipulated in its macronutrient content to address specific pathogen infection of honey bee colonies. During times of pollen dearth or in areas with low nutritional forage, beekeepers may need to provide their colonies with pollen supplements or substitutes to meet the nutritional needs of the colony. Further research into what nutrient components specifically define diet quality and are responsible for mitigating the effects of pathogen infection can lead to the creation of a pollen substitute that can be manipulated to better address colony stressors such as pathogen infection.

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APPENDIX A:

THE DETECTION OF HONEY BEE (*APIS MELLIFERA*)-ASSOCIATED VIRUSES IN ANTS

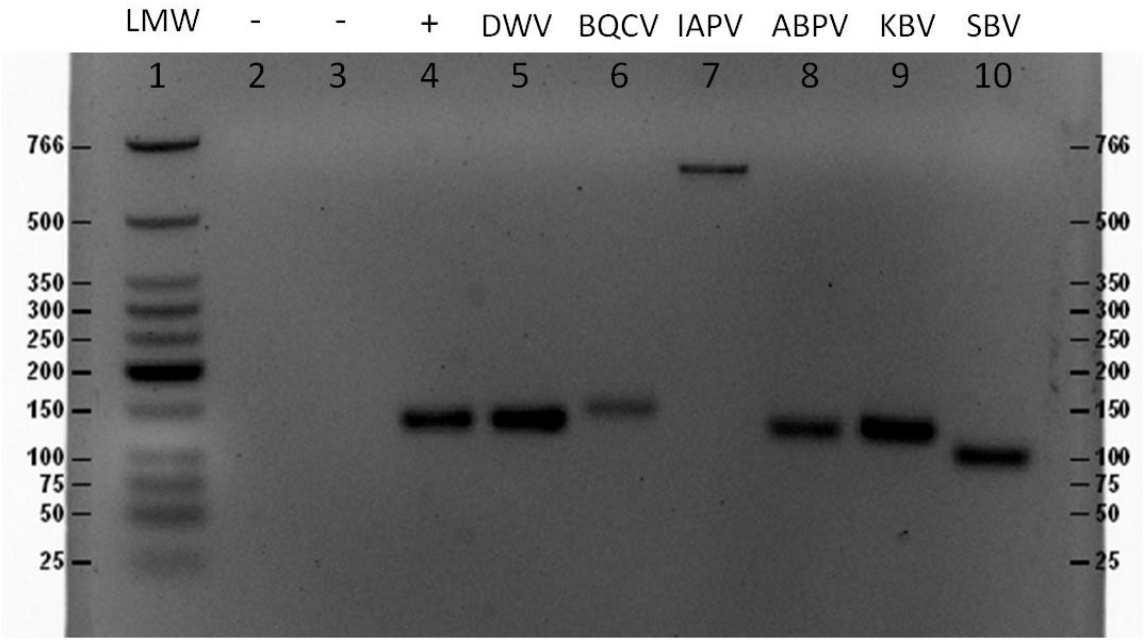

Figure 1: Positive controls used for diagnostic analyses

The bands represent cloned PCR products that were provided by the USDA-ARS Bee Research Laboratory in Beltsville, MD to serve as positive controls for our diagnostic analyses. Each control correlated to one of our six viruses of interest including Deformed wing virus (DWV; Lane 5), Black queen cell virus (BQCV; Lane 6), Israeli acute paralysis virus (IAPV; Lane 7), Acute bee paralysis virus (ABPV; Lane 8), Kashmir bee virus (KBV; Lane 9), and Sacbrood virus (SBV; Lane 10). A template-free reaction (Lane 2) and a RT-free reaction (Lane 3) served as negative controls when conducting all diagnostic RT-PCRs. Lane 4 consisted of a symptomatic honeybee

displaying the crumpled-wing phenotype of DWV and served as another positive control for this virus. All PCR products were run against a low molecular weight ladder (LMW; Lane 1) (New England Biolabs® Inc.). The amplicon sizes of each control were the following: DWV (130 bp), BQCV (140 bp), IAPV (587 bp), ABPV (124 bp), KBV (127 bp), and SBV (105 bp).

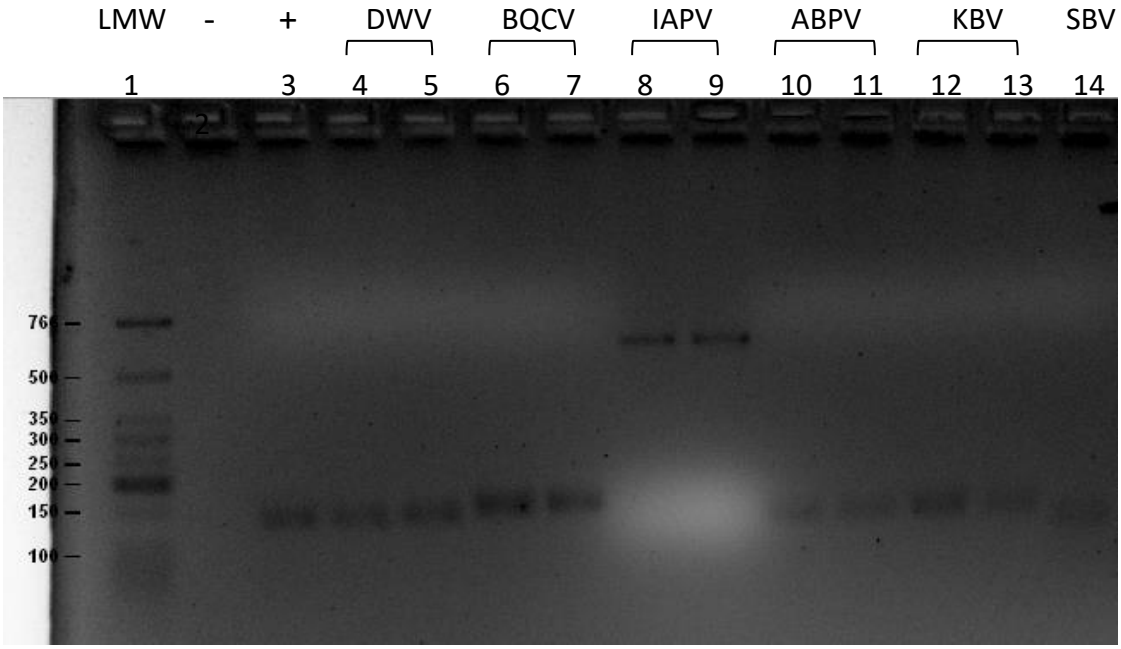

Figure 2: Subset of samples that tested positive for a virus

This included Deformed wing virus (DWV) in *Brachymyrmex* sp. (Lane 4) and in immature stages of *Crematogaster* sp. (Lane 5), Black queen cell virus (BQCV) in *Solenopsis invicta* (Lane 6) and in adult *Crematogaster* sp. (Lane 7), Israeli acute paralysis virus (IAPV) in *S. invicta* (Lane 8) and in *Pheidole* sp. (Lane 9), Acute bee paralysis virus (ABPV) in two samples of *Crematogaster* sp. (Lanes 10 and 11),

Kashmir bee virus (KBV) in two samples of *S. invicta* (Lanes 12 and 13), and Sacbrood virus (SBV) in a sample of *S. invicta* (Lane 14). A template-free reaction served as a negative control (Lane 2), and an adult honey bee with obvious symptomatic infection of DWV served as a positive control (Lane 3). All PCR products were run against a Low Molecular Weight ladder (LMW; Lane 1). The amplicon sizes of each product were the following: DWV (130 bp), BQCV (140 bp), IAPV (587 bp), ABPV (124 bp), KBV (127 bp), and SBV (105 bp). All PCR products were run against a Low Molecular Weight ladder (LMW; Lane 1) (New England Biolabs® Inc.).

APPENDIX B:

QUALITY OVER QUANTITY: HIGH-PROTEIN DIETS INCREASE PATHOGEN TOLERANCE OF HONEY BEES INFECTED WITH *NOSEMA CERANAE*

	Variable	Group 1	Group 2	Risk ratio	Confidence interval lower %- upper %	Prob > Chisq
	Infection status only	*Nosema*	NI	1.77	1.52-2.05	$p < 0.0001$*
	Diet groups only	30P:20L	40P:10L	1.52	1.20-1.92	$p = 0.0006$*
		20P:30L	40P:10L	3.68	2.96-4.58	$p < 0.0001$*
		20P:30L	30P:20L	2.43	1.98-2.97	$p < 0.0001$*
		20P:30L	No diet	1.07	0.89- 1.27	$p = 0.47$
		No diet	40P:10L	3.45	2.78-4.29	$p < 0.0001$*
		No diet	30P:20L	2.28	1.86-2.78	$p < 0.0001$*
Interactive effect of infection and diet group	Interactive comparison by infection group	*Nosema* 30P:20L	*Nosema* 40P:10L	1.87	1.38- 2.52	$p < 0.0001$*
		Nosema 20P:30L	*Nosema* 40P:10L	3.83	2.87- 5.12	$p < 0.0001$*
		Nosema 20P:30L	*Nosema* 30P:20L	2.05	1.58- 2.66	$p < 0.0001$*
		Nosema No diet	*Nosema* 40P:10L	5.31	3.99-7.07	$p < 0.0001$*
		Nosema No diet	*Nosema* 20P:30L	1.39	1.10- 1.75	$p = 0.0063$*
		Nosema No diet	*Nosema* 30P:20L	2.85	2.21- 3.67	$p < 0.0001$*
		NI 30P:20L	NI 40P:10L	1.22	0.85- 1.76	$p = 0.28$
		NI 20P:30L	NI 40P:10L	3.57	2.57- 4.95	$p < 0.0001$*
		NI 20P:30L	NI 30P:20L	2.92	2.14- 3.97	$p < 0.0001$*
		NI 20P:30L	NI No diet	1.55	1.19- 2.02	$p = 0.0011$*
		NI No diet	NI 40P:10L	2.3	1.66-3.2	$p < 0.0001$*
		NI No diet	NI 30P:20L	1.88	1.38- 2.57	$p < 0.0001$*
	Interactive comparison by diet group	*Nosema* 40P:10L	NI 40P:10L	1.31	0.92-1.87	$p = 0.13$
		Nosema 30P:20L	NI 30P:20L	1.93	1.41-2.65	$p < 0.0001$*
		Nosema 20P:30L	NI 20P:30L	1.25	0.97-1.61	$p = 0.085$
		Nosema No diet	NI No diet	3.32	2.58-4.28	$p < 0.0001$*

Table 1: Risk ratios based on infection status, diet group, and the interactive effect of infection status and diet group. Risk ratios are calculated as the fraction of Group 1 over Group 2. They determine the likelihood of death for bees belonging to the Group 1 treatment group compared to the Group 2 treatment group for the comparison of each row. All possible comparison are listed and sectioned based off of infection status only,

diet group only, the multivariate comparisons of infection status and diet group by infection status, and the multivariate comparisons of infection status and diet group based by diet type. Confidence intervals and *p* values are listed in the same row as each risk factor comparison. Asterisks next to the *p*-values show comparisons that that had a significantly higher likelihood of death for Group 1 compared to Group 2 ($\alpha = 0.05$).

APPENDIX C:

EFFECTS OF MACRONUTRIENT CONTENT IN HONEY BEE DIETS ON DEFORMED WING VIRUS INFECTION

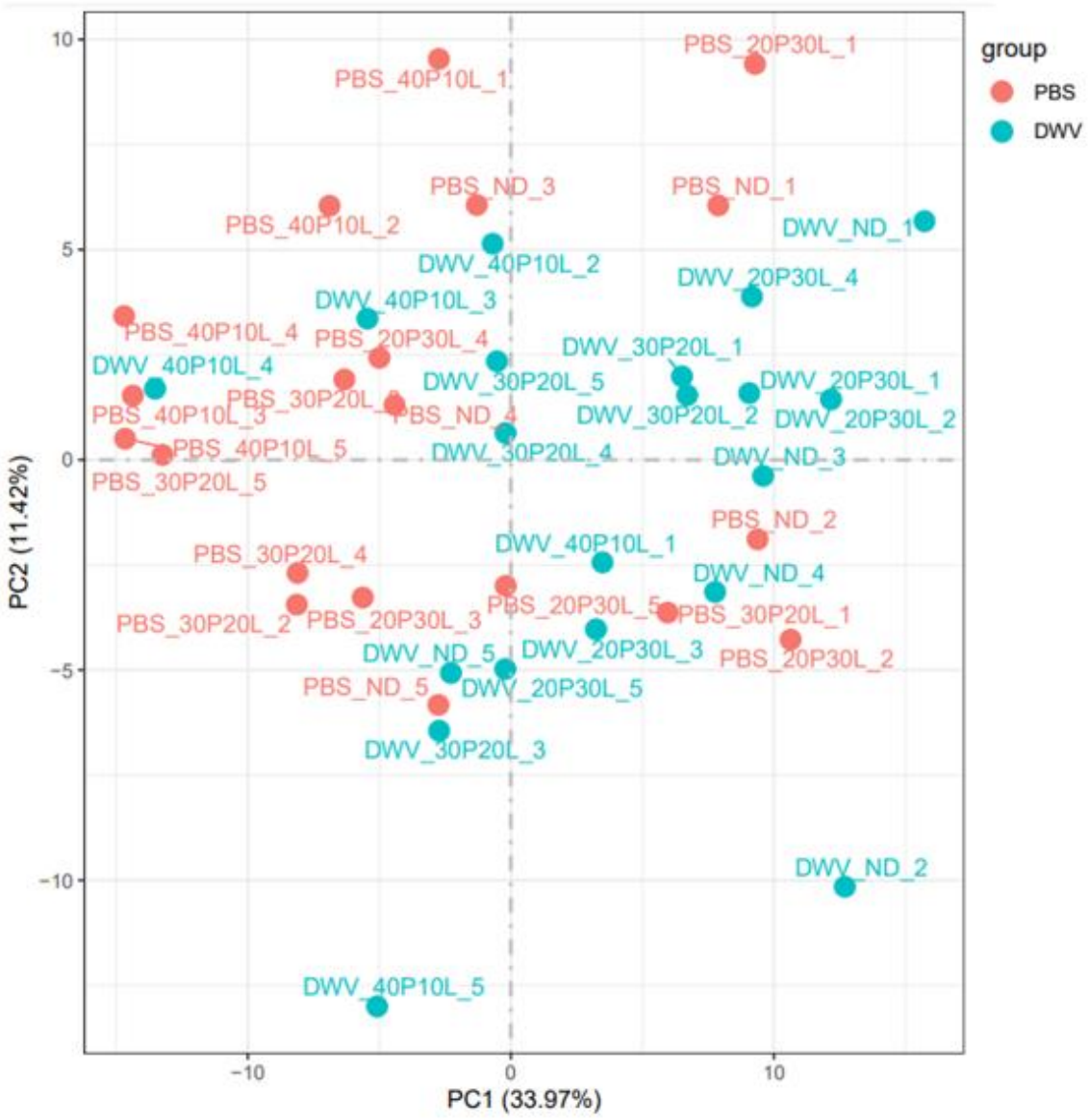

Figure 1: Principle component analysis (PCA) for all 40 RNA-seq samples. The PCA was constructed based on a PC1 of 33.97% and a PC2 of 11.42%. The samples are color

coordinated based on infection status, with samples consisting of bees mock infected and injected with phosphate buffered saline (PBS) shown in pink and samples consisting of bees experimentally infected with DWV shown in blue.

	Variable	Group 1	Group 2	Risk ratio	Confidence interval lower %- upper %	Prob > Chisq
	Infection groups only	DWV	NI	1.73	1.21- 2.46	*p*= 0.0024*****
		DWV	PBS	1.54	1.10- 2.14	*p*= 0.011*
		PBS	NI	1.12	0.78- 1.61	*p*= 0.53
	Diet groups only	40P:10L	30P:20L	1.64	1.12- 2.40	*p*= 0.012*
		40P:10L	20P:30L	1.31	0.91- 1.89	*p*= 0.14
		40P:10L	No diet	1.87	1.26- 2.77	*p*= 0.0019*
		30P:20L	No diet	1.14	0.74- 1.76	*p*= 0.56
		20P:30L	30P:20L	1.25	0.83- 1.88	*p*= 0.29
		20P:30L	No diet	1.42	0.93- 2.17	*p*= 0.10
Interactive effect of infection and diet group	Interactive comparison by infection group	DWV 40P:10L	DWV 30P:20L	6.96	3.29- 14.71	*p*< 0.0001*
		DWV 40P:10L	DWV No diet	2.63	1.55- 4.48	*p*= 0.0004*
		DWV 20P:30L	DWV 30P:20L	7.48	3.57- 15.64	*p*< 0.0001*
		DWV 20P:30L	DWV No diet	2.83	1.69- 4.74	*p*< 0.0001*
		DWV 20P:30L	DWV 40P:10L	1.07	0.73- 1.57	*p*= 0.71
		DWV No diet	DWV 30P:20L	2.64	1.16- 6.04	*p*= 0.021*
		NI 40P:10L	NI 20P:30L	2.3	1.09- 4.85	*p*= 0.029*
		NI 40P:10L	NI No diet	2.9	1.29- 6.52	*p*= 0.0099*
		NI 30P:20L	NI 20P:30L	3.03	1.48- 6.22	*p*= 0.0025*
		NI 30P:20L	NI No diet	3.83	1.75- 8.38	*p*= 0.0008*
		NI 30P:20L	NI 40P:10L	1.32	0.76- 2.30	*p*= 0.33
		NI 20P:30L	NI No diet	1.26	0.50- 3.20	*p*= 0.62
	Interactive comparison by diet group	DWV 40P:10L	NI 40P:10L	2.33	1.41- 3.87	*p*= 0.001*
		DWV 40P:10L	PBS 40P:10L	3.32	1.88- 5.84	*p*< 0.0001*

	NI 40P:10L	PBS 40P:10L	1.42	0.75- 2.71	p= 0.28
	NI 30P:20L	DWV 30P:20L	3.86	1.76- 8.44	p= 0.0007*
	PBS 30P:20L	DWV 30P:20L	2.38	1.03- 5.47	p= 0.041*
	NI 30P:20L	PBS 30P:20L	1.62	0.90- 2.92	p= 0.11
	DWV 20P:30L	NI 20P:30L	5.67	2.90- 11.07	p< 0.0001*
	DWV 20P:30L	PBS 20P:30L	3.66	2.08- 6.44	p< 0.0001*
	PBS 20P:30L	NI 20P:30L	1.55	0.70- 3.50	p= 0.28
	DWV No diet	NI No diet	2.57	1.13- 5.88	p= 0.025*
	DWV No diet	PBS No diet	1.1	0.58- 2.09	p= 0.78
	DWV No diet	PBS No diet	1.1	0.58- 2.09	p= 0.78
	PBS No diet	NI No diet	2.34	1.02- 5.39	p= 0.045*

Table 1: Risk ratios based on infection status, diet group, and the interactive effect of infection status and diet group. Risk ratios are calculated as the fraction of Group 1 over Group 2. They determine the likelihood of death for bees belonging to the Group 1 treatment group compared to the Group 2 treatment group for the comparison of each row. All possible comparison are listed and sectioned based off of infection status only, diet group only, the multivariate comparisons of infection status and diet group by infection status, and the multivariate comparisons of infection status and diet group based by diet type. Confidence intervals and *p* values are listed in the same row as each risk factor comparison. Asterisks next to the *p*-values show comparisons that that had a significantly higher likelihood of death for Group 1 compared to Group 2 ($\alpha = 0.05$).

Primer #	Primer name	Sequence (5'- 3')	Reference
1	DWV- A	CTCATTAACTGTGTCGTTGAT	
2	DWV- B	CTCATTAACTGAGTTGTTGTC	Kevill et al., 2017
3	DWV- C	ATAAGTTGCGTGGTTGAC	

4	DWV (negative-sense strand)	TCCATCAGGTTCTCCAATAACGG	Yue & Genersch, 2005
5	AKI (ABPV, KBV, IAPV)	ACCGACAAAGGGTATGATGC	Francis & Kryger, 2012
6	BQCV	TTTAGAGCGAATTCGGAAACA	Boncristiani et al., 2012
7	CBPV	CGCAAGTACGCCTTGATAAAGAAC	Blanchard et al., 2007
8	LSV	CGTGCGGACCTCATTTCTTCATGT	Daughenbaugh et al., 2015

Table 2: List of primers used for determining mapped read counts for honey bee-associated viruses within all sequenced libraries. DWV= deformed wing virus, ABPV= acute bee paralysis virus, KBV= Kashmir bee virus, IAPV= Iraeli acute bee paralysis virus, BQCV= black queen cell virus, CBPV= chronic bee paralysis virus, LSV= Lake Sanai virus. The different DWV primers target the three major variants of this virus a sequence that targets the negative-sense strand of the virus indicative of active replication.

	DWV-A	DWV-B	DWV-C	DWV (negative-sense strand)	AKI (ABPV, KBV, IAPV)	BQCV	CBPV	LSV
DWV 40P:10L 1	877	1423	0	135829	0	0	0	0
DWV 40P:10L 2	962	1502	0	96003	0	0	0	0
DWV 40P:10L 3	1313	526	0	70764	0	0	0	0
DWV 40P:10L 4	223	366	0	36058	0	0	0	0
DWV 40P:10L 5	1140	1343	0	132752	0	0	0	0
DWV 30P:20L 1	1350	1865	0	199005	0	0	0	0
DWV 30P:20L 2	1015	1452	0	109501	0	0	0	0
DWV 30P:20L 3	1004	2297	0	89976	0	0	0	0
DWV 30P:20L 4	3	969	0	357	0	0	0	0
DWV 30P:20L 5	895	1130	0	133902	0	0	0	0
DWV 20P:30L 1	547	1701	0	92321	0	0	0	0
DWV 20P:30L 2	1879	2309	0	160510	0	0	0	0
DWV 20P:30L 3	470	2298	0	36885	0	0	0	0
DWV 20P:30L 4	108	1281	0	25583	0	0	0	0
DWV 20P:30L 5	1545	1785	0	117780	0	0	0	0
DWV ND 1	1375	2287	0	195018	0	0	0	0
DWV ND 2	1636	2656	0	170440	0	0	0	0

DWV ND 3	1282	2385	0	113529	0	0	0	0
DWV ND 4	230	1298	0	26904	0	0	0	0
DWV ND 5	1181	582	0	104392	0	0	0	0
PBS 40P:10L 1	0	0	0	0	0	0	0	0
PBS 40P:10L 2	0	0	0	0	0	0	0	0
PBS 40P:10L 3	0	0	0	0	0	0	0	0
PBS 40P:10L 4	0	0	0	0	0	0	0	0
PBS 40P:10L 5	0	0	0	0	0	0	0	0
PBS 30P:20L 1	0	1997	0	0	0	0	0	0
PBS 30P:20L 2	0	0	0	0	0	0	0	0
PBS 30P:20L 3	0	0	0	0	0	0	0	0
PBS 30P:20L 4	0	0	0	0	0	0	0	0
PBS 30P:20L 5	0	0	0	0	0	0	0	0
PBS 20P:30L 1	0	0	0	0	0	0	0	0
PBS 20P:30L 2	0	0	0	0	0	0	0	0
PBS 20P:30L 3	0	0	0	0	0	0	0	0
PBS 20P:30L 4	0	0	0	0	0	0	0	0
PBS 20P:30L 5	0	0	0	0	0	0	0	0
PBS ND 1	0	0	0	0	0	0	0	0
PBS ND 2	0	0	0	0	0	0	0	0
PBS ND 3	0	0	0	0	0	0	0	0
PBS ND 4	0	0	0	0	0	0	0	0
PBS ND 5	0	0	0	0	0	0	0	0

Table 3: Mapped read counts for viruses of interest. We used previously published, virus-specific primers to determine the mapped read count for eight honey bee-associated viruses. This included three variants of DWV (DWV-A, B, C) and five other relatively common honey bee-associated viruses (including black queen cell virus-BQCV, acute bee paralysis virus-ABPV, Israeli acute paralysis virus-IAPV, Kashmir bee virus- KBV, chronic bee paralysis virus-CBPV, sacbrood virus-SBV, and Lake Sanai virus-LSV). Mapped read counts were performed on Texas A&M's Terra cluster.

Treatment comparison	Up/Down regulation	Category	GO:ID	Description	padj	Count
DWV vs PBS	Up	MF	GO:0009055	electron transfer activity	0.0166	10
		MF	GO:0016651	oxidoreductase activity, acting on NAD(P)H	0.0313	6
	Down	none				
DWV 40P:10L vs PBS 40P:10L	Up	BP	GO:0055114	oxidation-reduction process	0.0351	22
		MF	GO:0016651	oxidoreductase activity, acting on NAD(P)H	0.0002	6
		MF	GO:0016491	oxidoreductase activity	0.0035	27
	Down	none				
DWV 30P:20L vs PBS 30P:20L	Up	BP	GO:0007165	signal transduction	0.0199	29
		BP	GO:0007154	cell communication	0.0199	29
		BP	GO:0023052	signaling	0.0199	29
		BP	GO:0035556	intracellular signal transduction	0.0199	18
		BP	GO:0051716	cellular response to stimulus	0.0209	31
	Down	BP	GO:0007018	microtubule-based movement	0.0156	8
		BP	GO:0007017	microtubule-based process	0.0156	9
		BP	GO:0006928	movement of cell or subcellular component	0.0156	8
		MF	GO:0008017	microtubule binding	0.0006	8
		MF	GO:0015631	tubulin binding	0.0006	8
		MF	GO:0008092	cytoskeletal protein binding	0.0015	10
		MF	GO:0003777	microtubule motor activity	0.0028	8
		MF	GO:0003774	motor activity	0.0063	9
		MF	GO:0046982	protein heterodimerization activity	0.0153	7
DWV 20P:30L vs PBS 20P:30L	Up	none				
	Down	BP	GO:0007017	microtubule-based process	0.0003	12
		BP	GO:0007018	microtubule-based movement	0.0313	8
		BP	GO:0006928	movement of cell or subcellular component	0.0313	8
		CC	GO:0016021	integral component of membrane	0.0004	37
		CC	GO:0031224	intrinsic component of membrane	0.0004	37
		CC	GO:0044425	membrane part	0.0024	38
		MF	GO:0003777	microtubule motor activity	0.0255	8
		MF	GO:0003774	motor activity	0.0347	9
		MF	GO:0008017	microtubule binding	0.0347	6
		MF	GO:0015631	tubulin binding	0.0411	6
		MF	GO:0004930	G-protein coupled receptor activity	0.0440	10

DWV No diet vs PBS No diet	Up	MF	GO:0005216	ion channel activity	0.0399	10
		MF	GO:0015267	channel activity	0.0399	10
		MF	GO:0022803	passive transmembrane transporter activity	0.0399	10
		MF	GO:0022838	substrate-specific channel activity	0.0399	10
	Down	MF	GO:0008237	metallopeptidase activity	0.0111	10
		MF	GO:0016746	transferase activity, transferring acyl groups	0.0125	10
		MF	GO:0048037	cofactor binding	0.0187	18
		MF	GO:0070011	peptidase activity, acting on L-amino acid peptides	0.0227	18
		MF	GO:0008233	peptidase activity	0.0242	18
DWV 40P:10L vs DWV No diet	Up	BP	GO:0055114	oxidation-reduction process	0.0000	46
		BP	GO:0044281	small molecule metabolic process	0.0150	22
		CC	GO:0005576	extracellular region	0.0157	9
		MF	GO:0016491	oxidoreductase activity	0.0000	57
		MF	GO:0048037	cofactor binding	0.0000	27
		MF	GO:0050662	coenzyme binding	0.0000	18
		MF	GO:0016614	oxidoreductase activity, acting on CH-OH group of donors	0.0001	12
		MF	GO:0005506	iron ion binding	0.0001	12
		MF	GO:0050660	flavin adenine dinucleotide binding	0.0003	10
		MF	GO:0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	0.0003	7
		MF	GO:0016616	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	0.0028	7
		MF	GO:0070011	peptidase activity, acting on L-amino acid peptides	0.0070	20
		MF	GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	0.0078	9
		MF	GO:0008233	peptidase activity	0.0078	20
		MF	GO:0016903	oxidoreductase activity, acting on the aldehyde or oxo group of donors	0.0084	5
		MF	GO:0020037	heme binding	0.0107	9
		MF	GO:0046906	tetrapyrrole binding	0.0112	9
		MF	GO:0016627	oxidoreductase activity, acting on the CH-CH group of donors	0.0113	5
		MF	GO:0019842	vitamin binding	0.0322	6
		MF	GO:0008237	metallopeptidase activity	0.0330	8
		MF	GO:0016829	lyase activity	0.0419	7
	Down	BP	GO:0007154	cell communication	0.0233	21
		BP	GO:0023052	signaling	0.0233	21

		BP	GO:0007165	signal transduction	0.0372	20
		BP	GO:0050896	response to stimulus	0.0442	22
		BP	GO:0006820	anion transport	0.0442	5
DWV 30P:20L vs DWV No diet	Up	BP	GO:0006629	lipid metabolic process	0.0415	10
		BP	GO:0055114	oxidation-reduction process	0.0415	19
		MF	GO:0016491	oxidoreductase activity	0.0062	22
		MF	GO:0005506	iron ion binding	0.0360	7
	Down	none				
DWV 20P:30L vs DWV No diet	Up	MF	GO:0016491	oxidoreductase activity	0.0384	8
		MF	GO:0048037	cofactor binding	0.0384	6
	Down	BP	GO:0007186	G-protein coupled receptor signaling pathway	0.0071	7
		BP	GO:0007165	signal transduction	0.0071	14
		BP	GO:0007154	cell communication	0.0071	14
		BP	GO:0023052	signaling	0.0071	14
		BP	GO:0051716	cellular response to stimulus	0.0186	14
		BP	GO:0050896	response to stimulus	0.0286	14
		MF	GO:0004930	G-protein coupled receptor activity	0.0048	7
		MF	GO:0015291	secondary active transmembrane transporter activity	0.0157	4
		MF	GO:0015318	inorganic molecular entity transmembrane transporter activity	0.0283	8
		MF	GO:0015370	solute:sodium symporter activity	0.0335	3
		MF	GO:0015293	symporter activity	0.0374	3
		MF	GO:0015294	solute:cation symporter activity	0.0374	3
		MF	GO:0022804	active transmembrane transporter activity	0.0465	4
PBS 40P:10L vs PBS No diet	Up	CC	GO:0005694	chromosome	0.0464	7
		MF	GO:0008017	microtubule binding	0.0000	12
		MF	GO:0016491	oxidoreductase activity	0.0000	52
		MF	GO:0015631	tubulin binding	0.0000	12
		MF	GO:0008092	cytoskeletal protein binding	0.0035	13
		MF	GO:0003777	microtubule motor activity	0.0086	10
		MF	GO:0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	0.0186	7
		MF	GO:0048037	cofactor binding	0.0236	28
		MF	GO:0046982	protein heterodimerization activity	0.0313	9
		MF	GO:0050662	coenzyme binding	0.0366	18
		MF	GO:0005506	iron ion binding	0.0366	11
	Down	BP	GO:0006811	ion transport	0.0089	20

		BP	GO:0006810	transport	0.0089	45
		BP	GO:0051234	establishment of localization	0.0089	45
		BP	GO:0051179	localization	0.0096	45
		MF	GO:0005215	transporter activity	0.0000	39
		MF	GO:0022857	transmembrane transporter activity	0.0010	30
		MF	GO:0015075	ion transmembrane transporter activity	0.0010	24
		MF	GO:0015318	inorganic molecular entity transmembrane transporter activity	0.0013	22
		MF	GO:0005216	ion channel activity	0.0013	14
		MF	GO:0015267	channel activity	0.0013	14
		MF	GO:0022803	passive transmembrane transporter activity	0.0013	14
		MF	GO:0022838	substrate-specific channel activity	0.0013	14
		MF	GO:0022836	gated channel activity	0.0039	9
		MF	GO:0022839	ion gated channel activity	0.0039	9
		MF	GO:0030545	receptor regulator activity	0.0261	5
		MF	GO:0048018	receptor ligand activity	0.0261	5
		MF	GO:0005319	lipid transporter activity	0.0386	4
PBS 30P:20L vs PBS No diet	Up	BP	GO:0055114	oxidation-reduction process	0.0012	26
		BP	GO:0005975	carbohydrate metabolic process	0.0118	13
		CC	GO:0015629	actin cytoskeleton	0.0135	5
		MF	GO:0016491	oxidoreductase activity	0.0000	38
	Down	CC	GO:0016021	integral component of membrane	0.0209	30
		CC	GO:0031224	intrinsic component of membrane	0.0209	30
PBS 20P:30L vs PBS No diet	Up	none				
	Down	MF	GO:0005102	signaling receptor binding	0.0377	4
		MF	GO:0030545	receptor regulator activity	0.0377	3
		MF	GO:0048018	receptor ligand activity	0.0377	3

Table 4: List of significantly enriched gene ontology (GO) terms for differentially expressed up- and downregulated genes. Only terms that were significantly up or downregulated were included in the table. The table includes information on the comparison being made, the GO terms that were up- and down-regulated, the category to which the term belongs to (MF = molecular function, BP = biological process, CC =

cellular component), the GO ID number, a brief description of the GO term, the adjusted *p* value ($p < 0.05$) at which the GO term was significantly enriched for a particular comparison, and the gene count which is the number of DEGS associated with this term.

Treatment comparison	Up/Down regulation	KEGGID	Description	padj	Count
DWV vs PBS	Up	ame00190	Oxidative phosphorylation	6.32E-05	26
	Up	ame03040	Spliceosome	0.0269	22
	Up	ame04214	Apoptosis - fly	0.0269	13
	Down	ame04146	Peroxisome	0.0213	14
DWV 40P:10L vs PBS 40P:10L	Up	ame00190	Oxidative phosphorylation	1.10E-07	17
	Up	ame04745	Phototransduction - fly	0.0013	6
	Down	none			
DWV 30P:20L vs PBS 30P:20L	Up	none			
	Down	none			
DWV 20P:30L vs PBS 20P:30L	Up	ame04624	Toll and Imd signaling pathway	0.0344	6
	Up	ame04933	AGE-RAGE signaling pathway in diabetic complications	0.0344	5
	Up	ame04512	ECM-receptor interaction	0.0424	4
	Down	none			
DWV No diet vs PBS No diet	Up	ame04745	Phototransduction - fly	1.51E-06	9
	Down	ame00600	Sphingolipid metabolism	0.0221	5
	Down	ame00511	Other glycan degradation	0.0221	4
	Down	ame00520	Amino sugar and nucleotide sugar metabolism	0.0284	6
	Down	ame01212	Fatty acid metabolism	0.0284	6
	Down	ame00565	Ether lipid metabolism	0.0284	4
	Down	ame00564	Glycerophospholipid metabolism	0.0284	7
	Down	ame00592	alpha-Linolenic acid metabolism	0.0309	3
	Down	ame00561	Glycerolipid metabolism	0.0309	5
DWV 40P:10L vs DWV No diet	Up	ame00280	Valine, leucine and isoleucine degradation	1.07E-08	14
	Up	ame00380	Tryptophan metabolism	2.62E-05	9
	Up	ame00071	Fatty acid degradation	3.94E-05	9
	Up	ame01200	Carbon metabolism	3.94E-05	17
	Up	ame00030	Pentose phosphate pathway	5.60E-05	8

	Up	ame01212	Fatty acid metabolism	6.16E-05	11
	Up	ame00410	beta-Alanine metabolism	0.0003	7
	Up	ame00270	Cysteine and methionine metabolism	0.0013	9
	Up	ame00062	Fatty acid elongation	0.0030	5
	Up	ame00040	Pentose and glucuronate interconversions	0.0034	5
	Up	ame00350	Tyrosine metabolism	0.0034	5
	Up	ame04146	Peroxisome	0.0034	10
	Up	ame00310	Lysine degradation	0.0065	7
	Up	ame00053	Ascorbate and aldarate metabolism	0.0089	4
	Up	ame00670	One carbon pool by folate	0.0089	4
	Up	ame00981	Insect hormone biosynthesis	0.0089	4
	Up	ame00640	Propanoate metabolism	0.0104	6
	Up	ame00051	Fructose and mannose metabolism	0.0134	5
	Up	ame01230	Biosynthesis of amino acids	0.0144	8
	Up	ame00620	Pyruvate metabolism	0.0218	6
	Up	ame00630	Glyoxylate and dicarboxylate metabolism	0.0265	5
	Up	ame00590	Arachidonic acid metabolism	0.0277	4
	Up	ame01040	Biosynthesis of unsaturated fatty acids	0.0277	4
	Up	ame00260	Glycine, serine and threonine metabolism	0.0388	5
	Up	ame00592	alpha-Linolenic acid metabolism	0.0407	3
	Up	ame00790	Folate biosynthesis	0.0462	4
	Up	ame00220	Arginine biosynthesis	0.0498	3
	Down	ame04745	Phototransduction - fly	3.27E-11	10
DWV 30P:20L vs DWV No diet	Up	ame00561	Glycerolipid metabolism	0.0117	6
	Up	ame00564	Glycerophospholipid metabolism	0.0253	7
	Up	ame00592	alpha-Linolenic acid metabolism	0.0446	3
	Down	ame04745	Phototransduction - fly	6.24E-10	9
DWV 20P:30L vs DWV No diet	Up	none			
	Down	ame04745	Phototransduction - fly	4.98E-10	8
PBS 40P:10L vs PBS No diet	Up	ame00981	Insect hormone biosynthesis	0.0079	6
	Up	ame00380	Tryptophan metabolism	0.0088	8
	Up	ame00030	Pentose phosphate pathway	0.0155	7
	Up	ame00310	Lysine degradation	0.0155	9
	Up	ame00053	Ascorbate and aldarate metabolism	0.0155	5
	Up	ame00670	One carbon pool by folate	0.0155	5
	Up	ame01230	Biosynthesis of amino acids	0.0185	11
	Up	ame00071	Fatty acid degradation	0.0248	7
	Up	ame00270	Cysteine and methionine metabolism	0.0272	9
	Up	ame00040	Pentose and glucuronate interconversions	0.0272	5

	Up	ame00350	Tyrosine metabolism	0.0272	5
	Up	ame00280	Valine, leucine and isoleucine degradation	0.0272	8
	Up	ame00051	Fructose and mannose metabolism	0.0272	6
	Up	ame01200	Carbon metabolism	0.0289	15
	Up	ame01210	2-Oxocarboxylic acid metabolism	0.0308	5
	Up	ame00330	Arginine and proline metabolism	0.0467	6
	Up	ame00220	Arginine biosynthesis	0.0467	4
	Down	ame04745	Phototransduction - fly	1.42E-08	11
PBS 30P:20L vs PBS No diet	Up	ame00670	One carbon pool by folate	2.81E-06	7
	Up	ame00260	Glycine, serine and threonine metabolism	0.0002	8
	Up	ame00051	Fructose and mannose metabolism	0.0021	6
	Up	ame00380	Tryptophan metabolism	0.0028	6
	Up	ame00310	Lysine degradation	0.0028	7
	Up	ame00071	Fatty acid degradation	0.0031	6
	Up	ame00230	Purine metabolism	0.0034	10
	Up	ame01230	Biosynthesis of amino acids	0.0044	8
	Up	ame00053	Ascorbate and aldarate metabolism	0.0044	4
	Up	ame00981	Insect hormone biosynthesis	0.0044	4
	Up	ame00561	Glycerolipid metabolism	0.0088	6
	Up	ame00280	Valine, leucine and isoleucine degradation	0.0095	6
	Up	ame00330	Arginine and proline metabolism	0.0095	5
	Down	none			
PBS 20P:30L vs PBS No diet	Up	ame00670	One carbon pool by folate	0.0285	3
	Up	ame00230	Purine metabolism	0.0285	6
	Up	ame01040	Biosynthesis of unsaturated fatty acids	0.0399	3
	Down	ame04512	ECM-receptor interaction	0.0145	3

Table 5: List of significantly enriched KEGG pathways for differentially expressed up- and downregulated genes. Only pathways that were significantly up or downregulated were included in the table. The table includes information on the comparison being made, the KEGG pathway ID and whether it was up- or down-regulated, a description of the KEGG ID, the adjusted *p* value ($p < 0.05$) at which the GO term was significantly enriched for a particular comparison, and the gene count which is the number of DEGS associated with this pathway.