AMELIORATIVE EFFECTS OF PHYTOCHEMICAL INGESTION ON VIRAL INFECTION IN HONEY BEES

BY

EDWARD M. HSIEH

THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Entomology in the Graduate College of the University of Illinois at Urbana-Champaign, 2020

Urbana, Illinois

Master’s Committee:

Assistant Professor Adam Dolezal, Chair, Co-Director of Research
Professor May Berenbaum, Co-Director of Research
Assistant Professor Marianne Alleyne
ABSTRACT

Honey bee viruses, capable of causing a wide variety of detrimental effects, are an important dimension of honey bee health management. To date, there are no effective treatments for viral infection in honey bees and management relies largely on controlling Varroa mites, a honey bee pest capable of vectoring viruses. Plant-derived chemicals (phytochemicals) represent a broad range of substances that honey bees frequently encounter and consume, many of which are known to improve honey bee health, although their effect on bee viruses is largely unknown. This thesis project tested the therapeutic effectiveness of various phytochemicals on viral infection by measuring their ability to improve survivorship in honey bees inoculated with Israeli acute paralysis virus (IAPV).

In the absence of a viable cell line, virus particles were generated, purified, and extracted from honey bee pupae by pairing established protocols with “larval crawl-outs” a novel method of pupal extraction that greatly improved the efficiency of particle production. In the first part of this project, I screened for phytochemical effects by first inoculating honey bees with viral particles and then feeding them thymol, carvacrol, p-coumaric acid, quercetin, or caffeine in a series of high-throughput bioassays, all of which were chosen based on their immune-boosting properties in honey bees. Among these candidates, caffeine was the only phytochemical capable of significantly improving survivorship and was therefore selected for additional testing.

Initial screening showed that naturally-occurring concentrations of caffeine (25 ppm) were sufficient to produce an ameliorative effect on IAPV infection. Consequently, the second half of this project focused on determining the scope of caffeine effectiveness in bees inoculated and uninoculated with IAPV by performing the same type of high-throughput bioassay across a wider range of caffeine concentrations. My results indicated that caffeine may provide benefits that scale with concentration, to a point, and may partially compensate for seasonal nutritional deficits within a colony. However, the exact mechanism by which caffeine ingestion improves survivorship remains uncertain, as a comparison of viral titers between inoculated and uninoculated caffeine-fed bees revealed no significant difference between infection groupings.

My findings indicate that caffeine has potential to act as an accessible and inexpensive method of treating viral infections, while also serving as a tool to further understanding of honey bee-virus interactions at a physiological and molecular level.
ACKNOWLEDGMENTS

I would first like to thank both of my advisors, Drs. Adam Dolezal and May Berenbaum, for their unwavering support throughout the completion of my master’s thesis project. Their guidance and encouragement were invaluable during all stages of research and I am deeply grateful to them both. Additionally, I would like to thank my committee member Marianne Alleyne for her advice and perspective, as well as Dr. Juan Andrade and his lab members for their assistance with phytochemical production.

Specifically, I would like to thank Gyan Harwood, Lincoln Taylor, and Jake Torres for their help throughout the process and their willingness to occasionally endure sweltering incubator conditions on my behalf. In particular, I would like to express my gratitude to Alison Sankey for maintaining a smooth-running apiary operation and for aiding me during the innumerable times I requested bee yard assistance. I also must express my appreciation to my past mentors and all members of the Iowa State Bee Team for taking me in and fostering a fascination with the delightful insect that is the honey bee. Finally, I would like to thank my family and friends for their support and encouragement throughout this period of my life—I am forever grateful.
# TABLE OF CONTENTS

| Chapter | Title                                      | Page |
|---------|--------------------------------------------|------|
| 1       | INTRODUCTION                               | 1    |
| 2       | MATERIALS AND METHODS                      | 8    |
| 3       | RESULTS                                    | 17   |
| 4       | DISCUSSION                                 | 31   |
|         | REFERENCES                                 | 38   |
CHAPTER 1: INTRODUCTION

Honey bees are a keystone species in the modern crop pollination landscape, generating an estimated global annual value of over $215 billion USD in agricultural production (Gallai et al. 2009; Smith and Saunders 2016). These highly managed, charismatic insects have attracted global attention for their notable role in food production, as well as the apparent decline in their overall health. Although the global number of hives has actually risen by ~45% since 1961, this approximation is highly uneven across continents, with North America and Europe both experiencing precipitous declines in managed colony numbers while Asia, Africa, and South America all reported large increases (Aizen and Harder 2009; Potts et al. 2010). These declines cannot be attributed solely to poorer colony health, as fluctuating market demands have had significant impacts on overall colony numbers, but the fact that honey bees are currently suffering from a combination of biotic and abiotic stressors is undeniable (vanEngelsdorp and Mexiner 2009). Such stressors include pesticides, disease, poor forage, and the omnipresent Varroa mite (Varroa destructor) (vanEngelsdorp and Meixner 2009; Goulson et al. 2015), all of which can lead to high rates of hive loss and subsequent replacement (Steinhauer et al. 2014).

There are also an enormous variety of pathogens capable of infecting honey bees, including bacteria, viruses, and microsporidia and other fungi. Viruses are of particular concern due to their close association with Varroa, the most widespread and devastating parasite of honey bees known to date (Rosenkranz et al. 2010). A large number of honey bee viruses have already been characterized, with the count continually rising due to the onset of next-generation sequencing techniques (Chen et al. 2004; McMenamin and Flenniken 2018). Although there is some dispute over whether or not certain species can be classified as distinct or belonging to a closely related overall complex, the majority of these viruses share two important qualities: (1) they can be vectored by Varroa mites and (2) they can cause devastating effects that range from poor brood survival to physiological defects and total hive collapse (Grozinger and Flenniken 2019). One of the best known and comparatively well-studied examples is deformed wing virus (DWV), a virus that cripples wing functionality, reduces adult activity, and contributes to increased risk of overwinter hive mortality (Natsopoulou et al. 2017). Viruses belonging to the bee paralysis virus complex (acute bee paralysis virus, Kashmir bee virus, and Israeli acute paralysis virus) cause shivering wings and disorientation in workers before eventual paralysis.
and death (Chen et al. 2014; Amiri et al. 2019; Grozinger and Flenniken 2019). Still other viruses, such as sacbrood virus (SBV) and black queen cell virus (BQCV), target the larval stages of bees, impairing hive reproduction and productivity (Allen and Ball 1996). Although some developing technologies used to combat virus infection have been successful to some extent (Maori et al. 2009; Hunter et al. 2010; Leonard et al. 2020), much of the existing virus work focuses on identifying novel viral species (Chen et al. 2004; McMenamin and Flenniken 2018), with comparatively fewer studies examining their physiological impacts on honey bees. To date, there are currently no available treatments for any known viruses and management focuses mainly on reducing Varroa loads.

The first objective of my thesis project was to test potential treatments for honey bee virus infections. I selected Israeli acute paralysis virus (IAPV) as a model for its simplicity of phenotyping and ease-of-inclusion in the experimental workflow, as well as its importance as a pathogen of concern. Previous virus-based physiological research has generally used DWV as a model due to its ubiquity among honey bees and related Apis species. However, despite the fact that DWV-infected larvae typically eclose with the characteristic and easily recognizable crumpled wing defect, adult bee infections produce much more subtle behavioral phenotypes (Grozinger and Flenniken 2019). In contrast, signs of acute IAPV infection (lethargic movement, reduced reaction to stimulus, and eventual death) are readily discernible (Chen et al. 2014). Using IAPV allows for high-throughput bioassays, which aim to establish an effect before expanding to include a wider range of applicable virus models. Following confirmation of effect (or lack thereof), my secondary objective was to determine whether the mechanism of effect was through viral tolerance, an ability to sustain increased viral loads, or viral resistance, an active reduction in viral replication or overall titers.

As with all other factors that influence honey bee health, the effects of parasites and pathogens must be considered in the context of the ecology and behavior of honey bees. For example, the diversity and quality of pollens consumed by individual bees can have a significant effect on their ability to tolerate pathogenic infections (Alaux et al. 2010; Dolezal et al. 2019a, Negri et al. 2019). Additional investigations have taken a more granular approach by examining individual secondary plant metabolites found in nectar, pollen, and resins that bees naturally ingest. These secondary metabolites, or phytochemicals, are routinely collected by foragers and are typically present, albeit in varying concentrations, in honey, allowing for distribution
throughout the hive. Many of these phytochemicals have immune-boosting effects in *Apis* species and may serve as part of a self-medication strategy among social bees (Negri et al. 2019). Nicotine, a phytochemical common in species of Solanaceae, can reduce the parasite load and infection rate of *Crithidia bombi*, a bumble bee gut trypanosome (Baracchi et al. 2015). In fact, the infected bumble bees preferentially seek out nicotine-laced solutions, despite the fact that nicotine typically serves as a feeding deterrent (Köhler et al. 2012), lending further support to the idea of self-medication (Baracchi et al. 2015). In a series of experiments, Negri et al. (2015) and Szawarski et al. (2019) have demonstrated that abscisic acid (ABA), a phytohormone produced by both floral food sources and adult honey bees themselves, plays an important role in honey bee immunocompetence. ABA stimulates immune response to cold stress, improves wound healing and pesticide tolerance, and even reduces spore loads of *Nosema ceranae*, a microsporidian parasite, at a colony level (Negri et al. 2019). However, as Negri et al. (2019) noted in their review, many of these phytochemicals merit further investigation with respect to how they affect bee physiology at an individual and colony level. More specifically, there is currently a dearth of studies involving the physiological effects of these phytochemicals on honey bee viral infections.

I address this issue in my thesis by first screening for candidate phytochemicals with potential antiviral effect by using high-throughput cage experiments and then further investigating any phytochemicals that produce measurable benefits. Among the multitude of potential candidates, I selected several phytochemicals already known to have beneficial effects on bees, including thymol, carvacrol, *p*-coumaric acid (PCA), quercetin, and caffeine for viral treatment testing.

**Phytochemical Selection and Screening**

*Essential Oils: Thymol and Carvacrol*

In recent years, there has been rising interest in the use of essential oils as both nutraceuticals, i.e. food components that impart physiological benefits typically to humans, and antimicrobial control agents in other animals (Das et al. 2012, Salehi et al. 2018). Thyme oil, derived from the aromatic subshrub *Thymus vulgaris*, and oregano oil, originating from the Mediterranean herb *Origanum vulgare*, both confer immune-boosting benefits in humans.
including antibacterial, antifungal, antiparasitic, and antioxidative properties (Dauqan and Abdullah 2017; Gaur 2017; Salehi et al. 2018). The primary active components of both of these oils are the monoterpenes thymol and carvacrol. Independently administered, carvacrol increased the survivorship of bacteria-infected wax moths (Galleria mellonella) (Upadhyay and Venkitanarayanan 2016) and reduced cultures of Staphylococcus aureus in certain foods (Chang et al. 2017). Thymol, which inhibits growth of a wide variety of bacterial and fungal species and has been used for a broad range of traditional medicinal purposes (Salehi et al. 2018), is the active ingredient in the popular Varroa control treatments ApiGuard® and ApiLife VAR® (Floris et al. 2004; Gregorc and Planinc 2005). Palmer-Young et al. (2017b) examined the effects of thymol (among other phytochemicals) on adult honey bees, demonstrating that short-term consumption of thymol resulted in reduced levels of DWV in young bees while long-term consumption significantly increased antimicrobial peptide expression in older bees. Palmer-Young et al. (2017a) also observed that, as in the nicotine experiment performed by Barrachi et al. (2015), thymol suppresses the development of C. bombi and even interacts synergistically with other phytochemicals.

During the phytochemical screening process, I expanded upon the work performed by Palmer-Young et al. (2017b) by documenting the antiviral effects of thymol and carvacrol on Israeli acute paralysis virus (IAPV) infections in honey bee workers. Both of these essential oils have pungent odors in their pure form and can be difficult for humans and insects alike to ingest due to their topical irritant properties. In fact, thymol is naturally repellent to honey bees and overdosing with thymol-based mite treatments can cause them to abscond (Raffique et al. 2012; Colin et al. 2019). To administer what would otherwise be an intolerable dose, I first encapsulated thymol and carvacrol particles within β-cyclodextrin, a cyclic oligosaccharide, before feeding them to honey bees inoculated with IAPV to measure how these phytochemicals affect survivorship. This delivery method was based on work done by Gaur (2017), who formulated carvacrol and thymol for administering to children by masking the overpowering taste of carvacrol and thymol via encapsulation using β-cyclodextrin and then incorporating the capsules into nutritional supplements as a means of treating enteric parasitic infections.
p-Coumaric Acid and Quercetin

The phenolic acid p-coumaric acid (PCA) and the flavonol quercetin both belong to the group of phytochemicals that are effectively ubiquitous in pollen, propolis, and honey (Mao et al. 2009) and that provide an assortment of immune-boosting functions in honey bees. Bernklau et al. (2019) found that PCA improved the survivability of bees infected with *N. ceranae* by reducing the spore loads of the microsporidian parasite. Mao et al. (2013, 2015) demonstrated that PCA upregulates several families of cytochrome P450 genes (P450), genes responsible for the detoxification of xenobiotics, as well as several antimicrobial peptide genes when fed to adult and larval bees. Honey bees rely on these P450s to detoxify the broad range of defensive phytochemical compounds they encounter in their natural diet as well as pesticides and acaricides introduced into their hive environment (Mao et al. 2013; 2017). Liao et al. (2017) determined that both PCA and quercetin can extend the lifespan of bees exposed to pyrethroids and Wong et al. (2018) found similar survival-enhancing results when bees were fed the same phytochemicals in combination with imidacloprid, a neonicotinoid pesticide. It is unclear if these detoxification mechanisms provide protective benefits against infection.

Furthermore, PCA and quercetin both possess antiviral qualities, although, thus far, these studies have not included the use of pollinator virus models, i.e., they have been studied primarily in human pathogen models. Quercetin reduced the viral expression of Canine Distemper Virus and improved cellular viability in an *in vitro* mammalian cell culture assay (González-Búrquez et al. 2018) and PCA inhibited the replication of certain strains of human rhinovirus in infected human epithelial adenocarcinoma cervical cell cultures (Kwon et al. 2019). Human rhinovirus belongs to the order Picornavirales, to which many of the more frequently encountered bee viruses (DWV, IAPV, BQCV, and others) also belong (Grozinger and Flenniken 2019). These commonalities, in conjunction with the anti-DWV effect observed by Palmer-Young et al. (2017b), suggest that there may be a similar antiviral result when PCA and quercetin are administered to honey bees, even though previous viral studies have been conducted largely in mammalian cell lines. Despite some recent advances (Goblirsch et al. 203), there is currently no widely available honey bee continuous cell line, making the *in vitro* work like that of Kwon et al. (2019) or González-Búrquez et al. (2018) unfeasible in my phytochemical trials. Consequently, all of the bioassays in my study were conducted *in vivo*, examining viral effects at a physiological, rather than a cellular, level.
As part of the screening process, I studied the effects of PCA and quercetin on IAPV infections in honey bees using the same high-throughput cage assays employed in the essential oil trials with the same objectives of first establishing a direction of effect before examining the mechanism. Based on their established immune-boosting properties, I hypothesized that both phytochemicals would increase the survivorship of infected bees when supplemented into their diets.

Caffeine

Perhaps one of the most widely recognized plant secondary metabolites, caffeine is an alkaloid naturally found in the seeds, leaves, and berries of a wide variety of plant species including coffee (Coffea), cocoa (Theobroma), and tea (Camellia) (Nathanson 1984; Kretschmar and Baumann 1999). Apart from acting as an important stimulant for many humans, caffeine is capable of triggering a wide variety of behavioral and physiological reactions across a variety of taxa, including reducing the risk of Alzheimer’s disease in mice (Arendash et al. 2006), ameliorating oxidative stress in rats (Devasagayam et al. 1996), and even improving locomotory behavior in cockroaches (da Silva et al. 2018). Like nicotine, caffeine is toxic to many herbivorous insects, killing tobacco hornworm (Manduca sexta) larvae when ingested at naturally occurring levels, for example, indicative of its ecological function as an endogenous pesticide or feeding deterrent (Nathanson 1984).

In spite of its toxic effects, caffeine can also be found in the floral nectar and pollen of Coffea and citrus (Citrus) species (Kretschmar and Baumann 1999, Wright et al. 2013), where its defensive qualities do not appear to deter pollinator feeding. In fact, both honey bees and bumble bees prefer sucrose solutions containing higher concentrations of caffeine over lower concentrations or no caffeine at all (Singaravelan et al. 2005, Thomson et al. 2015). Si et al. (2005) observed that caffeine consumption increases the speed of olfactory learning in young bees and improves cognitive performance in complex learning tasks in older bees. Numerous studies have detailed the ways in which caffeine aids in the memory formation process (Mustard et al. 2012) with caffeine-fed bees being three times more likely to remember olfactory cues (Wright et al. 2013). In the field, bees stimulated by a caffeine reward intensify their recruitment behaviors, increasing foraging and waggle dance frequency, as well as persistence to target dance location (Couvillon et al. 2015). Curiously, these patterns of learning and recruitment are
not uniform across all pollinators; *Plebeia droryana*, a species of stingless bee that pollinates flowers in tropical habitats, have no behavioral response to caffeine ingestion (Peng et al. 2019).

The ecological function of secondary plant metabolites, such as caffeine, in nectar has long been a subject of debate. Among hypotheses advanced to account for the presence of a chemical that deters feeding by many herbivorous insects include reducing defense against robbing by non-specialists and defense against microbial contamination (Stevenson et al. 2017; Mustard 2020). However, Adler (2000) argues that there is no single overarching stimulus and the varied functions of plant secondary compounds such as caffeine are instead rooted in the fact that such traits are under multiple simultaneous selective pressures. For example, although caffeine negatively affects the herbivorous *Manduca sexta* larva (Nathanson 1984), it also has phagostimulatory effects on the pollinating adult hawk moth of the same species (Reiter et al. 2015). Stevenson et al. (2017) suggest that secondary metabolites found in nectar act as a coevolutionary force between plants and pollinators but also propose that their presence may be a byproduct of their pleiotropic effects elsewhere within the plant. By including viral infection into my caffeine investigations, I hope to attain a better understanding of the evolutionary relationship between honey bees, caffeine, and their plant hosts.

Despite the large body of research on behavioral effects of caffeine on honey bees, fewer studies have examined the physiological consequences of caffeine ingestion. Balieira et al. (2018) showed that caffeine helps to ameliorate the oxidative stress induced by the neonicotinoid insecticide imidacloprid. Both Strachecka et al. (2014) and Bernklau et al. (2019) found that caffeine increased the lifespan of honey bees infected by *N. ceranae* by actively reducing spore loads. However, no study has examined the effects of caffeine on viral infection, an issue that I addressed in this project. Using the same high-throughput cage assays, I set out to establish whether caffeine can improve honey bee survivorship in the presence of IAPV infection and to differentiate between viral tolerance versus resistance by measuring viral titers. I also generated caffeine dose-response curves with and without viral inoculation to provide a broader context for any beneficial effects of dietary caffeine.
CHAPTER 2: MATERIALS AND METHODS

Virus Particle Production

Large quantities of virus particles were produced and amplified inside honey bee pupae using methods similar to those compiled by de Miranda et al. (2015) and Carrillo-Tripp et al. (2016) with minor modifications. Pupae were collected using two different methods: (1) pupal excision and (2) larval crawl-outs.

For pupal excision, brood frames containing white-eye pupae were selected and removed from healthy hives in the University of Illinois at Urbana-Champaign (UIUC) apiary (Champaign County, IL). White-eye pupae were gently excised using forceps and arranged in groups of 8-10 in Petri dishes lined with filter paper. All excised pupae were then injected with 1 µl of a 1% virus particle solution suspended in phosphate-buffered saline (PBS). The stock virus particles were provided by Dr. Adam Dolezal at UIUC and were identical to those described in Geffre et al. (2020). Injections were performed between the third and fourth abdominal tergites using a Combitip injector and a 30G needle. The Petri dishes were then stacked in a Tupperware container and placed in an incubator maintained at 34 ºC and 75% relative humidity for approximately 3 to 5 days. Daily inspections were performed and dead or rotting pupae were removed before bacterial or fungal buildup could occur. After allowing sufficient time for the virus particles to propagate, the pupae were placed into 50 ml conical tubes, homogenized by vortexing, and stored in the -80 ºC freezer until ready for extraction and concentration.

Eventually, brood extractions were completed with a second method: larval crawl-outs. This approach results in much higher throughput with no measurable difference in viral production. Instead of selecting brood frames containing pupae of an approximate age, honey bee queens were caged on an empty frame and allowed to lay eggs for 24 hours. Following the egg-laying period, queens were released, and the egg frames were marked and allowed to develop normally within the colony. At exactly 192-hours after caging (the point right before 5th instar larvae are capped), marked frames were transferred to an incubator maintained at 34 ºC and 75% relative humidity and laid face-down (larval cells facing downwards) onto Tupperware containers lined with Kimwipes ®. Overnight, the food-seeking instinct of late-stage larvae drives them to crawl out of their cells and drop onto the padded containers below. The larvae were then removed from the containers and individually arranged onto shallow, plastic trays.
lined with Kimwipes and tented with aluminum foil to retain moisture. The trays were then returned to the incubator and larvae were allowed to develop for an additional 5 to 6 days until they reached the white-eye stage, whereupon they were injected and sampled following the steps outlined in the pupal excision protocol.

**Virus Particle Extraction and Concentration**

Virus particles were grown and extracted from 8 separate colonies across 16 total samplings. The homogenized pupae gathered during viral production were thawed, transferred to centrifuge bottles, and mixed with approximately three volumes of 1X PBS on a shaker at room temperature for 10 minutes. The bottles were then centrifuged using a Sorvall RC-5B ultracentrifuge equipped with a GSA Rotor at 15,000 x g for 5 minutes at 4 ºC, after which the supernatant was decanted and filtered through cheesecloth to remove remaining fat globules. The supernatant was extracted using 0.3 volumes of 24:1 chloroform:isoamyl alcohol and centrifuged at 21,000 x g for 20 minutes at 4 ºC. The subsequent aqueous phase was then decanted into a beaker and RNAse-free water was added to bring the total volume to 200 ml. Beakers were then transferred to a 4 ºC cold room and placed onto magnetic stir plates. 4.6 g NaCl and 14 g polyethylene glycol 8000 (PEG) were slowly added to each beaker under constant gentle stirring. The mixtures were stirred continually for an additional 5 hours in the cold room, after which they were incubated overnight to allow virus particles to precipitate. Following incubation, the mixtures were transferred to clean bottles and centrifuged at 15,000 x g for 30 minutes at 4 ºC to recover a PEG-particle pellet. The pellet was then resuspended in TES buffer (10 mM Tris-HCl pH 7.5, 2 mM EDTA, 150 mM NaCL) and passed through an 18G needle ten times before being aliquoted into 2-mL centrifuge tubes. The tubes were centrifuged at 13,000 x g for 15 minutes at 4 ºC, after which the supernatant was separated and centrifuged again at the same settings to ensure total removal of all PEG. The remaining supernatant was then concentrated to approximately 2 ml using Amicon® Ultra-4 Centrifugal Filter Unit (MilliporeSigma) via centrifugation at 14,000 x g for 10 minutes at room temperature. The concentrated particles were passed through a 26G needle and centrifuged one final time at 14,000 x g for 5 minutes at room temperature. The viscous supernatant was then separated and stored at -80 ºC until ready for use.
**Virus Particle Purification and Quantification**

RNA was extracted from the concentrated virus particles using TRIzol (Life Technologies) extraction and DNA was removed using DNase I (RNase-free) (New England BioLabs). Based on the protocols described in Carrillo-Tripp et al. (2016), fragments of the IAPV genome were amplified using one-step reverse transcription (RT)-qPCR with the Power SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystems) following an absolute quantification approach using 100 ng total RNA per sample. Amplification was performed using a 384-well Quantstudio 6 Flex Real-Time PCR System (Applied Biosystems) and programmed as follows: reverse transcription [48 ºC – 30 min], enzyme activation [95 ºC – 10 min], PCR – 40 cycles of [95 ºC – 15 sec, 60 ºC – 1 min], melt curve [95 ºC – 15 sec, 60 ºC – 1 min] – [stepwise increases of 0.05 ºC/sec from 60-95 ºC, hold for 15 min]. The final copy numbers are extrapolated using a 1:10 serially diluted RNA-based standard curve containing sequences of the viruses of interest (Carrillo-Tripp et al. 2016).

**Cage Assays: General Protocol**

The following protocol describes the universal processes shared by all subsequent cage assays. Specific details regarding experimental treatments, treatment concentrations, sample size, and testing duration can be found in their corresponding subsections.

Five+ frames of bees on the verge of eclosion (<24 hours) sourced from at least three separate hives located within the University of Illinois at Urbana Champaign (UIUC) apiary were placed into emergence boxes and stored inside a 34 ºC incubator set to 50% relative humidity. After 24 hours, all frames were removed and newly emerged bees were brushed into a single collection tub. In order to minimize the hive genetic effects of any individual colony, all bees were gently mixed to produce a homogeneous mixture before being separated into acrylic cages with 35 bees per cage. The cages were then transferred to the same incubator and randomly arranged to prevent any microclimate effects. Immediately following the transfer, each cage received a small weigh boat containing either 600 µl of 30% sucrose solution mixed with an IAPV inoculum or an equivalent quantity of sterile but otherwise untreated sucrose solutions (see associated subsections for concentrations of viral inocula employed in particular experiments). After allowing for total consumption of inocula within all cages (approximately 12-14 hours), feeder tubes (15 mL Falcon tubes with 18G needle holes poked in the bottom) were inserted into
each cage, the contents of which were provided *ad libitum* and refilled as necessary throughout the course of each experiment (see subsections for experiment-specific feeder solutions). Mortality within every cage was recorded at 12-hour intervals for the first 72 hours of each experiment, following which recordings were switched to 24-hour intervals. Dead bees were removed following each instance of mortality observation to avoid repeated count errors. Although bees were not sampled from every experiment, every experiment in which bees were sampled followed an identical protocol: three bees were haphazardly selected at 36-hours post-inoculation (hpi) and placed into centrifuge tubes on dry ice. Dead bees were given priority over live bees for sampling to minimize depopulating the cages. The 36-hpi timepoint was selected as past experimentation has shown that this tends to be the point at which IAPV titers are highest within the bee without inducing lethal effects (Dolezal et al. 2019).

**IAPV Yearly Dose Establishment**

1. *Virus Dose-Response (2018, 2019)*

   In order to ascertain which viral concentration would result in approximately 65% in-cage survival at 36-hpi (sufficient for detecting differences between treatments without inducing total-cage mortality too rapidly), IAPV dose-response curves were generated in both field seasons (summers of 2018 and 2019) prior to phytochemical experimentation.

   **2018:** The cages were split into six viral treatments and one control, resulting in 7 cages per treatment, for a total of 49 cages. The inocula used in viral treatments were generated using 1:10 serial dilutions ranging from \( \frac{1}{100} \) to \( \frac{1}{10,000,000} \) stock IAPV particles in 30% sucrose solution (denoted as \( 10^{-2} \) to \( 10^{-7} \)). Control treatments received an equivalent amount of unamended 30% sucrose solution. Following inocula ingestion, all cages received feeder tubes containing 30% sucrose solution and were monitored for 7 days. This dose-response trial was subsequently repeated using identical experimental parameters with the exception of excluding treatments \( 10^{-5} \), \( 10^{-6} \), and \( 10^{-7} \) due to their virtually indistinguishable results. No sampling was performed for either of these two trials.
2019: Novel dose-response curves were generated for the second field season instead of relying on previously established dosages in case of potential particle degradation. These trials followed identical protocols to the 2018 series with a few minor changes: only concentrations \(10^{-2}, 10^{-3}, 10^{-4}\), and a control were tested \((n = 21, 21, 14,\) and \(21,\) respectively) and the cages were monitored for 4 days instead of 7.

Table 1. Summary of the virus dose-response experiment parameters. Concentrations represent \(\frac{1}{100}\) to \(\frac{1}{10,000,000}\) IAPV particles in 30% sucrose solution (denoted as \(10^{-2}\) to \(10^{-7}\)); \(C\) = unamended sucrose control. Cages indicate number of cages per concentration per trial; final row in each year lists cages per concentration in order of concentration.

| Year | Trial # | Concentrations | Cages | Duration (days) |
|------|---------|----------------|-------|-----------------|
| 2018 | 1       | \(C, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}\) | 7      | 7               |
|      | 2       | \(C, 10^{-2}, 10^{-3}, 10^{-4}\) | 7      | 7               |
|      | Total per concentration | \(C, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}\) | \(14, 14, 14, 14, 14, 7, 7, 7\) | -               |
| 2019 | 1       | \(C, 10^{-2}, 10^{-3}\) | 7      | 4               |
|      | 2       | \(C, 10^{-2}, 10^{-3}, 10^{-4}\) | 7      | 4               |
|      | 3       | \(C, 10^{-2}, 10^{-3}, 10^{-4}\) | 7      | 4               |
|      | Total per concentration | \(C, 10^{-2}, 10^{-3}, 10^{-4}\) | \(21, 21, 21, 14\) | -               |

I. Phytochemical Screening

2. Carvacrol Dose-Response

To decide on an experimental carvacrol dose, I tested concentrations scaled around a previously tested concentration of thymol, an isomer of carvacrol (Palmer-Young et al. 2017b). This preliminary testing followed a simplified version of the standard cage protocol, which did not include a viral inoculation phase or bee sampling but instead provided cages with \textit{ad libitum} carvacrol solution for the entire duration of the trial. Six carvacrol concentrations \((160, 16, 1.6, 0.16,\) and \(0.016\) ppm) and a control were tested for 14 days; \(n = 5\) cages per treatment \((n = 30\) cages total). All carvacrol solutions used in this and subsequent experiments were produced by mixing 30% sucrose solution with encapsulated carvacrol particles provided by Dr. Juan Andrade and his laboratory using the methods detailed by Gaur (2017).
3. Carvacrol Moderate Dose Treatment

Based on the carvacrol dose response curve generated in Experiment 2, I tested whether a 160 ppm carvacrol solution affects IAPV infection using the standard cage assay protocol. The experiment consisted of four treatments (10⁻² IAPV inoculated and uninoculated controls, 10⁻² IAPV inoculated and uninoculated 160 ppm carvacrol), ran for 7 days (168-hpi), with 7 cages per treatment, and no sampling was performed. Note that this experiment ran concurrently with Trial 2 of 2018 from Experiment 1 due to field season time constraints.

4. Carvacrol Acute Rescue

Following the standard cage assay protocol, virally inoculated bees were fed elevated levels of carvacrol to determine whether the essential oil could act as therapeutic agent for cases of acute IAPV infection. The experiment consisted of an uninoculated control, two carvacrol concentrations (1,000 and 2,000 ppm) that were either inoculated or uninoculated with 10⁻² IAPV, ran for 3 days (72-hpi), with 7 cages per treatment (35 cages total), and no sampling was performed (Table 2).

Table 2. Summary of carvacrol acute rescue experiment details. IAPV column denotes presence (+) or absence (-) of a 0.01% IAPV inoculum.

| Diet Type          | Concentration | IAPV (10⁻³) |
|--------------------|---------------|-------------|
| Control (30% sucrose) | N/A           | -           |
| Carvacrol          | 1,000 ppm     | - +         |
|                    | 2,000 ppm     | - +         |

5. Thymol Acute Rescue, Prophylactic, and Regular Treatment

The effects of thymol supplementation were tested across three treatment forms: acute rescue response, ad libitum feeding, and prophylactic treatment. Both acute rescue (1,000 ppm thymol) and ad libitum (160 ppm thymol) feeding treatments with or without 10⁻² IAPV inoculation followed the standard cage assay protocol, running for 11 days (264-hpi), with 7 cages per treatment, and no sampling being performed (Table 3). The prophylactic treatments were tested using the same parameters but differed in their virus inoculation schedules. Instead of being fed a virus inoculum immediately after being separated into cages, bees in the prophylactic treatments received 160 ppm thymol-sucrose solution ad libitum (with controls...
instead receiving 30% sucrose) for 6 days before being fed the standard virus inoculum. Following inoculation, both treatments were switched to a sucrose-only diet and monitoring was performed as normal.

Table 3. Summary of thymol acute rescue experiment details. IAPV column denotes presence (+) or absence (-) of a 0.01% IAPV inoculum.

| Treatment             | Thymol concentration | IAPV (10⁻²) |
|-----------------------|----------------------|-------------|
| Control – standard    | 0 ppm                | -           |
| Ad libitum            | 160 ppm              | - +         |
| Acute Response        | 1,000 ppm            | - +         |
| Prophylactic – Control| 0 ppm; 0 ppm         | +           |
| Prophylactic – Inoculated | 160 ppm; 0 ppm     | +           |

II. Caffeine Investigation

6. Differentiating Caffeine in Multiple Phytochemical Trial

Following the standard cage assay protocol, virus inoculated bees were subjected to three different phytochemical treatments with or without 10⁻⁴ IAPV inoculation: p-coumaric acid (82 ppm), quercetin (75.6 ppm), and caffeine (25 ppm). All feeder solutions contained 0.25% DMSO in order to solubilize p-coumaric acid (PCA) and quercetin (Liao et al. 2017). Concentrations for PCA and quercetin were selected based on the natural range observed in local honey and beebread (Liao et al. 2017) and 25 ppm caffeine was selected based on the natural range in which the substance is found in Citrus and Coffea nectar (11.7 – 95 ppm) (Kretschmar and Baumann 1999; Wright et al. 2013). The experiment ran for five days (120-hpi), with 10 cages per treatment (80 cages total), and standard sampling was performed (see Table 4 for treatment details). All treatments were evenly divided into two concurrent trials initiated within 24 hours of one another for practical reasons.

Table 4. Summary of multi-phytochemical experiment details. IAPV column denotes presence (+) or absence (-) of a 0.0001% IAPV inoculum.

| Treatment          | Concentration | IAPV (10⁻⁴) |
|--------------------|---------------|-------------|
| Control            | N/A           | - +         |
| Quercetin          | 75.6 ppm      | - +         |
| p-Coumaric Acid    | 82 ppm        | - +         |
| Caffeine           | 25 ppm        | - +         |
7. **Caffeine Trial**

Focusing on the effects of caffeine on viral infection, a subcomponent of Experiment 6 was replicated by repeating only the control and caffeine treatments (Table 4). The sample sizes were increased to 20 cages per treatment (80 cages total), but all other experimental parameters were held constant. Treatments were split into two concurrent trials (see Experiment 6).

8. **Caffeine Dose Responses (8A, 8B)**

To determine honey bee reactions to a range of caffeine concentrations, two separate caffeine dose response curves were generated: one testing IAPV inoculated bees and the other testing un inoculated bees. The uninoculated dose response trial (Experiment 8A) followed the standard cage assay protocol and four caffeine concentrations (25, 100, 1000, and 10,000 ppm) and a control (30% sucrose), all of which were inoculated with $10^4$ IAPV, were tested. The test ran for 7 days (168-hpi), with 20 cages per treatment (100 cages total), and standard sampling was performed. The uninoculated dose response trial (Experiment 8B) followed an identical protocol to Experiment 8A with the following two exceptions: (1) all cages received a virus-free inoculum containing only sucrose solution and (2) testing ran for 12 days (288-hpi). Caffeine is sufficiently soluble in sucrose solution so DMSO was not used in any of the treatments, as in Experiments 6-7. Both Experiments 8A and 8B were split into two concurrent trials (see Experiment 6).

*Sampled Bee Homogenization for Viral Quantification*

To confirm infection and address the question of viral tolerance vs. resistance, I quantified the viral titers of the caffeine-fed bees and their control counterparts sampled from Experiments 6 and 7. I purposefully selected only dead bees from all treatments for quantification to guarantee the selection of a known IAPV phenotype (i.e., death). In order to homogenize the sampled bees across all collected treatments, 8 cages were randomly selected with the use of a random number generator from each of the four treatments (caffeine+, - and control+, -) pooled across Experiments 6 and 7; for a total of 32 cages. From each cage, 2 out of the 3 sampled bees were selected for quantification; $n = 64$ samples. Dead bees were ‘simulated’ in the treatments with fewer than necessary dead-upon-collection samples (i.e. the uninoculated
cages) by haphazardly selecting bees sourced from uninoculated treatments previously killed via freezing at -80 °C, placing them into empty cages, and transferring them to an incubator (34 °C, 50% RH). After 12 hours of incubation, the simulated dead bees were removed from their cages and immediately processed for viral quantification using the previously stated methodology.

Statistical Analyses

Survival analyses in Experiments 2-5 were conducted using Cox Proportional Hazard modeling performed in R using the survival (version 3.1-11) and survminer (version 0.4.6) packages. Experiments 6-8 were subdivided into two concurrent trials each that were initiated within 24 hours of one other. Consequently, these experiments were analyzed using mixed effects Cox models in the coxme package (version 2.2-16) to account for the trial factor. Subsequent pairwise comparisons within all Cox models were corrected using Benjamini-Hochberg corrections to reduce Type I error rate. Viral loads obtained from RT-qPCR were estimated using QuantStudio RealTime PCR Software (Applied Biosystems, version 1.3) and then analyzed with R using the dplyr package (version 0.8.3). Normality assumptions were not met despite Box-Cox transformations, and thus the data were analyzed using the Kruskal-Wallis rank sum test. Pairwise differences were determined using Wilcoxon rank sum and multiple comparisons were corrected using Benjamini-Hochberg corrections. All graphics were generated in R using the ggsurvplot function within the survminer package (version 0.4.6).
CHAPTER 3: RESULTS

IAPV Yearly Dose Establishment

All experiments involving viral infection used the same purified 99.9% IAPV particles produced in the summer of 2018 using the previously described virus isolation methods.

In 2018, the 1% virus solution ($10^{-2}$) was selected as the target concentration for all cage experiments conducted that year (Experiments 2-5) based on the 36-hpi survival rate (Figure 1A). In 2019, all tested treatments ($10^{-2} - 10^{-4}$) experienced a rise in mortality compared to 2018 (Figure 1B). As a result, the 0.01% virus solution ($10^{-4}$) was selected as the target concentration for all subsequent cage experiments (Experiments 6-8).

Figure 1. Dose-response survival curves of honey bee cages inoculated with IAPV in 2018 (A) and 2019 (B) at 0 hours post-infection. Treatments represent IAPV inocula concentrations with $10^{-2}$ to $10^{-7}$ denoting $\frac{1}{100}$ to $\frac{1}{10,000,000}$ stock IAPV particles in 30% sucrose solution; controls were uninoculated. All cages fed diets of 30% sucrose solution. $n = 77$ cages total in 2018 and in 2019.
I. Phytochemical Screening (Experiments 2-6)

*Carvacrol Screening*

In the carvacrol dose-response experiment, responses of bees in control cages fed unamended sucrose did not differ significantly from responses of bees in cages fed the lower concentrations of carvacrol tested (0.016, 0.16 ppm) (Figure 2). When compared to the control, consuming 16 or 160 ppm carvacrol significantly increased mortality risk by 37.9% and 62%, respectively, but neither of the two treatments were significantly different from one another (Table 5). As a result, the higher of the two concentrations, 160 ppm, was selected for additional screening to maximize the chances of discerning an effect.

![Figure 2](image)

**Figure 2.** Dose-response survival curves of honey bee cages fed varying concentrations of carvacrol in 30% sucrose solution, line colors indicative of treatment. *n* = 5 cages per concentration. NS, not significant (pairwise Cox proportionate hazards models, Benjamini-Hochberg correction).
Table 5. Hazard ratios (HR) of pairwise Cox proportionate hazards model comparisons between treatments in the carvacrol dose-response trial (Exp. 2). Blue and red HRs indicate significant increased and decreased risk of death, respectively (*p < 0.05, **p < 0.01, ***p < 0.001; Benjamini-Hochberg correction). Comparisons are read from left-row to top-column; e.g. Row 2 treatment increases or decreases risk of death by a factor of x when compared to Column 3. Control = sucrose only. n = 5 cages for all treatments.

|          | 0.016 ppm | 0.16 ppm | 1.6 ppm | 16 ppm | 160 ppm |
|----------|-----------|----------|---------|--------|---------|
| 0.016 ppm| N/A       | N/A      | N/A     | N/A    | N/A     |
| 0.16 ppm | 0.681     | N/A      | N/A     | N/A    | N/A     |
| 1.6 ppm  | 1.250*    | 2.234*** | N/A     | N/A    | N/A     |
| 16 ppm   | 1.262**   | 1.715*** | 1.327   | N/A    | N/A     |
| 160 ppm  | 1.201***  | 1.441*** | 1.181*  | 1.042  | N/A     |
| Control  | 0.950     | 1.030    | 0.785***| 0.621***| 0.380***|

Overall, none of the tested carvacrol treatments improved the survivorship of IAPV-inoculated bees relative to their control treatments. Neither the inoculated (HR = 1.01, p = 0.964) nor uninoculated (HR = 0.61, p = 0.319) 160 ppm carvacrol treatments significantly altered survival compared to controls (Figure 3, Table 6). Note that the inoculated control data set included in Experiment 3 are from the 2018 virus dose-response trials (Figure 1A) to provide a frame of reference, but these data were not produced concurrently with the remaining three treatments and are thus not present in the analysis. The higher concentrations used in the acute rescue trial (1,000 ppm, 2,000 ppm) not only failed to improve survivorship, they increased the mortality rate (Figure 4). Surprisingly, the bees in the inoculated 1,000 ppm treatment had a significantly higher hazard risk than those in the inoculated 2,000 ppm treatment (HR = 0.90, p < 0.001), but the survivorship of the bees in the uninoculated 1,000 ppm treatment was not significantly different from that of the control bees (HR = 1.68, p = 0.22) while the survivorship of bees in the uninoculated 2,000 ppm treatment was significantly different (HR = 2.03, p < 0.001).
**Figure 3.** Average survival curves of honey bee cages inoculated (+) or uninoculated (-) with IAPV and fed diets of 30% sucrose solution or 160 ppm carvacrol-sucrose solution. Note this Figure contains inoculated control data from a separate assay run under identical experimental conditions as a point of reference. *n* = 7 cages per treatment. NS, not significant (pairwise Cox proportionate hazards models, Benjamini-Hochberg correction).

**Table 6.** Hazard ratios (HR) of pairwise Cox proportionate hazards model comparisons between treatments in the carvacrol treatment trial (Exp. 3). Blue and red HRs indicate significant increased and decreased risk of death, respectively (**p < 0.001; Benjamini-Hochberg correction). Comparisons are read from left-row to top-column; e.g. Row 2 treatment increases or decreases risk of death by a factor of *x* when compared to Column 3. + (IAPV inoculated), - (uninoculated); Control = sucrose only, CV = 160 ppm carvacrol. *n* = 7 cages for all treatments.

|           | Control- | CV-   | CV+   |
|-----------|----------|-------|-------|
| Control-  | N/A      | N/A   | N/A   |
| CV-       | 0.606    | N/A   | N/A   |
| CV+       | 4.199*** | 28.465*** | N/A |
| Control+  | 2.580*** | 5.274*** | 1.005 |
**Figure 4.** Average survival curves of honey bee cages inoculated (+) or un inoculated (-) with IAPV and fed diets of 30% sucrose solution or high concentration carvacrol-sucrose solution. No difference was detected between survival of all uninoculated treatments except for 2000-, which experienced significantly lower survival than all other uninoculated treatments. $n = 7$ cages per concentration. NS, not significant; **p < 0.01, ***p < 0.001 (pairwise Cox proportionate hazards models, Benjamini-Hochberg correction).

**Table 7.** Hazard ratios (HR) of pairwise Cox proportionate hazards model comparisons between treatments in the carvacrol acute rescue trial (Exp. 4). Blue and red HRs indicate significant increased and decreased risk of death, respectively (**p < 0.01, ***p < 0.001; Benjamini-Hochberg correction). Comparisons are read from left-row to top-column; e.g. Row 2 treatment increases or decreases risk of death by a factor of $x$ when compared to Column 3. + (IAPV inoculated), - (uninoculated); Control = sucrose only. $n = 7$ cages for all treatments.

|          | Control | 1000 ppm- | 1000 ppm+ | 2000 ppm- |
|----------|---------|-----------|-----------|-----------|
| Control  | N/A     | N/A       | N/A       | N/A       |
| 1000 ppm-| 1.677   | N/A       | N/A       | N/A       |
| 1000 ppm+| 7.559***| 34.276*** | N/A       | N/A       |
| 2000 ppm-| 2.029***| 2.215***  | 0.111***  | N/A       |
| 2000 ppm+| 2.560***| 2.955***  | 0.897**   | 6.372***  |
None of the three thymol dosing strategies proved to be effective in improving survivorship of IAPV-inoculated bees. Although there was no significant difference detected between the uninoculated 160 ppm thymol treatment and the control, hazard modeling showed a very strong trend toward improved relative survivorship in the thymol-fed cages (HR = 0.66, p = 0.065). By contrast, the mortality in the inoculated 160 ppm thymol treatment was much higher when compared to the carvacrol treatment at the same dose (Figure 3, 5). Furthermore, the inoculated 1,000 ppm thymol experienced significantly lower survivorship than the uninoculated 1,000 ppm (HR = 2.69, p < 0.001), with bees in both treatments experiencing sharp declines in survivorship (~5% survival at Day 11), although this drop did not begin in the uninoculated group until Day 5 (Figure 5). Additionally, thymol does not appear to provide any prophylactic protective effect. After viral inoculation on Day 5, survival rates of bees in both prophylactic treatments rapidly dropped and the trial concluded with no significant difference between the two treatments (HR = 1.16, p = 0.168).

**Figure 5.** Average survival curves of honey bee cages inoculated (+) or uninoculated (-) with IAPV and fed varying concentrations of thymol-sucrose solution. “Pro” denotes prophylactic treatments in which inoculation did not occur until day 5. No difference was detected between the prophylactic treatments. n = 7 cages per treatment. NS, not significant; **p < 0.001** (pairwise Cox proportionate hazards models, Benjamini-Hochberg correction).
Table 8. Hazard ratios (HR) of pairwise Cox proportionate hazards model comparisons between treatments in the thymol trial (Exp. 5). Blue and red HRs indicate significant increased and decreased risk of death, respectively (**p < 0.01, ***p < 0.001; Benjamini-Hochberg correction). Comparisons are read from left-row to top-column; e.g. Row 2 treatment increases or decreases risk of death by a factor of x when compared to Column 3. + (IAPV inoculated), - (uninoculated); Control = sucrose only, ProCon = prophylactic fed sucrose only, ProTY = prophylactic fed thymol then sucrose; n = 7 cages for all other treatments.

|               | Control | 160 ppm- | 160 ppm+ | 1000 ppm- | 1000 ppm+ | ProCon+ |
|---------------|---------|----------|----------|-----------|-----------|---------|
| Control       | N/A     | N/A      | N/A      | N/A       | N/A       | N/A     |
| 160 ppm-      | 0.658   | N/A      | N/A      | N/A       | N/A       | N/A     |
| 160 ppm+      | 2.923***| 12.697***| N/A      | N/A       | N/A       | N/A     |
| 1000 ppm-     | 2.078***| 3.770***| 0.595*** | N/A       | N/A       | N/A     |
| 1000 ppm+     | 1.951***| 2.810***| 1.148**  | 2.690***  | N/A       | N/A     |
| ProCon+       | 1.433***| 1.773***| 0.790*** | 0.812***  | 0.296***  | N/A     |
| ProTY+        | 1.372***| 1.615***| 0.842*** | 0.910**   | 0.560***  | 1.157   |

Multi-phytochemical Screening

All of the treatments involving uninoculated bees in the multi-phytochemical trial were not significantly different from one another, with the exception of uninoculated bees consuming quercetin, which survived significantly better than uninoculated bees consuming caffeine (HR = 0.46, p < 0.001) (Figure 6). However, in the inoculated group, only mortality risk for caffeine-fed bees differed significantly from mortality risk in all other treatments, with their mortality risk reduced by 21.9% when compared to the inoculated control (HR = 0.78, p = 0.008) (Table 9). As a result, caffeine was selected as the only phytochemical eligible for continued investigation.
Figure 6. Average survival curves of honey bee cages inoculated (+) or uninoculated (-) with IAPV and fed sucrose solution (control), caffeine (caff), p-coumaric acid (PCA), or quercetin (quer). Caffeine- experienced significantly lower survival than quercetin-; all other (-) treatments were not significantly different from one another. Caffeine+ experienced significantly higher survival than all other (+) treatments; all other (+) treatments were not significantly different from one another. \( n = 10 \) cages per treatment. NS, not significant; *\( p < 0.05 \), **\( p < 0.01 \) (pairwise Cox proportionate hazards models, Benjamini-Hochberg correction).

Table 9. Hazard ratios (HR) of pairwise Cox mixed effects model comparisons between treatments in the multi-phytochemical trial (Exp. 6). Blue and red HRs indicate significant increased and decreased risk of death, respectively (*\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \); Benjamini-Hochberg correction). Comparisons are read from left-row to top-column; e.g. Row 2 treatment increases or decreases risk of death by a factor of \( x \) when compared to Column 3. + (IAPV inoculated), - ( uninoculated); Control = sucrose only, Caff = caffeine, PCA = p-coumaric acid, Quer = quercetin. \( n = 9 \) cages for Caff+; \( n = 10 \) cages for all other treatments.

|        | Control- | Control+ | Caff- | Caff+ | PCA- | PCA+ | Quer- |
|--------|----------|----------|-------|-------|------|------|-------|
| Control- | N/A      | N/A      | N/A   | N/A   | N/A  | N/A  | N/A   |
| Control+| 6.416*** | N/A      | N/A   | N/A   | N/A  | N/A  | N/A   |
| Caff-   | 1.101    | 0.185**  | N/A   | N/A   | N/A  | N/A  | N/A   |
| Caff+   | 1.561*** | 0.781**  | 3.254*** | N/A   | N/A  | N/A  | N/A   |
| PCA-    | 1.034    | 0.547*** | 0.920 | 0.228*** | N/A  | N/A  | N/A   |
| PCA+    | 1.440*** | 0.948    | 1.743*** | 1.245*** | 7.223*** | N/A  | N/A   |
| Quer-   | 0.870    | 0.585*** | 0.755* | 0.460*** | 0.637 | 0.058*** | N/A   |
| Quer+   | 1.297*** | 0.971    | 1.390*** | 1.112*** | 1.882*** | 0.978 | 15.884*** |
II. Caffeine Investigation

Caffeine Trials

The survival trends of the second set of caffeine trials (Exp. 7) remained consistent with those of the multi-phytochemical trial (Figure 6). Combining the results of both trials (Figure 7) does not significantly alter the magnitude of effect (HR = 0.86, p = 0.005), with the hazard ratios between inoculated caffeine and inoculated control treatments of both trials remaining within 95% confidence intervals of one another.

Figure 7. Average survival curves of honey bee cages inoculated (+) or uninoculated (-) with IAPV and fed sucrose solution or 25 ppm caffeine. Note this Figure contains control and caffeine data collected from a separate assay run under identical experimental conditions. No difference was detected between uninoculated treatments while caffeine+ experienced significantly higher survival than control+. n = 30 cages per treatment. NS, not significant; **p < 0.01 (pairwise Cox proportionate hazards models, Benjamini-Hochberg correction).
Table 10. Hazard ratios (HR) of pairwise Cox mixed effects model comparisons between treatments in the caffeine only trials (Exp. 7 + part of Exp. 6). Note this table contains the control and caffeine data from Table B. Blue and red HRs indicate significant increased and decreased risk of death, respectively (**p < 0.01, ***p < 0.001; Benjamini-Hochberg correction). Comparisons are read from left-row to top-column; e.g. Row 2 treatment increases or decreases risk of death by a factor of $x$ when compared to Column 3. + (IAPV inoculated), - (uninoculated); Control = sucrose only, Caff = 25 ppm caffeine. $n = 29$ cages for Caff+, 30 cages for all other treatments.

|       | Control- | Control+ | Caff-  |
|-------|----------|----------|-------|
| Control- | N/A      | N/A      | N/A   |
| Control+ | 6.423*** | N/A      | N/A   |
| Caff-   | 1.117    | 0.200*** | N/A   |
| Caff+   | 1.690*** | 0.863**  | 3.554*** |

Caffeine Dose-Response

In the caffeine dose-response trial with inoculated bees (Exp. 8A), 100 ppm caffeine significantly decreased mortality risk by 13.5% and 34.3% when compared to the inoculated control and 25 ppm caffeine, respectively (HR = 0.87, $p = 0.002$; HR = 0.66, $p < 0.001$) (Table 11). The control and 25 ppm treatment were not significantly different from one another but there was a trend for bees in the 25 ppm treatment to experience an increased mortality risk (HR = 1.61, $p = 0.083$), a result directly in contrast to the results observed in previous caffeine trials (Figures 7, 8). Both of the elevated dosages (1,000 ppm and 10,000 ppm) significantly increased mortality risk when compared to all other treatments, with all cages with 10,000 ppm caffeine experiencing complete mortality by Day 3 (Table 11).
Figure 8A. Average survival curves of honey bee cages inoculated with IAPV and fed varying concentrations of caffeine-sucrose solution (10k = 10,000 ppm). No difference was detected between control and 25 ppm; 100 ppm experienced significantly higher survival than both control and 25 ppm (**p < 0.01, ***p < 0.001, respectively). n = 20 cages per treatment. NS, not significant; pairwise Cox proportionate hazards models, Benjamini-Hochberg correction.

Table 11. Hazard ratios (HR) of pairwise Cox mixed effects model comparisons between treatments in the inoculated caffeine dose-response trial (Exp. 8). Blue and red HRs indicate significant increased and decreased risk of death, respectively (**p < 0.01, ***p < 0.001; Benjamini-Hochberg correction). Comparisons are read from left-row to top-column; e.g. Row 2 treatment increases or decreases risk of death by a factor of x when compared to Column 3. Control = sucrose only. n = 20 cages per treatment.

|        | Control | 25 ppm | 100 ppm | 1000 ppm |
|--------|---------|--------|---------|----------|
| Control| N/A     | N/A    | N/A     | N/A      |
| 25 ppm | 1.612   | N/A    | N/A     | N/A      |
| 100 ppm| 0.865** | 0.657***| N/A     | N/A      |
| 1000 ppm| 1.133***| 1.111**| 1.892***| N/A      |
| 10k ppm| 1.639***| 1.771***| 3.175***| 4.722***|

In the caffeine dose-response trial with uninoculated bees (Exp. 8B), the 25 ppm dose significantly decreased mortality risk by 21.6% when compared to the uninoculated control (HR
= 0.78, p = 0.012) (Figure 9). This differentiation between the control and 25 ppm was not observed in the caffeine trials (Exp. 6, 7) between the same treatments tested under the same conditions. The remaining treatments each increased the mortality risk in the order of 10,000 ppm > 1,000 ppm > 100 ppm > control, with 10,000 ppm dropping to near complete mortality in all cages by Day 3 (Table 12).

Figure 8B. Average survival curves of honey bee cages fed varying concentrations of caffeine-sucrose solution (10k = 10,000 ppm). 25 ppm experienced significantly higher survival than all other treatments; control experienced significantly higher survival than all treatments but 25 ppm. n = 20 cages per treatment. **p < 0.01 (pairwise Cox proportionate hazards models, Benjamini-Hochberg correction).
Table 12. Hazard ratios (HR) of pairwise Cox mixed effects model comparisons between treatments in the uninoculated caffeine dose-response trial (Exp. 8). Blue and red HRs indicate significant increased and decreased risk of death, respectively (*p < 0.05, **p < 0.01, ***p < 0.001; Benjamini-Hochberg correction). Comparisons are read from left-row to top-column; e.g. Row 2 treatment increases or decreases risk of death by a factor of x when compared to Column 3. Control = sucrose only. \( n = 20 \) cages per treatment.

|          | Control | 25 ppm | 100 ppm | 1000 ppm |
|----------|---------|--------|---------|----------|
| Control  | N/A     | N/A    | N/A     | N/A      |
| 25 ppm   | 0.784*  | N/A    | N/A     | N/A      |
| 100 ppm  | 1.105** | 1.516*** | N/A | N/A |
| 1000 ppm | 1.323*** | 1.686*** | 1.894*** | N/A |
| 10k ppm  | 1.947*** | 2.494*** | 3.346*** | 8.125*** |

**Caffeine Cages Viral Quantification**

RT-qPCR confirmed the presence of IAPV infection in the inoculated treatments (Figure 9). The bees sampled from both inoculated treatments contained significantly higher viral loads than the uninoculated bees of either diet type (caffeine \( p = 0.019 \); control \( p = 0.004 \)). However, no significant differences in viral loads were detected within the infection groups across the diet treatments (inoculated \( p = 0.645 \); uninoculated \( p = 0.641 \)) (Kruskal-Wallis ANOVA, \( \chi^2 = 17.03, df = 3, p < 0.001; \) Wilcoxon rank-sum post-hoc; Benjamini-Hochberg corrections). Three cages were discarded from final analysis due to contamination, resulting in total \( n = 29 \) cages (caffeine- = 6, caffeine+ = 8, control- = 7, control+ = 8).
Figure 9. IAPV loads (log estimated genome equivalents per 100 ng total RNA) of bees sampled from the caffeine survival trials (Exp. 6-7) using RT-qPCR. Bars represent averages of pooled samples of inoculated (+) and uninoculated (-) treatments across both diet types (± 1 standard error). Letters denote significant differences between groups, Kruskal-Wallis ANOVA, Wilcoxon rank-sum post-hoc test, Benjamini-Hochberg correction, p < 0.05. n = 8 for both inoculated treatments, n = 6 and 7 for caffeine- and control- treatments, respectively.

Table 13. Reference table describing all experiments and their associated figures and statistics.

| Experiment | Description | Figure | Table(s) |
|------------|-------------|--------|----------|
| Exp. 1     | Virus dose-response trial from 2018, 2019 | Fig. 1 | Table 1  |
| Exp. 2     | Carvacrol dose-response trial (0.016 – 160 ppm) | Fig. 2 | Table 5  |
| Exp. 3     | Carvacrol moderate dose treatments | Fig. 3 | Table 6  |
| Exp. 4     | Carvacrol acute dose treatments (1,000 – 2,000 ppm) | Fig. 4 | Tables 2, 7 |
| Exp. 5     | Thymol treatments (acute dose, prophylactic, 160 ppm) | Fig. 5 | Tables 3, 8 |
| Exp. 6     | Multi-phytochemical trial (quercetin, PCA, caffeine) | Fig. 6 | Tables 4, 9 |
| Exp. 7     | All caffeine 25 ppm treatments | Fig. 7 | Table 10 |
| Exp. 8A    | Inoculated caffeine dose-response trial | Fig. 8A | Table 11 |
| Exp. 8B    | Uninoculated caffeine dose-response trial | Fig. 8B | Table 12 |
| Virus Quant.| Viral titers of Experiments 6 and 7 via RT-qPCR | Fig. 9 | -        |
CHAPTER 4: DISCUSSION

Phytochemical Screening

Overall, phytochemical screening process determined that neither of the two tested essential oils in their encapsulated forms is capable of ameliorating the effects of IAPV infection. These results were reflected in all tests for both phytochemicals, regardless of concentration (Figures 3-5). Although carvacrol has been shown to have antibacterial properties (Upadhyay and Venkitanarayanan 2016, Chang et al. 2017), there has been little to no examination of carvacrol as an agent for pathogen treatment in honey bees. My results suggest that, at least in its encapsulated form, carvacrol does not possess antiviral properties. Thymol, on the other hand, has been shown to be an effective antipathogenic agent in *Apis* species, upregulate antimicrobial peptide expression in honey bees, and able to suppress *Crithidia bombi* in bumble bees (Palmer-Young et al. 2017a, b). The acaricidal properties of thymol are well-documented and their use for *Varroa* mite control has risen in popularity with formulations such as Apiguard® or ApiLife VAR® (Floris et al. 2004; Gregorc and Planinc 2005). The 160-ppm dose implemented in the thymol treatments trial (Exp. 5) falls within the range of thymol residue concentrations that can be found in hive wax after a standard two-week Apiguard® treatment (147.7 ± 188.9 ppm) (Floris et al. 2004). Despite this value being two orders of magnitude greater than the thymol concentration detected in honey in the same experiment (0.96 ± 0.61 ppm), a previous study has shown that honey bees can consume a similar thymol dose without negative effects. Indeed, Costa et al. (2009) showed that bees fed 100 ppm thymol syrup not only survived significantly longer than control bees, they also had significantly reduced *Nosema ceranae* spore loads. Although it is possible the uninoculated 160-ppm thymol treatment could have yielded a similar longevity-boosting result had the thymol treatment experiment been extended past 11 days (a non-significant trend is visible in Figure 5 between 160 ppm- and control), neither the 160- nor the 1,000-ppm concentration is capable of improving survival in the presence of IAPV infection (Figure 5). Furthermore, thymol does not appear to prevent viral infection, as suggested by the two prophylactic treatments. Bees in both treatments experienced decreases in survivorship at similar rates following inoculation on Day 5, but additional testing will be needed to fully assess any potential prophylactic qualities.
One possibility for future testing would be to reduce the amount of time during which both essential oils are consumed. The young bees observed to experience DWV-level reduction by Palmer-Young et al. (2017b) were fed thymol solution for only 24 hours before being released into a hive—suggesting that the antiviral qualities of thymol are effective only in the short term. Additionally, subsequent tests involving encapsulated essential oils should confirm whether or not the phytochemical was taken up by the honey bee itself. One major advantage of encapsulating essential oils is that the oligosaccharide coating masks the powerful odor of the phytochemical in its pure form, allowing for the administration of much higher doses than would otherwise be repellent to honey bees (Colin et al. 2019). Although the coating surrounding the particles is easily broken down within a human digestive system (Gaur 2017), it is possible that the honey bee digestive tract might not be as adept at releasing encapsulated phytochemicals, and, thus, the bees may not be actually gaining any therapeutic benefits. According to Ellis and Baxendale (1997), thymol is about 10 times more toxic to honey bees than carvacrol, yet the uninoculated bees in the 160- and 1,000-ppm carvacrol treatments had roughly the same survival rate as the equivalent thymol treatments at the same time points (Figures 3-5). However, the carvacrol and thymol treatments were never run concurrently with one another, so these similarities are based entirely on survival trends rather than an established lack of oligosaccharide release. In fact, the rapid decline of the uninoculated bees in the 1,000 ppm thymol-fed cages relative to the bees in the uninoculated control cages in Experiment 5 suggests that thymol was being released and subsequently overdosing the bees (Figure 5). Additionally, Ellis and Baxendale’s (1997) assay was performed using phytochemical fumigants instead of direct ingestion, meaning that the magnitude of toxicity may not be directly translatable to the system I tested. Further investigations will be needed to validate the results obtained in my experiments on encapsulated carvacrol and thymol and IAPV infection.

The screening process also revealed that neither $p$-coumaric acid nor quercetin were capable of improving the survivorship of honey bees inoculated with IAPV (Figure 6). Both of these phytochemicals possess multiple survivorship-boosting capabilities, including reducing *N. ceranae* spore load, upregulating detoxification genes, and increasing pesticide tolerance (Mao et al. 2013; Liao et al. 2017; Wong et al. 2018; Bernklau et al. 2019). Furthermore, they both inhibit the replication and production of a large number of viruses (Canine Distemper Virus, human rhinovirus, herpes simplex virus, Japanese encephalitis virus, among others) (Graziani et
al. 1983; Schnitzler et al. 2010; Johari et al. 2012; González-Búrquez et al. 2018; Kwon et al. 2019), although these tests have thus far been conducted only in mammalian systems. My current results do not support the hypothesis that these phytochemicals help combat IAPV infection, but it is difficult to rule out whether or not they activate any of the various defense mechanisms honey bees utilize to defend against viral infection without subsequent molecular analyses.

Caffeine Investigation

Caffeine, a defensive alkaloid that is capable of increasing pesticide tolerance (Balieira et al. 2018) and reducing Nosema spore count in honey bees (Strachecka et al. 2014; Bernklau et al. 2019), was identified by the phytochemical screening process as a potential agent for improving resilience against virus infection. The initial caffeine trials (Exp. 6, 7) clearly showed it significantly improves the survival of IAPV-inoculated honey bees, furthering our currently limited understanding of its physiological effects in honey bee systems. Most notably, 25 ppm, a concentration that bees can and do encounter in naturally occurring floral resources (Wright et al. 2013; Couvillon et al. 2015), was sufficient to generate a beneficial effect. With its ease-of-access and already established familiarity, caffeine has great potential for stakeholders and beekeeping communities as a relatively inexpensive and practical way of treating virus infections. However, it should be noted that, although caffeine clearly confers beneficial effects, it does not constitute a ‘medicine’ and therefore should not be counted on to completely clear viral infections. Instead, caffeine can serve as a useful instrument for furthering understanding of honey bee virus infections at the physiological and molecular level, while also acting as a potential antiviral dietary supplement.

The results of the caffeine dose-response curves (Exp. 8A, 8B) provide a foundation for understanding the range of effects of caffeine on IAPV infections. Bees in both 10,000 ppm treatments rapidly succumbed to the high dosages regardless of infection status, confirming the toxic caffeine range in bees measured by Detzel and Wink (1993) (Figures 8, 9). The 1,000-ppm treatments, although not as immediately lethal as the 10,000 ppm, was still detrimental for both inoculated and uninoculated bees and increased the mortality risk relative to all other treatments (except 10,000 ppm) in their respective experiments, albeit with differing magnitudes of effect (Tables 11, 12). At concentrations under 1,000 ppm, the results of the two experiments diverge. In the inoculated dose-response (Exp. 8A) 100 ppm significantly improved survivorship
compared to both the inoculated positive control and the 25-ppm treatment (Table 11). At 100 ppm, caffeine is still within its natural range found in Citrus flower nectar (Kretschmar and Baumann 1999) and it is reasonable to expect an increased dose will amplify the antiviral effect, to a point. However, the results of the inoculated dose-response trial exhibited some key differences from those observed in the initial caffeine trials (Figure 7). First, the increase in virus-inoculated survivorship induced by the 25-ppm caffeine treatments in Experiments 6 and 7 was not observed in the same treatments of Experiment 8A, despite the fact that these treatments shared identical physical parameters across all experiments. In fact, the 25-ppm treatment in Experiment 8A actually trended toward having a lower survival rate than the inoculated control, although this trend was ultimately not significant (Table 11). Overall, bees in both inoculated control and 25-ppm treatments in Experiments 6 and 7 had higher survival rates than bees in equivalent treatments in Experiment 8A at the same timepoints. The magnitude of effect between the 100-ppm treatment and the positive control in Experiment 8A was nearly identical to the effect measured between the 25-ppm treatment and the inoculated control of Experiment 7, reducing mortality risk by 13.7% and 13.5%, respectively. The 100-ppm treatment of Experiment 8A has essentially shifted to represent the same longevity-improving capabilities detected in Experiments 6 and 7.

One hypothesis for explaining this shift in treatment effectiveness could be the result of a seasonal factor—the bees tested in Experiments 6 and 7 were collected and assayed in late June and early July of 2019 whereas Experiments 8A and 8B did not occur until mid- to late August of the same year. It is possible that the hives from which Experiment 8’s bees were sourced had already begun to experience the nutritional deficit honey bee colonies can undergo in midwestern agricultural landscapes. Dolezal et al. (2019b) found that hives surrounded by such landscapes typically suffered from a lack of forage availability, reducing colony weight and individual fat stores, and eventually culminating in an overall poor colony nutritional state. Moreover, they observed that this decline consistently began in early August, which coincides with the seemingly mismatched survival rates I observed in Experiment 8A. I posit that this potential forage dearth could have contributed to the change in caffeine-virus interaction observed in the inoculated bees in the caffeine dose-response trial, with the nutritionally stressed bees requiring a stronger dose of caffeine to achieve the same level of survivorship improvement seen earlier in the season. There is, of course, always the possibility that aberrations in typical honey bee
survival patterns can be attributed to hive genetics or intense pathogen pressure. However, the method used in all cage assays was purposefully designed to minimize these possibilities and measuring genetic differences or parasitic load after the assays was beyond the scope of this project.

The bees in the control and 25-ppm treatments of the uninoculated caffeine dose-response assay (Exp. 8B) also yielded somewhat contrary results when compared to the same treatments tested in Experiments 6 and 7. The initial caffeine trials yielded no significant difference between the survival rates of bees in the control and 25-ppm treatments but Experiment 8B revealed that 25 ppm significantly decreased mortality risk by 21.6% compared to control (Figure 8B, Table 12). However, Experiment 8B also extends lifespan for the longest period of time among all caffeine trials (over twice that of Experiments 6 and 7), and it is possible that 25-ppm caffeine does not produce a measurable effect until after 120-hpi (5 days), the point at which the initial caffeine trials were terminated. Alternatively, it is also possible that the inconsistent survival detected in Experiment 8B could have been caused by background virus infection. Most managed honey bee colonies are infected by a large number of different viruses, which can often persist throughout the hive without manifesting acute symptoms (Chen et al. 2004; Carrillo-Tripp et al. 2016). As previously discussed, the bees used in Experiment 8B were collected in late August, the same point at which *Varroa* populations typically tend to rise (Rosenkranz et al. 2010), further increasing the chances of an elevated viral load not mediated by humans. Undetected viral infections could have depressed the survivorship of the supposedly uninoculated control bees and the 25-ppm caffeine dose may have been just enough to ameliorate the viral effects without also poisoning the bees, as in the case of the 10,000 ppm treatments. Future experimentation examining caffeine dose-response of inoculated and uninoculated bees should include repeated tests across a broader temporal range to take into account any seasonal variation, as well as testing a narrower range of caffeine concentrations under 1,000 ppm to obtain a clearer image of the stratification of dosage on antiviral effect.

Experiments 8A and 8B laid the groundwork for research in several promising directions. In all of the cage assays, I intentionally excluded the addition of an external protein source as a means of maximizing the chances of observing an effect. Pollen is a critical component of the bee diet, affecting many aspects of bee biology (Wright et al. 2018), including immunocompetence (Alaux et al. 2010), pesticide tolerance (Liao et al. 2017), and response to
virus infection. Dolezal et al. (2019a) showed that even the quality of pollen can influence mortality rates of bees infected with viruses. The exclusion of dietary pollen therefore prevents the accidental masking or augmentation of any potential effects incurred by phytochemical consumption, an observation that was shared by Palmer-Young et al. (2017b) when discussing the effects of phytochemicals on pathogen infection. However, following the confirmation of caffeine’s ameliorative qualities, subsequent studies should introduce a protein supplement to examine how it affects the caffeine-virus interaction.

Lastly, RT-qPCR analysis confirmed the presence of viral infection in the expected groups across Experiments 6 and 7, with the inoculated treatments of both diet types having significantly greater viral loads than their uninoculated counterparts (Figure 9). These data are important in addressing the question of whether caffeine influences viral tolerance or viral resistance, but the conclusions that can be drawn are not definitive. The viral titers are not significantly different from one another within infection groups across both diet types, suggesting that caffeine does not actively increase viral resistance by reducing titers or hindering replication. However, this does not necessarily validate the presence of a tolerance mechanism, either. The titers are restricted to a single timepoint and thus only a snapshot of the infection progression is available. Moreover, I collected data only on the bees that died of infection, all of which had similar IAPV levels, suggesting that similar levels are linked to mortality. However, future experiments that perform additional sampling conducted at multiple timepoints on live and dead bees are necessary to understand this phenomenon. Caffeine-treated bees may be able to tolerate higher virus titers (although my data on bees that died of infection does not support this possibility), or they may be better able to resist infection by suppressing IAPV replication. In the case of the latter, I would predict that randomly-collected live bees from the caffeine-treated groups will exhibit lower IAPV levels than infected controls.

The survivorship-enhancing effects I documented provide a useful comparison to caffeine tested in other insect systems, highlighting the range of coevolutionary relationships that bees have with the plants they use as nectar sources, relative to other insect nectarivores. Multiple studies have shown that caffeine possesses larvicidal properties when administered to *Aedes* mosquitoes (Laranja et al. 2003; Eastep et al. 2012), and Njoroge (dissertation in progress) demonstrated that concentrations similar to those that improved IAPV-inoculated bee survivorship (100, 200 ppm) actually significantly reduced the lifespan of *A. albopictus*. Eastep
et al. (2012) found that caffeine did not significantly alter the titers of the La Crosse virus (a mosquito-borne virus capable of causing encephalitis in humans) in A. albopictus, a result similar to that of IAPV measured in my experiments.

Eventually, these caffeine treatments could be used to scale the project up to semi-field conditions to test effects on whole colonies and use gene expression measurements to characterize the genes responsible for resistance to viral pathogens. Ascertaining the full scope of caffeine’s antiviral qualities can help craft recommendations for reducing virus pressure in managed honey bee colonies, provide understanding of basic mechanisms by which bees reduce pathogen infection, and enrich the understanding of the coevolution between plant nectar sources and pollinators.

Conclusion

Through a series of screening experiments, I have shown that p-coumaric acid, quercetin, and encapsulated thymol and carvacrol do not have a significant effect on honey bee survivorship during IAPV infection. While this finding is not consistent with the hypothesis that these chemicals provide protection against virus infection, it is important to note that the high-throughput bioassay screens I performed does not account for all possible scenarios. Most notably, I have demonstrated that caffeine consumed at a naturally occurring level can significantly increase honey bee survival when they are infected with IAPV. Furthermore, I have expanded upon my initial caffeine results by establishing caffeine dose-response curves in honey bees to determine the scope of caffeine’s effect at different concentrations with or without viral infection. These dose-response curves show that 100 ppm caffeine has a similar effect to that of 25 ppm in IAPV-inoculated bees but only 25 ppm improves survivorship relative to controls fed unamended sucrose in the absence of IAPV inoculation. To attain a more granular understanding of caffeine’s effects on virus infection, I would recommend future research along four additional pathways: (1) testing a narrower dose range under 1,000 ppm across a wider temporal range, (2) including dietary pollen in regular test diet, (3) increasing bee sampling for a clearer molecular picture of virus propagation rates, and (4) eventually expanding into testing additional viruses, such as the ubiquitous DWV. Exploration into these areas can both allow for a better grasp of how caffeine interacts with virus and improve comprehension of virus-phytochemical interactions in general.
REFERENCES

Adler, L. S. (2000). The ecological significance of toxic nectar. *Oikos, 91*(3), 409–420. https://doi.org/10.1034/j.1600-0706.2000.910301.x

Aizen, M. A., & Harder, L. D. (2009). The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. *Current Biology, 19*(11), 915–918. https://doi.org/10.1016/j.cub.2009.03.071

Alaux, C., Ducloz, F., Crauser, D., & Le Conte, Y. (2010). Diet effects on honeybee immunocompetence. *Biology Letters, 6*(4), 562–565. https://doi.org/10.1098/rsbl.2009.0986

Allen, M., & Ball, B. (1996). The incidence and world distribution of honey bee viruses. *Bee World, 77*(3), 141–162. https://doi.org/10.1080/0005772X.1996.11099306

Amiri, E., Seddon, G., Smith, W. Z., Strand, M. K., Tarpy, D. R., & Rueppell, O. (2019). Israeli acute paralysis virus: Honey bee queen–worker interaction and potential virus transmission pathways. *Insects, 10*(1). https://doi.org/10.3390/insects10010009

Balieira, K. V. B., Mazzo, M., Bizerra, P. F. V., Guimarães, A. R. de J. S., Nicodemo, D., & Mingatto, F. E. (2018). Imidacloprid-induced oxidative stress in honey bees and the antioxidant action of caffeine. *Apidologie, 49*(5), 562–572. https://doi.org/10.1007/s13592-018-0583-1

Baracchi, D., Brown, M. J. F., & Chittka, L. (2015). Weak and contradictory effects of self-medication with nectar nicotine by parasitized bumblebees. *F1000Research, 4*, 73. https://doi.org/10.12688/f1000research.6262.1

Bernklau, E., Bjostad, L., Hogeboom, A., Carlisle, A., & Arathi, H. S. (2019). Dietary phytochemicals, honey bee longevity and pathogen tolerance. *Insects, 10*(1), 1–12. https://doi.org/10.3390/insects10010014

Carrillo-Tripp, J., Dolezal, A. G., Goblirsch, M. J., Miller, W. A., Toth, A. L., & Bonning, B. C. (2016). *In vivo and in vitro* infection dynamics of honey bee viruses. *Scientific Reports, 6*(November 2015), 1–12. https://doi.org/10.1038/srep22265

Chang, Y., Yoon, H., Kang, D. H., Chang, P. S., & Ryu, S. (2017). Endolysin LysSA97 is synergistic with carvacrol in controlling *Staphylococcus aureus* in foods. *International Journal of Food Microbiology, 244*, 19–26. https://doi.org/10.1016/j.ijfoodmicro.2016.12.007

Chen, Y. P., Pettis, J. S., Corona, M., Chen, W. P., Li, C. J., Spivak, M., Visscher, P. K., DeGrandi-Hoffman, G., Boncristiani, H., Zhao, Y., VanEngelsdorp, D., Delaplane, K., Solter, L., Drummond, F., Kramer, M., Lipkin, W. I., Palacios, G., Hamilton, M. C., Smith, B., … Evans, J. D. (2014). Israeli acute paralysis virus: Epidemiology, pathogenesis and
implications for honey bee health. *PLoS Pathogens*, 10(7).
https://doi.org/10.1371/journal.ppat.1004261

Chen, Y., Zhao, Y., Hammond, J., Hsu, H. T., Evans, J., & Feldlaufer, M. (2004). Multiple virus infections in the honey bee and genome divergence of honey bee viruses. *Journal of Invertebrate Pathology*, 87(2–3), 84–93. https://doi.org/10.1016/j.jip.2004.07.005

Colin, T., Lim, M. Y., Quarrell, S. R., Allen, G. R., & Barron, A. B. (2019). Effects of thymol on European honey bee hygienic behaviour. *Apidologie*, 50(2), 141–152.
https://doi.org/10.1007/s13592-018-0625-8

Costa, C., Lodesani, M., & Maistrello, L. (2009). Effect of thymol and resveratrol administered with candy or syrup on the development of *Nosema ceranae* and on the longevity of honeybees (*Apis mellifera* L.) in laboratory conditions. *Apidologie*, 41(2), 141–150.
https://doi.org/10.1051/apido/2009070

Couvillon, M. J., Al Toufailia, H., Butterfield, T. M., Schrell, F., Ratnieks, F. L. W., & Schürch, R. (2015). Caffeinated forage tricks honeybees into increasing foraging and recruitment behaviors. *Current Biology*, 25(21), 2815–2818. https://doi.org/10.1016/j.cub.2015.08.052

da Silva, C. S., de Cássia Goçalves de Lima, R., Elekofehinti, O. O., Ogunbolude, Y., Duarte, A. E., Rocha, J. B. T., Alencar de Menezes, I. R., Barros, L. M., Tsopmo, A., Lukong, K. E., & Kamdem, J. P. (2018). Caffeine-supplemented diet modulates oxidative stress markers and improves locomotor behavior in the lobster cockroach *Nauphoeta cinerea*. *Chemico-Biological Interactions*, 282, 77–84. https://doi.org/10.1016/j.cbi.2018.01.011

Das, L., Bhaumik, E., Raychaudhuri, U., & Chakraborty, R. (2012). Role of nutraceuticals in human health. *Journal of Food Science and Technology*, 49(2), 173–183.
https://doi.org/10.1007/s13197-011-0269-4

Dauqan, E. M. A., & Abdullah, A. (2017). Medicinal and functional values of thyme (*Thymus vulgaris* L.) herb. *Journal of Applied Biology & Biotechnology*, 5(02), 17–22.
https://doi.org/10.7324/jabb.2017.50203

de Miranda, J. R., Bailey, L., Ball, B. V, Blanchard, P., Budge, G. E., Chejanovsky, N., Chen, Y.-P., Gauthier, L., Genersch, E., de Graaf, D. C., Ribière, M., Ryabov, E., De Smet, L., & M van der Steen, J. J. (2013). Standard methods for virus research in *Apis mellifera*. *Journal of Apicultural Research*, 52, 1–56. https://doi.org/10.3896/IBRA.1.52.4.22

Detzel, A., & Wink, M. (1993). Attraction, deterrence or intoxication of bees (*Apis mellifera*) by plant allelochemicals. *Chemoecology*, 4(1), 8–18. https://doi.org/10.1007/BF01245891

Devasagayam, T. P. A., Kamat, J. P., Mohan, H., & Kesavan, P. C. (1996). Caffeine as an antioxidant: Inhibition of lipid peroxidation induced by reactive oxygen species. *Biochimica et Biophysica Acta - Biomembranes*, 1282(1), 63–70. https://doi.org/10.1016/0005-2736(96)00040-5
Dolezal, A. G., Carrillo-Tripp, J., Judd, T. M., Allen Miller, W., Bonning, B. C., & Toth, A. L. (2019). Interacting stressors matter: Diet quality and virus infection in honeybee health. *Royal Society Open Science, 6*(2). https://doi.org/10.1098/rsos.181803

Dolezal, A. G., Clair, A. L. S., Zhang, G., Toth, A. L., & O’Neal, M. E. (2019). Native habitat mitigates feast–famine conditions faced by honey bees in an agricultural landscape. *Proceedings of the National Academy of Sciences of the United States of America, 116*(50), 25147–25155. https://doi.org/10.1073/pnas.1912801116

Eastep, N. E., Albert, R. E., & Anderson, J. R. (2012). Modulation of La Crosse virus infection in *Aedes albopictus* mosquitoes following larval exposure to coffee extracts. *Frontiers in Physiology, 3*(66). https://doi.org/10.3389/fphys.2012.00066

Ellis, M. D., & Baxendale, F. P. (1997). Toxicity of seven monoterpenoids to tracheal mites (Acari: Tarsenemidae) and their honey bee (Hymenoptera: Apidae) hosts when applied as fumigants. *Journal of Economic Entomology, 90*(5), 1087–1091. https://doi.org/10.1093/jee/90.5.1087

Floris, I., Satta, A., Cabras, P., Garau, V. L., & Angioni, A. (2004). Comparison between two thymol formulations in the control of *Varroa destructor*: Effectiveness, persistence, and residues. *Journal of Economic Entomology, 97*(2), 187–191. https://doi.org/10.1093/jee/97.2.187

Gaur, S. (2017). *Lipid-based nutrient supplement to address child undernutrition and enteric parasitic infections in India*. [Doctoral dissertation, University of Illinois at Urbana-Champaign] Retrieved from https://www.ideals.illinois.edu/handle/2142/98235

Geffre, A. C., Gernat, T., Harwood, G. P., Jones, B. M., Morselli Gysi, D., Hamilton, A. R., Bonning, B. C., Toth, A. L., Robinson, G. E., & Dolezal, A. G. (2020). Honey bee virus causes context-dependent changes in host social behavior. *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.2002268117

Goblirsch, M. J., Spivak, M. S., & Kurtti, T. J. (2013). A cell line resource derived from honey bee (*Apis mellifera*) embryonic tissues. *PLoS ONE, 8*(7). https://doi.org/10.1371/journal.pone.0069831

González-Búrquez, M. D. J., González-Díaz, F. R., García-Tovar, C. G., Carrillo-Miranda, L., Soto-Zárate, C. I., Canales-Martínez, M. M., Penieres-Carrillo, J. G., Crúz-Sánchez, T. A., & Fonseca-Coronado, S. (2018). Comparison between *in vitro* antiviral effect of Mexican propolis and three commercial flavonoids against canine distemper virus. *Evidence-Based Complementary and Alternative Medicine, 2018*. https://doi.org/10.1155/2018/7092416

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science, 347*(6229), 1255957. https://doi.org/10.1126/science.1255957
Graziani, Y., Erikson, E., & Erikson, R. L. (1983). The effect of quercetin on the phosphorylation activity of the Rous sarcoma virus transforming gene product in vitro and in vivo. *European Journal of Biochemistry, 135*(3), 583–589. https://doi.org/10.1111/j.1432-1033.1983.tb07692.x

Gregorc, A., & Planinc, I. (2005). The control of *Varroa destructor* in honey bee colonies using the thymol-based acaricide - Apiguard. *American Bee Journal, 145*(8), 672–675.

Grozinger, C. M., & Flenniken, M. L. (2019). Bee viruses: Ecology, pathogenicity, and impacts. *Annual Review of Entomology, 64*, 205–226. https://doi.org/10.1146/annurev-ento-011118-111942

Hunter, W., Ellis, J., Vanengelsdorp, D., Hayes, J., Westervelt, D., Glick, E., Williams, M., Sela, I., Maori, E., Pettis, J., Cox-Foster, D., & Paldi, N. (2010). Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (*Apis mellifera*, Hymenoptera: Apidae). *PLoS Pathogens, 6*(12), 1001160. https://doi.org/10.1371/journal.ppat.1001160

Johari, J., Kianmehr, A., Mustafa, M. R., Abubakar, S., & Zandi, K. (2012). Antiviral activity of baicalein and quercetin against the Japanese encephalitis virus. *International Journal of Molecular Sciences, 13*(12), 16020–16045. https://doi.org/10.3390/ijms131216785

Köhler, A., Pirk, C. W. W., & Nicolson, S. W. (2012). Honeybees and nectar nicotine: Deterrence and reduced survival versus potential health benefits. *Journal of Insect Physiology, 58*(2), 286–292. https://doi.org/10.1016/j.jinsphys.2011.12.002

Kretschmar, J. A., & Baumann, T. W. (1999). Caffeine in *Citrus* flowers. *Phytochemistry, 52*(1), 19–23. https://doi.org/10.1016/S0031-9422(99)00119-3

Kwon, M. J., Shin, H. M., Perumalsamy, H., Wang, X., & Ahn, Y. J. (2019). Antiviral effects and possible mechanisms of action of constituents from Brazilian propolis and related compounds. *Journal of Apicultural Research*. https://doi.org/10.1080/00218839.2019.1695715

Laranja, A. T., Manzatto, A. J., & de Campos Bicudo, H. E. M. (2003). Effects of caffeine and used coffee grounds on biological features of *Aedes aegypti* (Diptera, Culicidae) and their possible use in alternative control. *Genetics and Molecular Biology, 26*(4), 419–429. https://doi.org/10.1590/S1415-47572003000400004

Leonard, S. P., Powell, J. E., Perutka, J., Geng, P., Heckmann, L. C., Horak, R. D., Davies, B. W., Ellington, A. D., Barrick, J. E., & Moran, N. A. (2020). Engineered symbionts activate honey bee immunity and limit pathogens. *Science, 367*(6477), 573–576. https://doi.org/10.1126/science.aax9039
Liao, L. H., Wu, W. Y., & Berenbaum, M. R. (2017). Impacts of dietary phytochemicals in the presence and absence of pesticides on longevity of honey bees (Apis mellifera). Insects, 8(1). https://doi.org/10.3390/insects8010022

Mao, W., Schuler, M. A., & Berenbaum, M. R. (2013). Honey constituents up-regulate detoxification and immunity genes in the western honey bee Apis mellifera. Proceedings of the National Academy of Sciences, 110(22), 8842–8846. https://doi.org/10.1073/pnas.1303884110

Mao, W., Rupasinghe, S. G., Johnson, R. M., Zangerl, A. R., Schuler, M. A., & Berenbaum, M. R. (2009). Quercetin-metabolizing CYP6AS enzymes of the pollinator Apis mellifera (Hymenoptera: Apidae). Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology, 154(4), 427–434. https://doi.org/10.1016/j.cbpb.2009.08.008

Mao, W., Schuler, M. A., & Berenbaum, M. R. (2015). A dietary phytochemical alters caste-associated gene expression in honey bees. Science Advances, 1(7), e1500795. https://doi.org/10.1126/sciadv.1500795

Maori, E., Paldi, N., Shafir, S., Kalev, H., Tsur, E., Glick, E., & Sela, I. (2009). IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. Insect Molecular Biology, 18(1), 55–60. https://doi.org/10.1111/j.1365-2583.2009.00847.x

McMenamin, A. J., & Flenniken, M. L. (2018). Recently identified bee viruses and their impact on bee pollinators. In Current Opinion in Insect Science (Vol. 26, pp. 120–129). Elsevier Inc. https://doi.org/10.1016/j.cois.2018.02.009

Mustard, J. A. (2020). Neuroactive nectar: compounds in nectar that interact with neurons. Arthropod-Plant Interactions, 14, 151–159. https://doi.org/10.1007/s11829-020-09743-y

Mustard, J. A., Dews, L., Brugato, A., Dey, K., & Wright, G. A. (2012). Consumption of an acute dose of caffeine reduces acquisition but not memory in the honey bee. Behavioural Brain Research, 232(1), 217–224. https://doi.org/10.1016/j.bbr.2012.04.014

Nathanson, J. A. (1984). Caffeine and related methylxanthines: Possible naturally occurring pesticides. Science, 226(4671), 184–187. https://doi.org/10.1126/science.6207592

Natsopoulou, M. E., McMahon, D. P., Doublet, V., Frey, E., Rosenkranz, P., & Paxton, R. J. (2017). The virulent, emerging genotype B of Deformed wing virus is closely linked to overwinter honeybee worker loss. Scientific Reports, 7(1). https://doi.org/10.1038/s41598-017-05596-3

Negri, P., Villalobos, E., Szawarski, N., Damiani, N., Gende, L., Garrido, M., Maggi, M., Quintana, S., Lamattina, L., & Eguaras, M. (2019). Towards precision nutrition: A novel concept linking phytochemicals, immune response and honey bee health. Insects, 10(11). https://doi.org/10.3390/insects10110401
Njoroge, T. (2020). *Evaluation of the role of plant-derived chemicals on mosquito ecology and control*. [Unpublished doctoral dissertation]. University of Illinois at Urbana-Champaign.

Palmer-Young, E. C., Sadd, B. M., Irwin, R. E., & Adler, L. S. (2017). Synergistic effects of floral phytochemicals against a bumble bee parasite. *Ecology and Evolution, 7*(6), 1836–1849. https://doi.org/10.1002/ece3.2794

Palmer-Young, E. C., Tozkar, C. O., Schwarz, R. S., Chen, Y., Irwin, R. E., Adler, L. S., & Evans, J. D. (2017). Nectar and pollen phytochemicals stimulate honey bee (Hymenoptera: Apidae) immunity to viral infection. *Journal of Economic Entomology, 110*(5), 1959–1972. https://doi.org/10.1093/jee/tox193

Peng, T., Segers, F. H. I. D., Nascimento, F., & Grüter, C. (2019). Resource profitability, but not caffeine, affects individual and collective foraging in the stingless bee *Plebeia droryana*. *Journal of Experimental Biology, 222*(10). https://doi.org/10.1242/jeb.195503

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. In *Trends in Ecology and Evolution* (Vol. 25, Issue 6, pp. 345–353). https://doi.org/10.1016/j.tree.2010.01.007

Raffique, M. K., Mahmood, R., Aslam, M., & Sarwar, G. (2012). Control of *Tropilaelaps clareae* mite by using formic acid and thymol in honey bee *Apis mellifera* L. colonies. *Pakistan Journal of Zoology, 44*(4), 1129–1135.

Reiter, S., Rodriguez, C. C., Sun, K., & Stopfer, M. (2015). Spatiotemporal coding of individual chemicals by the gustatory system. *Journal of Neuroscience, 35*(35), 12309–12321. https://doi.org/10.1523/JNEUROSCI.3802-14.2015

Rosenkranz, P., Aumeier, P., & Ziegelmann, B. (2010). Biology and control of *Varroa destructor*. *Journal of Invertebrate Pathology, 103*(SUPPL. 1), S96–S119. https://doi.org/10.1016/j.jip.2009.07.016

Salehi, B., Mishra, A. P., Shukla, I., Sharifi-Rad, M., Contreras, M. del M., Segura-Carretero, A., Fathi, H., Nasrabadi, N. N., Kobarfard, F., & Sharifi-Rad, J. (2018). Thymol, thyme, and other plant sources: Health and potential uses. *Phytotherapy Research, 32*(9), 1688–1706. https://doi.org/10.1002/ptr.6109

Schnitzler, P., Neuner, A., Nolkemper, S., Zundel, C., Nowack, H., Sensch, K. H., & Reichling, J. (2010). Antiviral activity and mode of action of propolis extracts and selected compounds. *Phytotherapy Research, 24*(1), S20–S28. https://doi.org/10.1002/ptr.2868

Si, A., Zhang, S. W., & Maleszka, R. (2005). Effects of caffeine on olfactory and visual learning in the honey bee (*Apis mellifera*). *Pharmacology Biochemistry and Behavior, 82*(4), 664–672. https://doi.org/10.1016/j.pbb.2005.11.009
Singaravelan, N., Nee’man, G., Inbar, M., & Izhaki, I. (2005). Feeding responses of free-flying honeybees to secondary compounds mimicking floral nectars. *Journal of Chemical Ecology, 31*(12), 2791–2804. https://doi.org/10.1007/s10886-005-8394-z

Smith, T. J., & Saunders, M. E. (2016). Honey bees: the queens of mass media, despite minority rule among insect pollinators. *Insect Conservation and Diversity, 9*(5), 384–390. https://doi.org/10.1111/icad.12178

Steinhauer, N. A., Rennich, K., Wilson, M. E., Caron, D. M., Lengerich, E. J., Pettis, J. S., Rose, R., Skinner, J. A., Tarpy, D. R., Wilkes, J. T., & VanEngelsdorp, D. (2014). A national survey of managed honey bee 2012-2013 annual colony losses in the USA: Results from the Bee Informed Partnership. *Journal of Apicultural Research, 53*(1), 1–18. https://doi.org/10.3896/IBRA.1.53.1.01

Stevenson, P. C., Nicolson, S. W., & Wright, G. A. (2017). Plant secondary metabolites in nectar: Impacts on pollinators and ecological functions. *Functional Ecology, 31*(1), 65–75. https://doi.org/10.1111/1365-2435.12761

Strachecka, A., Krauze, M., Olszewski, K., Borsuk, G., Paleolog, J., Merska, M., Chobotow, J., Bajda, M., & Grzywnowicz, K. (2014). Unexpectedly strong effect of caffeine on the vitality of western honeybees (*Apis mellifera*). *Biochemistry (Moscow), 79*(11), 1192–1201. https://doi.org/10.1134/S0006297914110066

Szawarski, N., Saez, A., Domínguez, E., Dickson, R., de Matteis, Á., Eciolaza, C., Justel, M., Aliano, A., Solar, P., Bergara, I., Pons, C., Bolognesi, A., Carna, G., Garcia, W., Garcia, O., Eguaras, M., Lamattina, L., Maggi, M., & Negri, P. (2019). Effect of abscisic acid (ABA) combined with two different beekeeping nutritional strategies to confront overwintering: Studies on honey bees’ population dynamics and nosemosis. *Insects, 10*(10). https://doi.org/10.3390/insects10100329

Thomson, J. D., Draguleasa, M. A., & Tan, M. G. (2015). Flowers with caffeinated nectar receive more pollination. *Arthropod-Plant Interactions, 9*(1). https://doi.org/10.1007/s11829-014-9350-z

Upadhyay, A., & Venkitanarayanan, K. (2016). *In vivo* efficacy of trans-cinnamaldehyde, carvacrol, and thymol in attenuating *Listeria monocytogenes* infection in a *Galleria mellonella* model. *Journal of Natural Medicines, 70*(3), 667–672. https://doi.org/10.1007/s11418-016-0990-4

vanEngelsdorp, D., & Meixner, M. D. (2010). A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *Journal of Invertebrate Pathology, 103*(SUPPL. 1), S80–S95. https://doi.org/10.1016/j.jip.2009.06.011

Wong, M. J., Liao, L. H., & Berenbaum, M. R. (2018). Biphasic concentration-dependent interaction between imidacloprid and dietary phytochemicals in honey bees (*Apis mellifera*). *PLoS ONE, 13*(11). https://doi.org/10.1371/journal.pone.0206625
Wright, G. A., Baker, D. D., Palmer, M. J., Stabler, D., Mustard, J. A., Power, E. F., Borland, A. M., & Stevenson, P. C. (2013). Caffeine in floral nectar enhances a pollinator’s memory of reward. *Science, 339*(6124), 1202–1204. https://doi.org/10.1126/science.1228806

Wright, G. A., Nicolson, S. W., & Shafir, S. (2018). Nutritional physiology and ecology of honey bees. *Annual Review of Entomology, 63*, 87–103. https://doi.org/10.1146/annurev-ento-020117-043423