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European honey bees (*Apis mellifera)* are economically and ecologically important due to the large-scale pollination service they offer a myriad of plant species. In 2015, honey bee pollination augmented crop value in the United States by 15 billion dollars. A high density of individuals combined with a homeostatic nest environment facilitates the impact of pathogens and parasites on honey bee health. Among these parasites is *Varroa destructor*, a vector for Israeli acute paralysis virus (IAPV) and other viruses, which harm honey bee health. Since its discovery in 2004, IAPV is largely understudied in regards to queen-virus interactions. Therefore, my study focused on the impact of IAPV on the queens' attractiveness, transmission routes, and the potential of trans-generational immune priming. IAPV prevalence was surveyed in experimental colonies and IAPV was present in three life history stages: egg, capped brood, and adult, suggesting vertical and horizontal transmission routes of IAPV. No experimental evidence for an influence of IAPV on the queens' attractiveness and for trans-generational immune priming was found. Due to the lack of effective methods to determine viral infection level of live queens, the IAPV status of treatment queens was evaluated at the end of the experiment. IAPV infection of the inoculated queens was highly variable. My study is one of the few studies that focused on queen health and lays the foundation for future studies. Such future studies are required to conclusively evaluate immune priming and the impact of IAPV on queens' attractiveness.

ISRAELI ACUTE PARALYSIS VIRUS IMPACT ON *APIS MELLIFERA* QUEENS' ATTRACTIVENESS, TRANSMISSION ROUTES, AND TRANS-GENERATIONAL IMMUNE PRIMING

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Honey Bees' Ecological and Economical Importance

Among the animal pollinators worldwide, the European honey bees (*Apis mellifera*) are the ones primarily domesticated and utilized to increase agricultural production (Potts et al., 2010). Honey bees are native to Europe, the Middle East, and Africa and were introduced to the Americas in 1622 to be managed by beekeepers (Calderone, 2012; Seeley, 1985). Honey bees are well suited to help pollinate crop monocultures as a result of their large colony size (30 to 80 thousand individuals), they can be fed artificial diets to increase their population size, and they can be transported over large distances (vanEngelsdorp & Meixner, 2010; Wilson-Rich, 2014). Honey bees are economically very valuable due to the large-scale pollination services they offer a myriad of plant species (Bauer & Wing, 2010; Calderone, 2012). In 2015, they augmented crop value by 15 billion dollars in the United States (U.S.), which is an increase from 11.68 billion dollars in 2009 (Calderone, 2012; USDA, 2016). Honey bee pollination increases the commercial value of some fruits by improving their commercially marketable traits. For example, honey bee pollinated strawberries exhibit better fruit shape, weight, and shelf life, increasing the commercial value by 38.6% relative to wind pollination (Klatt et al., 2014). Furthermore, honey bee pollination increases yields of nuts, fruits, and vegetables—specifically almonds, kiwifruit, apples,

cherries, pears, blueberries, and cucumbers (Rucker et al., 2012). Almonds require a substantial pollination service relative to the total number of United States honey bee managed colonies. Almond producers employed about 5% of U.S. honey bee colonies before the mid 1960's, about 15% in the late 1970's, and up to 60% in 2010 (Rucker et al., 2012).

Most wild plant species (80%) are also directly dependent on insect pollination to produce seeds and fruits; honey bees possess multiple ecologically relevant traits required for maintaining the biodiversity of plants (Bauer & Wing, 2010; National Academy of Sciences, 2007; Potts et al., 2010). For example, they are generalist pollinators, so they are behaviorally and/or morphologically capable of pollinating a multitude of plants species (Motten et al., 2009; National Academy of Sciences, 2007). Other traits include their populous perennial colonies, which are able to pollinate flowers year-round and forage up to a 12 kilometer area, so they can readily pollinate seasonal blooms (National Academy of Sciences, 2007; Seeley, 1985). Additionally, honey bee foragers are able to communicate with other hive members in regards to location of food resources by using the waggle dance, which is a repetition of figure-of-eights configuration (Griffin et al., 1968; National Academy of Sciences, 2007). Honey bees mold natural ecosystems by allowing gene diversity and distribution in angiosperms, which is the most thriving and diverse plant taxon (Evans & Schwarz, 2011). A decrease in pollinators could potentially decrease the reproductive fitness of plants and lead to an extinction of plants and animals. Thus, pollinator declines could affect the wild plant diversity and ecosystem stability by impacting species survivability (Kevan & Viana, 2003; Potts et al., 2010).

Moreover, honey bees pollinate hay crops, such as alfalfa and clover, which can supply livestock and thus meat, dairy, and other products (National Academy of Sciences, 2007). The agriculture in the developing world is 50% more reliant on pollinator-dependent crops than in the developed world (Aizen et al., 2009). A healthy and abundant population of honey bees is essential to meet the demand of a global increase of pollinator-dependent crops and production of other honey bee products (Aizen et al., 2008; Chen & Siede, 2007). A significant decrease in pollinators' health and population size may lead to concerns for food security, reduce economic development, and have potential consequences to human health since insect-pollinated crops contribute to essential micronutrients (vitamins, antioxidants, and folic acid) (Bauer & Wing 2010; Vanbergen & Insect Pollinators Initiative, 2013).

In addition to improving crop value, honey bees are able to make other consumable and non-consumable products, such as honey, resins, propolis, royal jelly, bees wax, which further enhances their economic importance (vanEngelsdorp & Meixner, 2010; Wilson-Rich, 2014). Unprocessed honey has several antibacterial properties that permit it to have medical applications, such as cleaning dead tissues from ulcers and killing various organisms that cause wound infections (Efem & Iwara, 1992).

Honey Bee Biology

Honey bees belong to the order Hymenoptera, which also includes wasps and ants, and they are highly eusocial organisms (Bourke, 1988; Hölldobler & Wilson, 2009). Eusocial insects possess three main characteristics: united effort in caring for the young; division of tasks based on reproductive abilities in which the non-reproductive members

take care of the nest in order to contribute to the prosperity of the reproductive members; and overlap of at least two generations within their dwelling (Wilson, 1971; Wilson & Hölldobler 2005; Hölldobler & Wilson, 2009). Honey bee colonies are typically composed of a single reproductive queen, 30 to 80 thousand non-reproductive female workers, and zero to a few thousand reproductive male drones based on the time of year (Page & Peng, 2001; Seeley, 1985; Wilson-Rich, 2014). At different developmental rates, queens, workers, and drones undergo four life history stages: egg, larva, pupa, and adult. The development averages 16 days for queens, 24 days for drones, and 21 days for workers (Winston, 1987). Once workers emerge they have a malleable system of age determined labor specialization or "age polytheism" that generally starts in the nest and then shifts to the field (Seeley, 1985; Winston, 1987).

The adult workers' tasks start with cell cleaning in early life, then attending brood and food storage midlife and finally foraging (Seeley, 1985). The worker's anatomy has evolved to carry out these tasks (Winston, 1987). It consists of the head (mouthparts and sensory organs), thorax (mostly muscles to move the wings and jointed legs), and the abdomen (poison sting, organs to digest and carry out other physiological functions) (Seeley, 1985; Wilson-Rich, 2014). Workers have a proboscis to absorb liquids, principally honey and nectar, which can function as a food exchange mechanism between worker and worker, worker and queen, and worker and drones (Winston, 1987). Honey bees utilize their pollen baskets to collect pollen, which nourishes them with amino acids, vitamins, and fat deposits (Seeley, 1995). In addition to workers who maintain the nest hygiene, there is the drone male reproductive caste whose sole purpose is to mate with an

available queen from another colony. Their orientation, flight, and mating-related structures are highly developed in order to travel to drone congregation sites, which are essential for the success of drones (Seeley, 1985).

The third and final caste is the honey bee queen, the only female reproductive member of the colony, which makes her the most vital individual and longest lived, with an average of three years (Laidlaw & Page, 1996; Seeley, 1985). The life span of a queen is extensive compared to workers who average 15-38 days and drones average 21-32 days during the summer, which further reflects the importance of the queen's role of securing the survival of future generations (Winston, 1987). The queen becomes the mother of all the colony members during her lifetime and she becomes the vessel for all the inherited traits of the colony, accumulated from multiple mating. Therefore, the queen is responsible for the color of the bees, resistance to disease, degree of gentleness, and other colony and individual characteristics (Laidlaw & Page, 1996). A queen is able to regulate the sex of the offspring by a simple system: males develop from unfertilized eggs (haploid) and females develop from fertilized eggs (diploid); females remain sterile as long as the queen is present (Laidlaw & Page, 1996; Seeley, 1985). The presence of the queen is vital to the normal functioning of the colony and is constantly signaled to the workers by a perpetual dissemination of the pheromone, (E)-9-oxo-2-decenoic acid (9-ODA) by workers who have contact with the queen. This pheromone is secreted by the queen's mandibular glands and it motivates workers to maintain the integrity of the colony (Laidlaw & Page, 1996; Seeley, 1985).

The queens and workers have the same genetic material and initially all fertilized eggs for the first three days are given the same composition of food, but on the fourth day the larva destined to be queens will be given food called “royal jelly” (Seeley, 1985; Wilson-Rich, 2014; Winston, 1987). Royal jelly contains more mandibular gland secretions and 35% more hexose sugars than worker food and it is given in larger quantities (Seeley, 1985; Winston, 1987). Queens are reared naturally in queen cells, which protrude downward from the middle or edges of comb (Laidlaw & Page, 1996). As the sole female reproductive member, the queen mates with a multitude of drones, up to 29 has been recorded, storing the sperm for several years (Page & Peng, 2001; Wilson-Rich, 2014).

The queen’s physiology is modified compared to worker in the form of a reduced proboscis, absence of pollen baskets, retractable sting with two to three times more volume of venom (Winston, 1987). Moreover, her ovaries are highly developed, consisting of 150-180 egg-producing ovarioles, which take up most of the space in the abdominal cavity. In contrast worker ovaries only have 2-12 ovarioles (Winston, 1987). The queen has a spermatheca, which is the structure that encloses all the sperm, able to hold up to seven million sperm (Laidlaw & Page, 1996). These highly developed reproductive structures facilitate the queen’s ability to lay over 1 million eggs during her lifetime until she is replaced by a new fertile queen (Laidlaw & Page, 1996; Winston, 1987). Vitellogenin is one component in the eggs laid by queens, which is a yolk precursor protein (Guidugli et al, 2005). It has evolved and displays various functions in all three honey bee castes (Dainat et al., 2012). For example, nurse bees use vitellogenin

to prepare proteins in the form of royal jelly to nourish the larva (Dainat et al., 2012; Guidugli et al., 2005). Vitellogenin is a 180-kDa protein made in fat body cells and discharged into the haemolymph of insects in order to eliminate harmful oxidative molecules (Corona et al., 2007; Wilson-Rich, 2014). Therefore, vitellogenin has been suggested to play a crucial role in honey bee immunity and queen and worker longevity (Dainat et al., 2012).

Not only are queens physiologically different from workers, but they are also different behaviorally. Even though she is the mother of the colony, she does not participate in caring for the eggs she lays (Laidlaw & Page, 1996). The queen is attractive to workers via a chemical regulation of the mandibular glands; therefore she usually has a circle of six to ten alternating attendants that take care of her (Gary, 1961; Winston, 1987). The worker visits are short, usually last an average of one minute, and they analyze the queen with their antennae, lick her with their tongues, and make foreleg contact to groom her and to transmit her pheromone to the rest of the colony (Allen, 1960; Winston, 1987). Workers constantly offer food to the queen via trophallaxis (i.e. mouth-to mouth transfer of food) with an increased frequency during summer to stimulate an increased egg-laying behavior (Farina, 1996; Gary, 1961; Winston, 1987). Workers feeding the queen is a way to control the egg-laying activity and coordinate the activities of the colony with the season and food availability (Laidlaw & Page, 1996). Secluded queens are able to feed themselves for weeks, but queens in colonies rarely have the need to feed themselves (Winston, 1987).

Pathogens Affecting Honey Bee Health

Honey bee populations are highly vulnerable to numerous environmental factors, such as starvation, queen loss or queen failure, poor wintering conditions and a multitude of pathogens and parasites (Evans & Schwarz, 2011; Genersch, 2010; Lee et al., 2015). Therefore, it is normal for a 10% loss of colonies during the winter, but in 2007 losses in the U.S. were high, closer to 38%, and widespread, affecting 22 states (vanEngelsdorp et al., 2008). This event was not isolated to the U.S. and it has been reported by European beekeepers as well (Oldroyd, 2007). These colony losses in the last decade surged general public awareness and scientific community interest regarding pollinators' decline and the event was termed Colony Collapse Disorder (CCD) (Chen et al., 2014; Ellis et al., 2010). The disorder itself has occurred for a century under various labels and it has resurfaced in the last decade (Champetier et al., 2015; Evans & Schwarz, 2011). The symptoms associated with collapsed (dead) colonies affected by CCD include the dearth of adult bees, existence of capped brood, and for undetermined reasons there are food stores present that repel robber honey bees or other pests (Cox-Foster et al., 2007; Ellis et al., 2010). CCD is a disorder that highlights the general decline in honey bee health. Factors that impact honey bee health include two major bacterial diseases and a fungal disease which target brood: European Foul Brood caused by *Melissococcus plutonius*, American Foul Brood caused by *Paenibacillus larvae*, and the fungal disease of *Ascosphaera apis* (Oldroyd, 2007). In addition, chemicals applied to hives, such as in-hive acaricides, are prevalent in combs and wax foundations. These neurotoxicants at high levels can reduce honey bee fitness and even at sub-lethal levels interfere with

honey bees' learning abilities and suppresses their immune systems (Desneux et al., 2007; Mullin et al., 2010). Other factors include the parasitic mite *Varroa destructor* (*Varroa*), which vectors honey bee RNA viruses that in the past 50 years has led to the deaths of millions of honey bees (Martin, 2001; Martin et al., 2012; Neumann & Carreck, 2010). Among these pathogens, the least understood are viruses due to the scarcity of studies on the dynamics of virus and host interactions (Chen et al, 2006b). At least 23 honey bee viruses have been described and among them, 20 have positive-strand RNA genomes belonging to the families *Dicistroviridae* and *Iflaviridae* (Boncristiani et al., 2013; Evans & Schwarz, 2011; Gisder & Genersch, 2015). Among those viruses is Israeli acute paralysis virus (IAPV), which was the main focus of my study.

Honey Bee Viruses

The colony structure and social behavior is crucial to the overall propagation and success of honey bee viruses. Honey bee workers collaborate in a network composed of thousands of individuals, which creates a high density environment vulnerable to a many pathogens (Fries & Camazine, 2001; Singh et al., 2010; Wilson, 1971). This close interaction combined with a homeostatic nest environment and availability of stored food allows pathogens to thrive and quickly infect multiple individuals in small enclosed spaces (Evans et al., 2006; Fouks et al., 2011). In parallel with other animals, many viruses can impact honey bee health (Chen & Siede, 2007; Chen et al., 2006a). Viruses can infect all three honey bee castes largely focusing on infection of the larval or pupa stage, while the symptoms are more prominent in adult bees (Moore et al., 2015). A large portion of the individuals in the colony need to be affected by the virus to threaten

the integrity of the colony (Martin, 2001). Frequently the symptoms stay dormant until specific conditions are met, such as high pathogen presence, then the virus replicates leading to noticeable symptoms and colony losses (Chen et al., 2006a; Singh et al., 2010).

Colony life of eusocial insects allows viruses to utilize several transmission routes. Understanding the mode of transmission, regardless if it is occurring vertically, horizontally or both, is essential to understanding the dynamics of host-pathogen interactions (Chen et al., 2006a). Vertical transmission happens within or on the surface of the eggs when queens transmit viruses directly to their offspring via their germline or when drones transmit viruses through their semen (Carter & Saunders, 2007; Evans & Schwarz, 2011). Vertical transmission is a very effective route for viruses since queens lay thousands of eggs during their lifetime (Evans & Schwarz, 2011). Horizontal transmission occurs when one individual is infected from another individual, excluding transmission routes from parents to offspring, and can occur directly or indirectly (Chen et al., 2006a; Fries & Camazine, 2001). Horizontal transmission can be just as effective as vertical transmission due to the frequency of the worker to worker interactions that can potentially spread the virus (Evans & Schwarz, 2011). Horizontal transmission is proposed to select for more virulent pathogens than vertical transmission, since it does not depend on its hosts' fitness (Lindström et al., 2008). Direct transmission modes involve air-borne, food-borne, or venereal infection. Food-borne infections occur by honey bees ingesting pathogen-contaminated food or cannibalizing pathogen-ridden brood, then carrying out behaviors such as defecating virus containing feces, feeding brood and queen, grooming the queen, packing pollen, and handling nectar (Chen et al.,

2006a; Evans & Schwarz, 2011). In addition, robbing behavior of strong honey bee colonies on the weaker neighboring colonies contribute to the spread of viruses already present in those food sources (Evans & Schwarz, 2011; Fries & Camazine, 2001; Lindström et al., 2008). This robbing behavior is particularly relevant in apiaries due to the high colony density (Fries & Camazine, 2001). An indirect method requires an intermediate biological host, such as the mite *Varroa*, an obligate parasite that feeds on bee haemolymph by using its piercing mouth parts (Chen et al., 2006a; Dietemann et al., 2012; Martin, 2001). The introduction of *Varroa* mites and other mites in the mid 1980's into the US, increased the winter losses from 5-10% to 15-25% (vanEngelsdorp et al., 2008). Prior to the introduction of the *Varroa* mite, honeybee viral pathogens prevailed as covert infections, but the mite has been reported to have an association with overt infections usually leading to high adult mortality rates (Rosenkranz et al., 2010; Yue & Genersch, 2005). *Varroa* serves as a vector to transmit several viruses among them deformed wing virus (DWV), Kashmir bee virus (KBV), sacbrood virus (SBV), acute bee paralysis virus (ABPV) and Israeli acute paralysis virus (IAPV) (Chen et al., 2006b; Di Prisco et al., 2011; Rosenkranz et al., 2010).

Adults can be hosts to multiple viruses. For example, queens can be co-infected with the following viruses: black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), DWV, KBV, and SBV (Chen et al., 2005b; Chen et al., 2006b; De Miranda & Fries, 2008). Several of these viruses have been found to be vertically transmitted from queens to offspring, including KBV, SBV and DWV, and horizontally transmitted to larvae via larva food infected with the virus (De Miranda & Fries, 2008; De Miranda &

Genersch, 2010; Möckel et al., 2011; Yue et al., 2007). In a study done with 86 queens, 56 queens were infected with the AKI virus complex (consisting of ABPV, KBV, and IAPV). A further analysis of which of the three viruses were actually present was not conducted due to low virus titers (Francis et al., 2013). In another study, a survey of 78 egg samples varying in the number of eggs in each sample from 1-10 eggs, found high levels of DWV (40%), SBV (42%), Lake Sinai Virus (LSV) (28%) and ABPV (14%), but IAPV was not prevalent in the eggs sampled (Ravoet et al., 2015). Both the studies conducted by Francis et al. (2013) and Ravoet et al. (2015) found IAPV not prevalent, but a study done by Chen et al. (2014) found IAPV to be third most common virus in the bee colonies surveyed. These studies surveyed presence or absence of IAPV, but none of the studies dealt with establishing an effective dose and delivery of IAPV to ensure the virus is in the individual, which allows for a controlled study to understand IAPV transmission routes. In addition, a great portion of the virus studies are centered around honey bee workers based on the rational if the workers are dying at a faster rate than they can be replaced, this will lead to colony death (vanEngelsdorp et al., 2009). Ultimately, honey bee workers are more abundant, have shorter life spans, and are more inclined to get viral infections and display symptoms than queens. In contrast, queens are expensive, both in time and resources expended in raising them, and they seem to be asymptomatic (Francis et al., 2013). Incomplete knowledge on honey bee viruses jeopardizes our capacity to evaluate their impact and develop management practices. IAPV is understudied in queens compared to workers. Therefore, my study endeavored to establish an effective

dose and delivery method of IAPV in order to evaluate how viruses impact queens' attractiveness and the potential viral transmission routes in the colony.

Israeli Acute Paralysis Virus

IAPV has a RNA genome comprised of 9,487 nucleotides and it is similar to other viruses in the *Dicistroviridae*, with KBV and ABPV as its closest relatives (Maori et al., 2007a). All three viruses are acutely virulent when injected at the pupae or adult stage leading to 80% death (De Miranda et al., 2010; Maori et al., 2007a). The family *Dicistroviridae* is characterized by two open reading frames (ORFs) contained in a linear positive-strand RNA genome of about 9 kb (Chen et al., 2014; Genersch & Aubert, 2010; Maori et al., 2007a; Maori et al., 2007b). There is a 5' untranslated region (UTR) followed by the two ORFs, which are separated by an intergenic untranslated region (IGR). In addition, there is a UTR of variable length at the 3' end of the genome. All three viruses share highly similar ORFs sequences, but IAPV UTRs are different from KBV and ABPV (Maori et al., 2007a). The virions are approximately 30 nm, non-enveloped with icosahedral symmetry and composed of viral capsids (VP1-VP4) (Figure 1A) (Chen et al., 2014; Maori et al., 2007a). The ORF near the 5' end of the genome codes for non-structural proteins functioning in viral replication, and the ORF nearest to the 3' end codes for the structural proteins (Figure 1B) (Maori et al., 2007a). Translation is initiated by internal ribosomal entry sites (IRES), one found in the 5' UTR and the other in the IGR (Maori et al., 2007a).

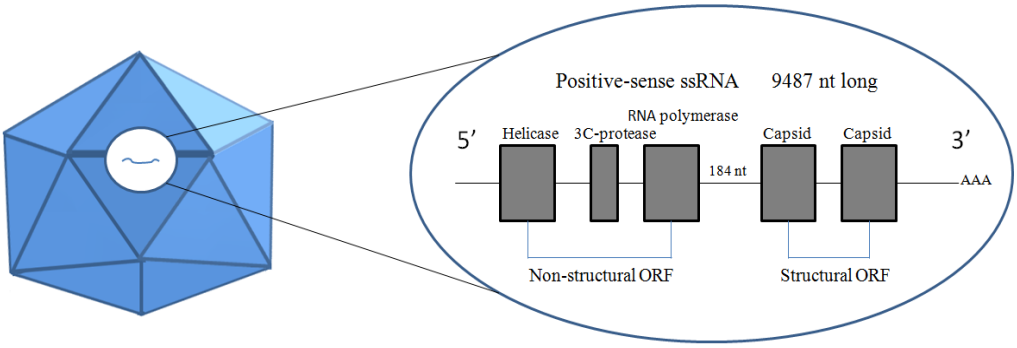

Figure 1. IAPV Capsid and Genome. **A**. Non-enveloped icosahedral capsid. **B**. An illustration of the IAPV genome. It has two ORFs, which are separated by a 184-nucleotide-long region.

IAPV was discovered in Israel in 2004 by inoculating healthy-looking larvae with the homogenate of a single dead bee that showed symptoms similar to those infected by ABPV. In the early stage of the infection there was a darkening of the abdomen tip, which spread to the thorax, and the behavior of the bees changed to walking in circles aimlessly, and flight and feeding decreased. As the infection spread, both the abdomen and thorax darkened while the thorax became hairless until finally the bees experienced spasms prior to their deaths (Maori et al., 2007a). However, these symptoms are rarely observed in the field, since highly infected individuals are promptly killed (Francis et al., 2013). IAPV not only infects honey bees, but has been found in other pollinator species, such as tricolored bumble bee, yellow jacket wasp, sweat bee, and the non-pollinating yellow-legged hornet (Levitt et al., 2013; Singh et al., 2010; Yañez et al., 2012b). Since 2004, IAPV has been sequenced and three major groups have been proposed: group 1 is the western strain which encompasses the western U.S. , group 2 incorporate samples

from Israel, and group 3 includes samples from eastern U.S. and Canada (Palacios et al., 2008; Singh et al., 2010).

IAPV infects every developmental stage of all castes, ranging from eggs, larvae, pupae, to adult workers, drones, and queens and can trigger a systemic infection (Chen et al., 2014). IAPV was also identified in royal jelly, honey, pollen, queen feces and drone semen (Chen et al., 2014). IAPV in association with *Varroa* mite may increase infection levels of IAPV of brood and adult workers, which may lead to weaker colonies collapsing before the end of winter (Chen et al., 2014; Genersch & Aubert, 2010). The IAPV and *Varroa* association has a synergistic detrimental effect to the host immunity and augments IAPV viral titer levels (Chen et al., 2014). Most of the virus studies conducted have been surveys, so they are limited by the location they surveyed. These studies have not used purified IAPV to establish a delivery method to introduce an effective viral dose in queens in order to analyze routes of transmission and impact on queens' attractiveness. Understanding the pathogen-host interactions is important to the development of management practices. Overall, the current information about viral infections in queens is limited, especially the transmission and symptoms of IAPV (Francis et al., 2013). Therefore, my study attempted to understand how IAPV affects honey bee queens and tested vertical and horizontal transmission of this important honey bee pathogen.

Honey Bee Immunity

Honey bees are not defenseless to all these pathogens and have innate defenses that are well studied and include the Toll, immune deficiency (IMD), Janus kinase/Signal

Transducer and Activator of Transcription (Jak/STAT) and RNA interference (RNAi) pathways (Brutscher & Daughenbaugh, 2015; Evans et al., 2006). The Toll and IMD pathways play an essential part in the regulation of genes that code for antimicrobial peptides (AMPs). AMPs found in the haemolymph of honey bees include Apidaecin, Hymenoptaecin, Abaecin, and Defensin (Gätschenberger et al., 2013). Apidaecin is upregulated during an IAPV infection, but the main pathway involved in antiviral defense is the RNAi pathway (Galbraith et al., 2015). The RNAi response is initiated by Dicer-2 detecting the presence of dsRNA due to RNA viral replication. Dicer-2 cleaves the dsRNA and loads the siRNA into Argonaute, which is the catalytic part of the RNA Induced Silencing Complex (RISC) (Brutscher & Daughenbaugh, 2015). Another defense mechanism is vitellogenin, which is linked to immunity and displays antioxidant effects in the haemolymph (Corona et al., 2007; Wyatt, 1961; Dainat et al., 2012).

Invertebrates are thought to depend exclusively on innate defenses without acquired immunity (Kurtz, 2005). However, several studies indicate that immune priming offers protection from a previously exposed pathogen, for example in *Bombus terrestris* and *Tribolium castaneum* (Roth et al., 2009; Sadd & Schmid-Hempel, 2006). Honey bees lack the adaptive immunity of vertebrates, but queens may be able to immune prime their offspring (Hernández López et al., 2014). Honey bee queens previously exposed to *Paenibacillus larvae*, the cause of American Foulbrood, increase the resistance of direct offspring to that pathogen (Hernández López et al., 2014; Moret, 2006; Sadd & Schmid-Hempel, 2006). However, the concept of immune priming in insects is still disputed and needs to be further studied, especially in regards to the

transfer of immunity against viral infection (Hernández López et al., 2014; Sadd & Schmid-Hempel, 2006). Therefore, my study endeavored to evaluate the potential of immune priming of IAPV in offspring.

Overall, my study was based on three hypotheses that led to three predictions, and my aims were designed to test these predictions as described below.

Hypotheses

1) IAPV infection affects the queens' attractiveness in the colony.
2) IAPV has effective horizontal and vertical transmission routes.
3) IAPV affects the survival rate of progeny from infected queens in contrast to non-infected queens.

Predictions

1) IAPV-infected queens have reduced attractiveness to workers, limiting the attention and care they receive.
2) IAPV-infected queens produce IAPV-infected offspring and infect adult workers by social interactions.
3) Progeny from an IAPV-infected queen survives IAPV infection better than progeny from a non-infected queen.

Aims

The first aim was to study the effects of IAPV on queens' attractiveness to workers. Worker selection between infected and non-infected queens was observed using a 2-choice olfactometer bioassay.

The second aim was to evaluate IAPV vertical and horizontal transmission routes from infected queens in honey bee colonies. Adults and brood of different ages were surveyed with qPCR assay for the presence of IAPV in a series of experimental colonies.

The third aim was to **e**xplore the potential of queens immune priming their offspring against IAPV. The progeny from infected and non-infected queens were exposed to an effective IAPV dose. The survival rates of progeny from infected queens and non-infected queens were analyzed to determine the potential of an immune priming effect.

CHAPTER II

METHODS

General Methods

Rearing Queens

Rearing experimental queens was integral to the success of my study. The grafting method to raise new queens from IAPV free colonies was employed (Laidlaw & Page , 1996). Grafting took place in a shed near the University of North Carolina at Greensboro (UNCG) apiary in order have a temperature controlled environment. A strong queenless colony was established with extra food stores, capped brood, and adult workers. This was done one day prior to grafting in order to prepare the workers to build newly grafted queen cells. A brood frame was selected from a colony that contained first and second instar larvae, which are roughly 4-5 days old. The larvae were transferred from their wax comb cells into manufactured wax queen cells (Figure 2A) by using a metal grafting tool (Laidlaw & Page , 1996). The empty wax cell was primed with a droplet of royal jelly prior to grafting the larvae in order to increase larvae survival by reducing the drying out process (Büchler et al., 2013). Special care was taken not to damage the larvae by situating them in the correct orientation in the primed wax cells, so they could breathe through their spiracles (Winston, 1987). Grafted queen wax cells were turned upside down and inserted into cell cup holding frames (Figure 2B). The frames were placed in the queenless colony so the nurse workers could

take care of the larvae by feeding it royal jelly, drawing out the wax queen cell, and capping the cells (Figure 2C).

Figure 2. Queen Grafting Process. **A**. Wax queen cells constructed from melted wax and a mold with the dimensions of commercial base mount queen cell cups (Mann Lake Ltd., Hackensack, MN). **B**. Cell cup holding frame with drawn out queen cells. **C**. Capped grafted queen cell.

The queen cells were checked six to seven days post-grafting to verify that they were fully capped. Small queenless nuclear hives (nucs) were set up with workers from various colonies in the apiary. The nucs were small (10.5 in x 11.8 in) with a divider in the middle splitting the nuc into two colonies each with their own small entrance. Each capped queen cell was placed into a queenless nuc at least one week prior to their emergence since queens at emergence kill neighboring developing queens (Laidlaw & Page, 1996). Rearing queens allowed us to select for IAPV free larvae to be reared in IAPV free colonies. In addition, it facilitated a selection of queens grafted on the same day to reduce variations due to queens' age or colony rearing.

Quantitative Real Time PCR (qPCR)

RNA was extracted from eggs, capped brood, adults, and queens samples using TRIzol® (Invitrogen, Carlsbad, CA) adhering to the manufacturer's established protocol. The isolated RNA pellets were resuspended with nuclease-free molecular grade water. Extracted RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). The cDNA was synthesized using a SensiFAST ™ cDNA Synthesis Kit (Bioline, Taunton, Massachusetts). A total of 2,000 ng of RNA diluted in 8 µl nuclease-free molecular grade water was used for cDNA synthesis. This mixture was added to 0.5 ml thermocycler tubes and an additional 2.2 µl of DNase solution (1 µl of 10X DNase buffer (Thermo Scientific, Wilmington, DE) + 1µl of DNase (1 unit/ µl, Thermo Scientific, Wilmington, DE)(1 unit of enzyme degrades 1 µg of DNA) + 0.2 µl RNase Out™ (Invitrogen, Carlsbad, CA)) was added to each sample to degrade contaminating DNA. The samples were incubated in a thermocycler at 37 °C for 1 hour and 75 °C for 10 minutes to denature the DNase enzyme. Then 9.8 µl of cDNA synthesis master mix was added to each sample. The master mix consisted of 4 µl of 5x TransAmp Buffer, 1 µl of Reverse Transcriptase, and 4.8 µl of molecular grade water. Samples were incubated in the thermocycler with the following program: 25 °C for 10 minutes (primer annealing), 42 °C for 15 minutes (reverse transcription), and 85 °C for 5 minutes (inactivation).

Two microliters of the product of the cDNA reaction were used for qPCR using Sensifast SYBR™ Green kit (Bioline, Taunton, Massachusetts). The qPCR reactions were done using a StepOnePlus™ (Applied Biosystems, Foster City, CA) real-time

thermocycler. The protocol consisted of a holding stage of 3 minutes at 95 °C, then 35 cycles of three steps of 95 °C for 20 seconds, 60 °C for 30 seconds, and 72 °C for 1 minute. Fluorescence was measured during the last 72 °C step for each cycle (Boncristiani et al., 2013). Each sample was assayed to ascertain presence or absence of IAPV in duplicates by using the geometric mean of C_T (cycle threshold) values and normalized against the geometric mean C_T values of a reference gene. Samples that did not amplify for target and reference genes were excluded from further evaluation.

Primers for the specific targets of the qPCR are listed below in Table 1.

Table 1. Oligonucleotide Primers for qPCR Assay

Target	Primers (5' to 3')	Source
A. mellifera actin	AMActin F TTGTATGCCAACACTGTCCTTT AMActin R TGGCGCGATGATCTTAATTT	Simone et al., 2009
Ribosomal protein S5	RPS5 F AATTATTTGGTCGCTGGAATTG RPS5 R TAACGTCCAGCAGAATGTGGTA	Evans & Pettis, 2005
IAPV virus protein 2 (203-bp)	IAPV EVA F CCATGCCTGGCGATTCAC IAPV EVA R CTGAATAATACTGTGCGTATC	De Miranda et al., 2010
IAPV (586-bp)	IAPV1 F GCGGAGAATATAAGGCTCAG IAPV1 R CTTGCAAGATAAGAAAGGGGG	Di Prisco et al., 2011; Gregorc et al., 2012 EU224279
IAPV virus protein 3 (110-bp)	IAPV B4S0427_L17 CGAACTTGGTGACTTGAAGG IAPV B4S0427_R130 GCATCAGTCGTCTTCCAGGT	Cox-Foster et al, 2007
DWV RNA-dependent RNA polymerase (6526-6772) (302-bp)	DWV1 F GAGATTGAAGCGCATGAACA DWV1 R TGAATTCAGTGTCGCCCATA	Zhang et al, 2012
DWV	DWV Q F TAGTGCTGGTTTTCCTTTGTC DWV Q R CTGTGTCGTTGATAATTGAATCTC	Oliveira, 2013
DWV (702-bp)	DWV-Long F ATCAGCGCTTAGTGGAGGAA DWV-Long R TCGACAATTTTCGGACATCA	Chen et al., 2005 a; accession no. NC_004830
DWV RNA dependent RNA polymerase (136-bp)	DWVQ-F8668 TTCATTAAAGCCACCTGGAACATC DWVQ- B8757 TTTCCTCATTAACTGTGTCGTTGA	Yañez et al., 2012a; Forsgren et al., 2009

Methods Specific to Each Experiment

Preliminary Experiments of 2015 and 2016

The 2015 and 2016 preliminary experiments were performed with the following experimental steps: 1) survey of source colonies 2) survey of experimental nucs for IAPV and DWV 3) evaluation of three delivery methods of IAPV**:** feeding, injection, and topical application.

Survey of Source Colonies

In the summer of 2015, the IAPV and DWV status of the UNCG apiary source colonies was studied by sampling two capped brood items from each of the six colonies. After removal from the brood frame, the samples were kept on dry ice and stored immediately in a -80 °C freezer. Their RNA was extracted followed by cDNA synthesis and qPCR using IAPV1 and DWV1 primers (Table 1). In the summer of 2016, six new source colonies were established. The IAPV-free status of these colonies was confirmed by a qPCR assay of a pooled sample of 20-30 nurses per colony (De Miranda & Fries, 2008). The primer IAPV EVA was used in conjunction with the housekeeping gene primer AMActin (Table 1). Both IAPV1 and IAPV EVA primers target different sequences within the IAPV genome (Table 1). DWV was not monitored because the almost ubiquitous presence of DWV had already been confirmed by the 2015 preliminary experiments. Due to the overall high presence of DWV in the U.S. (Ellis & Munn, 2005) excluding samples infected with DWV was not feasible.

Initially, IAPV1 and DWV1 primers were used to evaluate the IAPV and DWV status of source colonies in 2015 (Table 1). However, due to the numerous IAPV and

DWV strains present in the U.S. (Martin et al., 2012; Singh et al., 2010) it was useful to optimize the primers to identify the specific strain of virus in our virus inoculum and in the apiary. Three different IAPV primers and four different DWV primers (Table 1) from various studies were tested using a qPCR assay to amplify for the targeted genes in IAPV fed and injected, and control 2-day old workers. Upon evaluation of consistent mean of melt curves and consistent geometric means of C_T, IAPV EVA and DWV Q primers were selected for the subsequent experiments.

Survey of Experimental Nucs for IAPV and DWV

A survey of the experimental nucs was conducted in the 2015 summer by analyzing 12 nucs placed at the Joint School of Nanoscience and Nanoengineering in Greensboro, the experimental site for both 2015 and 2016 summers. Nucs were assembled with workers from IAPV-free source colonies. Ten queens were grafted, one was a naturally raised queen, and one queen was an older mated queen from the apiary (age unknown). All virgin queens were given approximately one month to mature, mate, and start laying eggs. Once queens were mated and had established an acceptable egg laying pattern, two pooled samples of 25 eggs each were collected. Five capped brood and five adult nurses were collected in tandem one to three weeks post egg collection based on capped brood availability since adult nurses were constantly present in a colony. RNA was extracted from these samples, followed by cDNA synthesis, and qPCR to detect IAPV and reference gene ribosomal protein S5 with IAPV EVA and RPS5 primers (Table 1). In order to evaluate co-infection of DWV and IAPV in samples, samples that tested positive for IAPV were subsequently tested for DWV using DWVQ primer

(Table 1). A Pearson correlation analysis was conducted using IBM SPSS to assess the relationship between the levels of IAPV and DWV.

The RPS5 and the AMActin primers for the reference genes ribosomal protein S5 and *A. mellifera* actin were evaluated after the summer 2015 preliminary experiment to survey the experimental nucs for IAPV and DWV, to determine which reference gene was optimal to normalize the targeted genes. The primers for both genes were used in a qPCR assay. After evaluating the consistent mean of melt curves and consistent geometric means of C_T of both reference genes, AMActin primer was selected to be used for further experiments.

Evaluation of Three Delivery Methods of IAPV: Feeding, Injection, and Topical Application

Feeding and Injection

In the summer of 2015, preliminary experiments were conducted on 2-day old workers to compare feeding and injection methods to establish an effective dose that was later administered to queens to cause an infection. Workers were used instead of queens due to the lack of success in grafting experimental queens. The stock solution of IAPV for both delivery methods consisted of previously isolated infectious and non-infectious virus particles diluted in PBS (Boncristiani et al., 2013). The stock solution was diluted in PBS in a series of dilutions ranging from 10^{-1} to 10^{-7}. These dilutions were the ones used for the preliminary and main experiments.

For both feeding and injection methods, comb with emerging brood was collected from an IAPV free colony and transported to the incubator set to 34 °C and 60 % relative

humidity (RH). Emerging adult workers were collected and allowed to mature for two days in 9.9 cm x 9.9 cm x 10.5 cm plexiglass cages with an *ad libitum* diet of dark corn syrup mixed with powdered sugar and a separate receptacle of water (Figure 3A).

For the feeding delivery method, the workers were starved in plexiglass cages for three hours. Starved workers were inserted into prepared 1.5 ml microcentrifuge tubes with an opening and fed via a micropipette with 2 µl of 30 % sucrose mixed with 1 µl of PBS containing IAPV dilutions of 10^{-2}, 10^{-3}, or 10^{-4} of our IAPV stock solution (Figure 3B). The control group was fed with 3 µl of 30 % sucrose solution mixed with PBS. The PBS control was always performed in tandem with an IAPV treated group. Each dilution was administered one time in the individual's lifespan.

For the injection delivery, the IAPV was injected using a micro-injector at a 0.1 µl/sec infusion flow rate (NanoJet, Chemyx Inc, Stafford, TX). A standard microliter syringe with a 10 µl cemented needle was inserted between the second and third anterior abdominal plates (Figure 3C). Workers were sedated in a freezer (-20 °C) until they were immobile preceding an injection, then injected with 1 µl of IAPV dilutions of 10^{-4}, 10^{-5}, 10^{-6}, or 10^{-7} of our IAPV stock solution. The control group was injected with 1 µl of PBS.

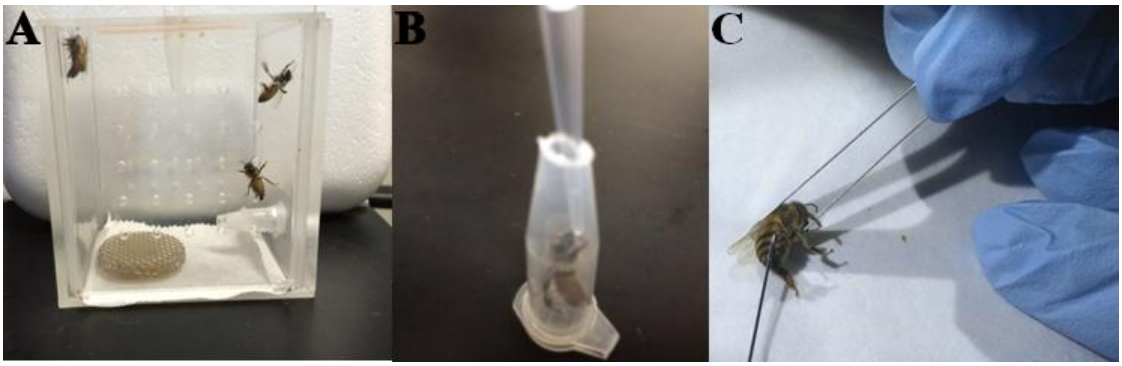

Figure 3. Infection of Honey Bee Workers Via Feeding and Injection. **A**. Workers in plexiglass cages supplied with *ad libitum* diet of dark corn syrup mixed with powdered sugar and a separate receptacle of water. **B**. Worker in a microcentrifuge tube fed by a micropipette tip. **C**. Worker injected with a microliter syringe between the second and third anterior abdominal plates.

Both IAPV treated and control groups of workers, which consisted of five workers per treatment group, were placed in plexiglass cages in an incubator set to 34 °C and 60 % RH in complete darkness provided the same diet mentioned previously. Mortality was monitored daily for both fed and injection groups for a week to evaluate their response to the dosage. Statistical analysis was performed using Graph Pad Prism (Graph Pad Software, CA, USA). Multiple 2 x 2 contingency tests were used to determine if there was difference in survival rates of PBS and IAPV pairings done in tandem. Significance was determined using a two-tailed Chi-square statistics with Yates' correction. This statistic could not be computed for pairings without any mortality, but in those cases there was obviously no difference in mortality.

Injection: Testing Established Effective Dose

Subsequently, the established effective dose for injecting workers of 1 µl of a 10^{-6} dilution of the IAPV stock solution was tested on eight queens. The queens were placed

in a refrigerator at 4 °C until immobile. Then, they were injected with IAPV, adhering to the same procedure applied to 2-day old workers. After injection, the queens were placed back in their respective nuc colonies. Any colony that had a surviving queen three days post-injection was checked for an adequate supply of workers and food resources, then sealed for a week to allow for egg-laying.

Topical Method

Due to the availability of queens in the summer of 2016 and unsatisfactory results from the other incubation procedures in 2015, a topical application for IAPV inoculation was tested directly on queens. Twenty naturally mated queens were purchased from Roberts Bee Company- H & Apiaries in Georgia in order to start evaluating the topical method and establish an effective IAPV dose early in the spring of 2016. Prior to the inoculation, the following samples were tested to assess the IAPV status of the purchased queens: two individual queens, two pooled samples of 16-20 workers accompanying the queens in their cages. In addition, two control queens that were shaved and inoculated with PBS as described below, were also assessed in parallel to the IAPV inoculated queens. The RNA was extracted from the samples, followed by cDNA synthesis and qPCR to detect IAPV using the IAPV EVA primer normalized with the reference gene *A. mellifera* actin (Table 1).

For the actual evaluation of the topical method experiment, the remaining 18 queens were anesthetized in glass vials placed in an ice container. A latex free syringe with a Precision Glide^{TM} Needle (Becton Dickinson and Co., Franklin Lakes, NJ) was used to shave hairs from a small area on the thorax to which 2 µl of inoculum solution

was applied (Figure 4) (Amiri et al., 2014). The inoculum solution after being placed on the queen's thorax would form a tiny bubble, which needed to dry. Therefore, queens were placed in wooden cages upright to prevent loss of treatment solution. The queens were divided into the following five treatment groups: undiluted IAPV inoculum, IAPV 10^{-1}, IAPV 10^{-2}, IAPV 10^{-3}, and PBS control (Table 2). The queens were kept in wooden cages with mesh wire in a sealed observation hive to be fed by workers. During the experimental period, the hive had to be opened twice to allow workers to defecate outside. The workers obstructed queens visually in the cages even after the queens' deaths, so mortality data of queens could not be collected. Instead, queens were left 5-7 days in the observation hive, and then assessed for IAPV titer levels. Random samples from each group were tested for IAPV presence, excluding the undiluted IAPV group (Table 2). Based on the qPCR analysis of the presence of IAPV in the tested three dilutions, the IAPV 10^{-1} dilution was selected as the dose to use for experimental queens in the main experiments.

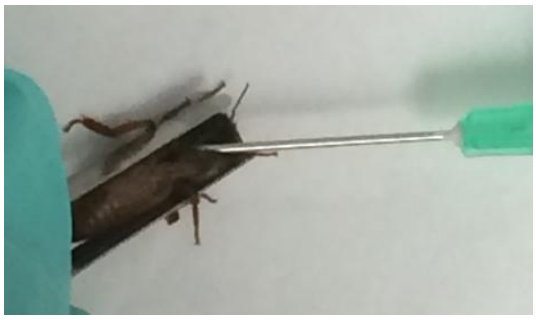

Figure 4. Shaving Procedure to Remove Hairs on the Queens' Thoraces.

Table 2. Sample Sizes for Preliminary Tests of Topical IAPV Application on Queens

Treatment	N- applied the treatment	qPCR- samples tested for presence of IAPV
Undiluted IAPV	3	0
10^{-1} dilution of IAPV stock	6	3
10^{-2} dilution of IAPV stock	3	3
10^{-3} dilution of IAPV stock	2	2
PBS (control)	4	2

Experiments 1 and 2: Experimental Queens

All the queens were supplied with workers from IAPV free source colonies and all queens were allowed to mate naturally. There were two set of experimental queens, group one consisted of a total of 18 queens that had their thoraces shaved and the 10^{-1} IAPV dilution was applied to 11 of the queens and PBS was applied to the other seven. In this case, experimental queens were treated in the laboratory and thus need to be transported for 15 minutes from and to the experimental site. The second experimental group of queens was treated directly at the experimental site with a lower dilution (10^{-2}) of IAPV inoculum due to the high mortality rate in the first group. The second group consisted of 19 queens that had IAPV applied to them and 11 queens that had PBS applied to them. These experimental queens were used for experiment 1 and 2. Group one was grafted at the North Carolina State University while group two was grafted at UNCG.

The three specific aims were achieved in two separate experiments that directly compared IAPV-inoculated with control queens. The first experiment investigated queen–worker interactions in an attraction bioassay addressing the first aim. The second experiment addressed the transmission dynamics of IAPV from infected queens in

separate nucs and studied trans-generational immune priming focused on the second and third aims.

Experiment 1 (Aim 1): 2-Choice Olfactometer Attraction Bioassay

For the first experiment a 2-way choice olfactometer attraction bioassay (Figure 5) was developed and used to test the prediction that IAPV-infected queens would have reduced attractiveness to workers.

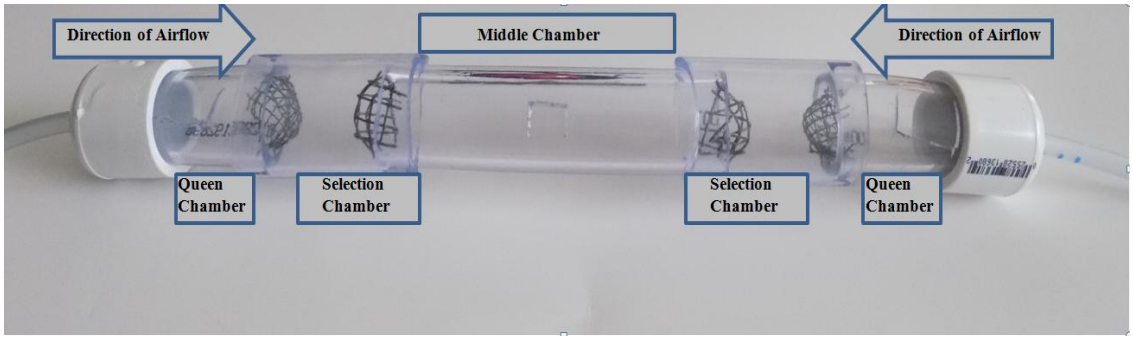

Figure 5. The Five-chamber Olfactometer Used to Measure Worker Preference Towards IAPV Infected or Control Queens. A steady stream of air flowed from both sides towards the middle chamber. This allowed the pheromone smell from both queens to be directed towards the tested worker in the center. The worker made a selection for one queen when she crossed from the middle chamber through a wire mesh into either selection chamber.

The olfactometer consisted of five chambers (left chamber 5 cm in length, 2.5 cm outer diameter, and 1.9 cm inner diameter; next chamber 5 cm in length, 3.2 cm outer diameter, and 2.5 cm inner diameter; middle chamber 10 cm in length, 2.5 cm outer diameter, 1.9 cm inner diameter; the last two chamber are symmetrical in dimensions to the first two chambers). Each chamber was made of polyvinyl chloride (PVC) pipe. An aquarium pump with a two-way splitter attached to two tubes provided even air flow from both sides from the outer chambers towards the middle chamber. The queens were

placed in the outer chambers, so their pheromones flowed towards the middle chamber where each tested worker was placed. A flap made in the middle chamber allowed workers to be inserted into the center chamber of the apparatus. Only one worker was tested per trial. Trials were performed in a dark room with only red light shining symmetrically on the apparatus to allow the observer to see the choice made by each worker. The worker selected either a control or IAPV treated queen by moving from the middle chamber, passing through a queen excluder, which consisted of mesh wire with holes large enough to allow a worker to squeeze through into a selection chamber (Figure 5). The queen was isolated in her own chamber by a mesh wire with holes that prevented her from leaving her chamber and a worker entering her chamber. Workers were two to four-days old from a separate colony to reduce any bias towards nestmates. The outer chambers were interchangeable, and the control and infected queen were switched after each trial to reduce any directional bias. All assays were conducted by one researcher to reduce any inter-trial variation. Before each bioassay the apparatus was completely disassembled and cleaned with bleach, 70% ethanol, rinsed with water, and dried.

IAPV-infected queens were randomly paired with control queens and transported 2-12 days after IAPV application, two at a time to the laboratory site to conduct the 2-choice olfactometer assay as described above. A total of 12 randomly assembled pairs from a total of 19 IAPV and PBS treated experimental queens were analyzed. Four of the controls were used twice in the bioassay. Ten trials were conducted in 10 out of the 12 pairs in which one trial consisted of one worker's selection of treatment queen. The two remaining pairs, one had three trials while the other pair had nine trials. The recorded

observations of workers choosing IAPV-infected queens against workers choosing PBS applied control queens were analyzed by using the Wilcoxon signed rank test in the statistical software IBM SPSS. This analysis was based on treatment since the actual IAPV infection status of queens could not be verified without killing the queens.

Experiment 2 (Aims 2 and 3)

The main experimental steps were 1) gathering different members of colonies with IAPV-infected queen to determine their IAPV infection status and infer virus transmission routes, 2) evaluating the potential of immune primed offspring, and 3) verifying the IAPV infection status of experimental queens at the end of the experiment.

Virus Transmission Routes

In order to evaluate horizontal and vertical transmission routes of IAPV from a sick queen, the following life history stages were collected from nucs with IAPV- and PBS-inoculated queens: eggs, capped brood, and adults. A total of 17 colonies were sampled, nine colonies were in the IAPV treatment group while eight were in the PBS treatment group. Eggs, capped brood, and adults were collected from all 17 colonies (Table 3). The IAPV-infected queens had two or three sample collection points ranging from one week post-application to two weeks post-application, then a final collection when the colony was disassembled after one month. The PBS group only had one collection when the colony was disassembled after one month. The samples were collected and stored on dry ice at the experimental site and then transported to a -80 °C freezer for later analysis.

Table 3. Collection of Eggs, Capped Brood, and Adults from Experimental Colonies

IAPV Applied Colonies				PBS Applied Colonies			
Colony	Eggs*	Capped Brood^	Adults^	Colony	Eggs*	Capped Brood^	Adults^
C1	2,2,2[a]	5,8,8	5,8	C12	2	8	8
C2	2,2,2	5,8,8	5,8	C13	0	8	8
C3	2,2,2	5,8,8	5,8	C14	2	8	8
C4	2,2	5,8,8	5,8	C15	2	8	8
C5	2,2	8,8	8,8	C16	2	8	8
C6	2,2	8,8	8,8	C17	2	8	8
C7	2,2	8,8	8,8	C18	2	8	8
C8	2,2	8,8	8,8	C19	2	8	8
C9	2,2	8,8	8,8				

***** There are 25 eggs pooled into one sample
^ Each sample constitutes one individual
[a] Each comma separates a different collection date

Immune Priming

Experimental colonies that remained alive after one month included eight IAPV treated and eight PBS treated nucs. Two capped brood frames per colony were placed in separate wired cages after they were brushed to clear all adults, checked for food, and emerging adults. IAPV treated wired cages were separated from the PBS treated wired cages and both groups of cages were placed in separate incubators set to 34 °C. Adults were left to emerge and mature for four days. Subsequently, ten to twelve workers were collected from each of the 16 wired cages and placed in plexiglass cages fed with an *ad libitum* diet of dark corn syrup mixed with powdered sugar and a separate container of water.

To test whether worker offspring from IAPV-exposed queens survived a subsequent IAPV challenge better than offspring from naïve queens, the worker offspring from the 16 nucs were compared for their survival after IAPV inoculation. The shaving and application was done on progeny from alternating IAPV and PBS treated queens. The workers were anesthetized in individual glass vials placed in ice, shaved, and applied with 2 µl of IAPV 10^{-2} following the same procedure as queens. Then, they were placed back into new plexiglass cages as described above. All 16 cages were placed in the same incubator at 34 °C. The mortality data was recorded twice a day, once in the morning and once in the evening for one week. The progeny of IAPV treated queens was referred to as "primed", while the progeny of control queens was referred to as "non-primed". The data was analyzed for a difference in survival data between the primed and non-primed individuals using a Mantel-Cox test in IBM SPSS. This analysis was based on treatment since the IAPV status of queens during offspring production could not be verified without killing them.

Verifying the IAPV Status of Experimental Queens at the End of the Experiment

The queens used for the 2-choice olfactometer bioassay and the immune priming experiments were evaluated by qPCR for the presence of IAPV using IAPV EVA primers in conjunction with reference gene *A. mellifera* actin (Table 4). A total of 22 colonies were analyzed. Three of those colonies had no queens left at the time of analysis, therefore a pooled sample of eight capped brood were sampled in their place. The 19 queens were removed from the -80 °C freezer and kept on dry ice during dissection into three segments: head, thorax, and abdomen. The head and abdomen underwent RNA

extraction, cDNA synthesis, and qPCR analysis for 17 out of 19 queens. In the last two queens only the abdomen was analyzed. The thorax was excluded from analysis due to the possibility of residual virus from the inoculum being present on it. Delta C_T values were calculated for all three categories (head, thorax, and capped brood) by mean C_T of reference gene minus mean C_T of targeted gene. The higher positive value of delta C_T the higher the concentration of the targeted gene (Chen et al., 2005a). A t-test analysis was conducted using IBM SPSS to assess the difference between IAPV titer levels of IAPV and PBS treated queens' heads and thoraces. Any values that did not amplify for targeted gene are "undetermined" values and classified as delta $C_T = 40$ in the analysis.

Table 4. Samples That Were Tested for IAPV Via qPCR and Whether They Were Used for the Olfactometer Assay , Immune Priming, or Both

IAPV Applied Colonies					
Colony	Head	Abdomen	Capped Brood	Olfactometer Assay	Immune Priming
C1	T*	T	NT**	T	T
C2	T	T	NT	T	T
C3	T	T	NT	T	T
C4	NT	NT	T	T	NT
C5	T	T	NT	T	T
C6	T	T	NT	T	T
C7	T	T	NT	T	T
C8	T	T	NT	T	T
C9	T	T	NT	T	T
C10	T	T	NT	T	NT
C11	T	T	NT	T	NT
C12	T	T	NT	T	NT
PBS Applied Colonies					
Colony	Head	Abdomen	Capped Brood	Olfactometer	Immune Priming
C13	T	T	NT	T	T
C14	T	T	NT	T	T
C15	T	T	NT	T	T
C16	NT	T	NT	NT	T
C17	T	T	NT	T	T
C18	T	T	NT	T	T
C19	T	T	NT	T	T
C20	NT	T	NT	NT	T
C21	NT	NT	T	T	NT
C22	NT	NT	T	T	NT

*T= Tested, **NT= Not Tested

CHAPTER III

RESULTS

Preliminary Experiments of 2015 and 2016

Survey of Source Colonies

In the summer of 2015, the six selected source colonies at the UNCG apiary were screened for IAPV and DWV. The qPCR results indicated IAPV in one of the six colonies while DWV was detected in five colonies (Table 5). Colony F was the only IAPV-infected colony and it was subsequently excluded from the remaining preliminary experiment. In the summer of 2016 all six new source colonies tested negative for the presence of IAPV with a clear amplification of the reference gene *A. mellifera* actin. The colonies were used to supply emerged workers to the experimental nucs and the 2-choice olfactometer bioassay.

Table 5. Presence of DWV and IAPV in Capped Brood from Experimental Colonies in 2015

Colony Name	IAPV	DWV
Colony A	0/2	1/2
Colony B	0/2	1/2
Colony C	0/2	1/2
Colony D	0/2	0/2
Colony E	0/2	2/2
Colony F	1/2	2/2

Survey of Experimental Nucs for IAPV and DWV

The following preliminary experiment was implemented to establish the natural IAPV virus titer levels at the experimental site without experimental IAPV inoculation. A total of 12 colonies were assessed via qPCR assay, only two colonies were IAPV free in the three life history stages of egg, capped brood, and adult (Figure 6). IAPV was detected in 50% of the colonies in the egg and capped brood stages, although IAPV was not necessarily present in both stages within one colony. IAPV was detected at a lower percentage of 42% in adults within colonies. There were only two colonies positive for IAPV in all three life history stages.

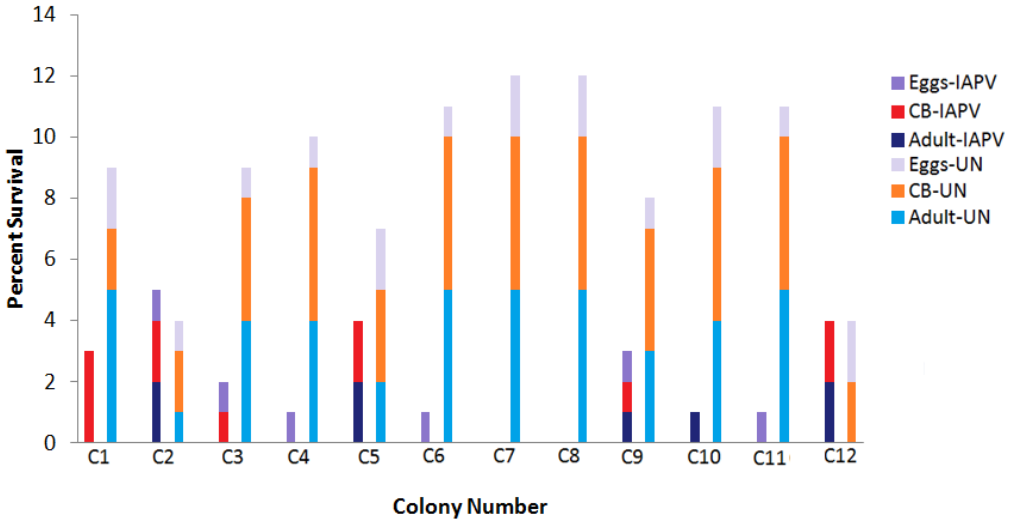

Figure 6. Results of IAPV Survey in Eggs, Capped Brood (CB), and Adults of 12 Experimental Colonies. Only two colonies were IAPV free (UN), while two colonies had IAPV present in all three life history stages.

All IAPV positive samples were additionally screened for presence of the DWV in conjunction with the reference gene *A. mellifera* actin. Across life history stages, total

of 23 samples across nine colonies amplified for all three transcripts (Figure 7). All colonies were co-infected with DWV and IAPV in at least one of the life history stages and two of the colonies had co-infection in all three life history stages. Across these samples, the IAPV and DWV delta C_T values were positively correlated based on the Pearson correlation (r=0.415, p=0.001, with a R^2=0.172).

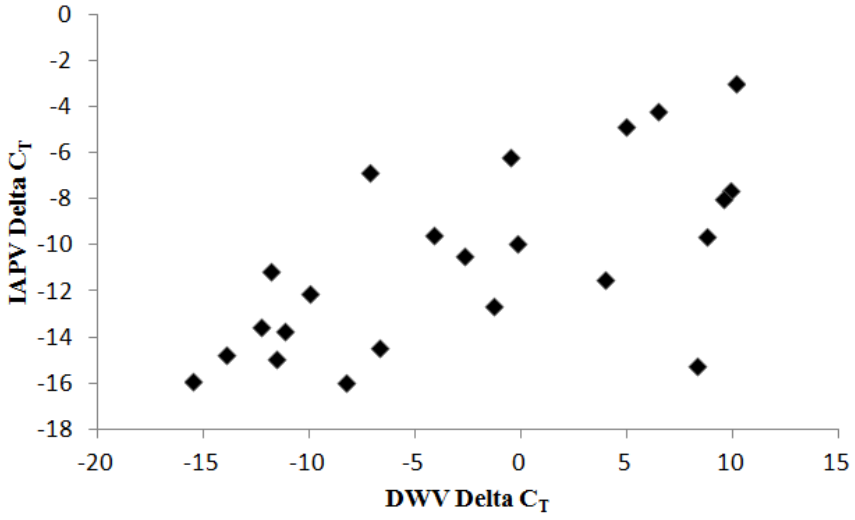

Figure 7. Twenty-three Samples Tested for DWV and IAPV Across Nine Colonies. Nine colonies were co-infected with DWV and IAPV in at least one of the life history stages. Delta C_T represents mean C_T of reference gene minus mean C_T of targeted gene. The higher positive value of delta C_T the higher the concentration of the targeted gene.

Evaluation of Three Delivery Methods of IAPV: Feeding, Injection and Topical Application

In the absence of any reliable information on effective inoculation of honey bees with IAPV, preliminary methods were established in workers via feeding and injection that were then transferred to queens. Due to the availability of queens in the 2016 summer, the topical method was tested directly on queens.

Feeding

The survival rate of 2-day old workers fed with 10^{-2}, 10^{-3}, or 10^{-4} dilutions of IAPV was analyzed. Only the 10^{-3} dilution had deaths in the IAPV treated groups with an 85% survival rate in the four trials conducted (Figure 8). Chi-square analysis resulted in no statistically significant differences between survival rates of PBS and IAPV pairings.

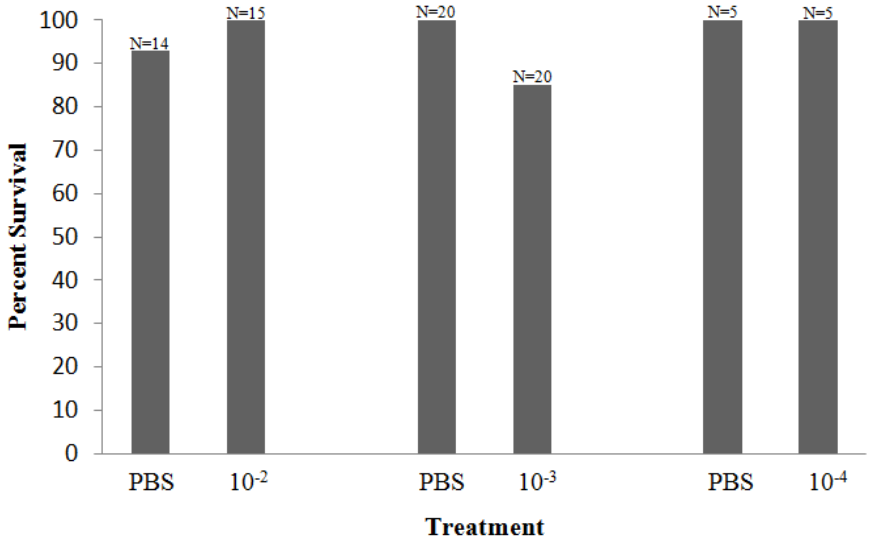

Figure 8. One week Survivorship of 2-day Old Workers Fed IAPV. Chi-square analysis resulted in no statistically significant differences in survival rates between PBS and IAPV treated groups: PBS vs. 10^{-2} p=0.972, PBS vs. 10^{-3} p= 0.230 and PBS vs. 10^{-4} could not be calculated.

Injection

The injection survival rates over a span of one week of 2-day old workers injected with 10^{-4}, 10^{-5}, 10^{-6}, or 10^{-7} dilutions of IAPV paired with PBS controls were recorded, and then the PBS and IAPV pairings were analyzed statistically for differences in

survival rates. The IAPV 10^{-5} dilution with two trials performed had the lowest survival rate of 20% (Figure 9). The next dilution of IAPV 10^{-6} had two trials and had a 72.2% survival rate. The chi-square analysis resulted in a significant difference between survival rates of both IAPV 10^{-4} and IAPV 10^{-5} pairings (Figure 9). The IAPV 10^{-6} pairing had no significant difference in survival rates while the IAPV 10^{-7} pairing could not be calculated. Within the injection method, the dilution selected was IAPV 10^{-6} with a 72.7% survival rate as the effective dose to test on queens.

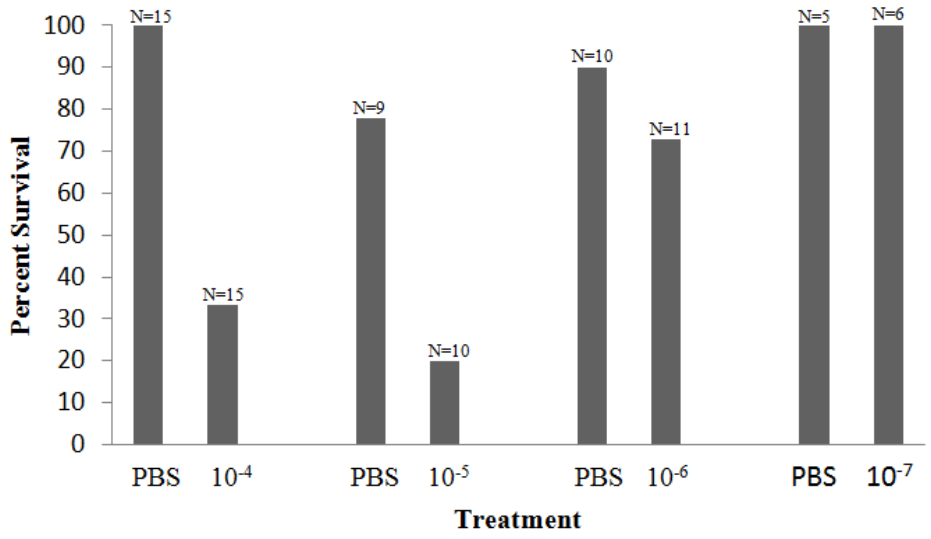

Figure 9. One Week Survival Rates of 2-day Old Workers Injected with IAPV. High IAPV concentrations resulted in significantly lower survival rates than control injections (PBS vs. 10^{-4} p=0.0005, PBS vs. 10^{-5} p= 0.040). The PBS and 10^{-6} pairing was not significantly different p= 0.6524. In the last pairing of PBS and 10^{-7} the P value could not be calculated.

Injection: Testing Established Effective Dose on Queens

When the effective injection dose of IAPV 10^{-6} was tested on eight queens, six queens died two days post-infection. The last two queens died in less than a week, but

the exact time of death is unknown since they were sealed in nucs with workers attending them. When the nucs were opened a week later the whole colony was dead. The injection method resulted in the death of all eight queens one-week post-injection.

Topical: Establishing an Effective Dose

The IAPV status of purchased naturally mated queens was determined by qPCR of two pooled samples of accompanying workers in the shipping cages and two queens. One pooled sample and the two queens tested positive for low levels of natural IAPV.

The test of topical IAPV application onto the shaved thorax (shaving method) resulted in IAPV detection in all eight inoculated queens and none in the two PBS controls. The level of infection varied within the same treatment group regardless of concentration (Figure 10). Based on the high delta C_T values of IAPV and the slightly higher success of viral introduction at that value, the 10^{-1} dilution was selected as the effective dose.

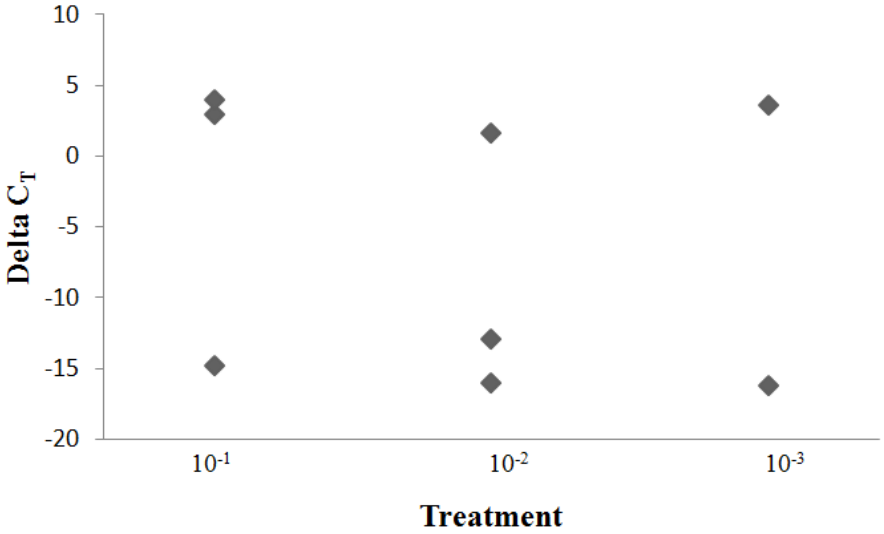

Figure 10. Delta C_T Values of Eight Queens' Topical Application of IAPV 10^{-1}, 10^{-2}, and 10^{-3} Dilutions. The PBS group composed of 2 queens had no presence of IAPV, so it was excluded from the graph. 50% of the queens had IAPV levels that were very high compared to the reference gene. Delta C_T represents mean C_T of reference gene minus mean C_T of targeted gene. The higher positive value of Delta C_T the higher the concentration of the targeted gene.

Experiment 1 and 2: Experimental Queens

The first group of experimental queens were applied the selected IAPV 10^{-1} topical effective dose and consisted of 11 IAPV and seven PBS treated queens. Five of the IAPV treated queens died less than1 hour post-application (Figure 11). Overall, the mortality rate after one week for the IAPV group was 90.1% and the PBS group was a 42.9%. As a result of this high mortality, this group of experimental queens was not used in experiment 1 and 2. The second group of queens were applied a lower dilution of IAPV 10^{-2} and consisted of 19 IAPV and 11 PBS treated queens. Less than one hour post-application only one IAPV treated queen died from the total 19 IAPV treated queens

(Figure 12). A total of eight IAPV treated queens died 0.5-7 days later. However, three of those queens constitute accidental deaths or went missing from the hive. The overall mortality rate from 90.1% in the IAPV 10^{-1} group (Figure 11) dropped to 26.3% in the IAPV 10^{-2} group (Figure 12). The PBS treated queens had an overall 0% mortality rate in the second experimental group.

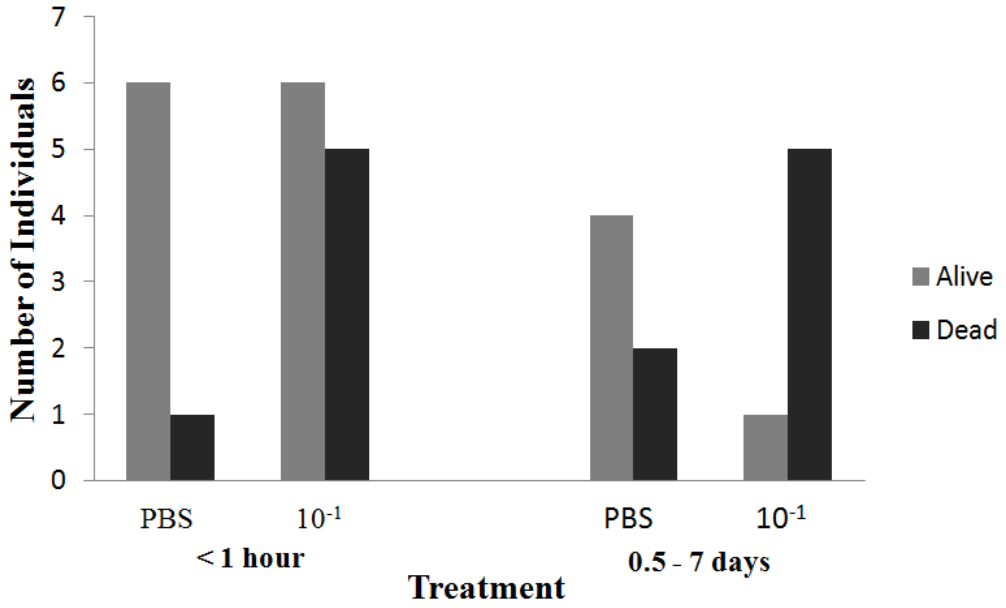

Figure 11. Record of the Deaths and Survival of the First Group of Experimental Queens Inoculated with IAPV 10^{-1}. IAPV 10^{-1} was applied to 11 queens and PBS was applied to seven queens. The bars on the left are PBS and IAPV treated queens that died in less than one hour after application. Any queens that died less than one hour were excluded from the bars on the right, which display PBS and IAPV queens that died 0.5-7 days post-application.

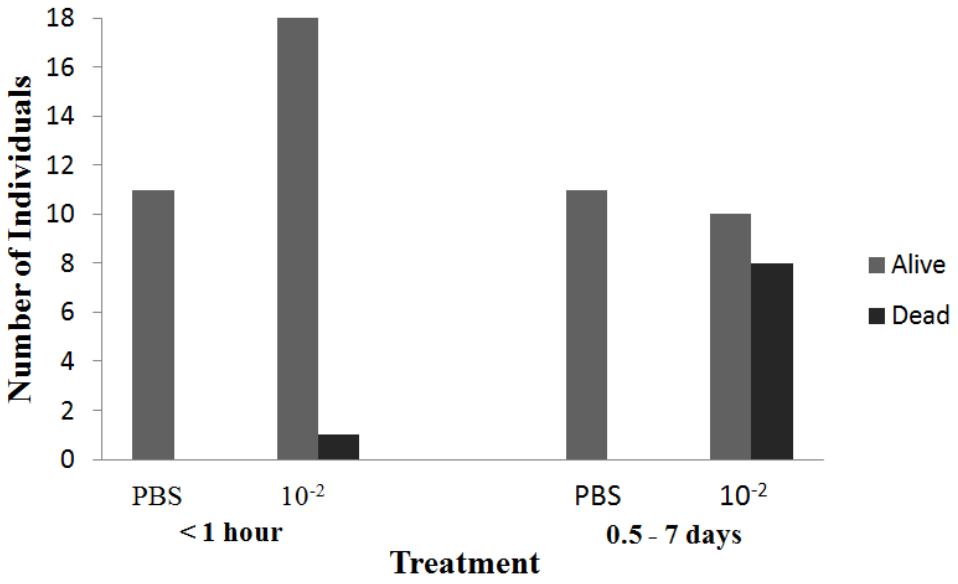

Figure 12. Record of the Deaths and Survival of the Second Group of Experimental Queens Applied IAPV 10^{-2}. Experimental group 2 consisted of 19 queens that had IAPV 10^{-2} applied to them and 11 queens that had PBS applied to them. The bars on the left are queens both in PBS and IAPV groups that died less than hour post-application. Any queens that died in less than one hour are excluded from the bars on the right, which display PBS and IAPV queens that died 0.5-7 days post-application Three of the IAPV applied queens in the 0.5-7 days group died due to an accident or went missing.

2-Choice Olfactomer Attraction Bioassay

To assess the potential impact of IAPV on the queens' attractiveness to individual workers, a 2-choice olfactometer bioassay was built and 12 pairs of queens were tested. There were two pairs of queens in which workers had no preference for either queen (five selected IAPV and five selected control) (Figure 13). The worker's selection of infected versus control queens was not significant (Wilcoxon signed rank test: p=0.645; Figure 13).

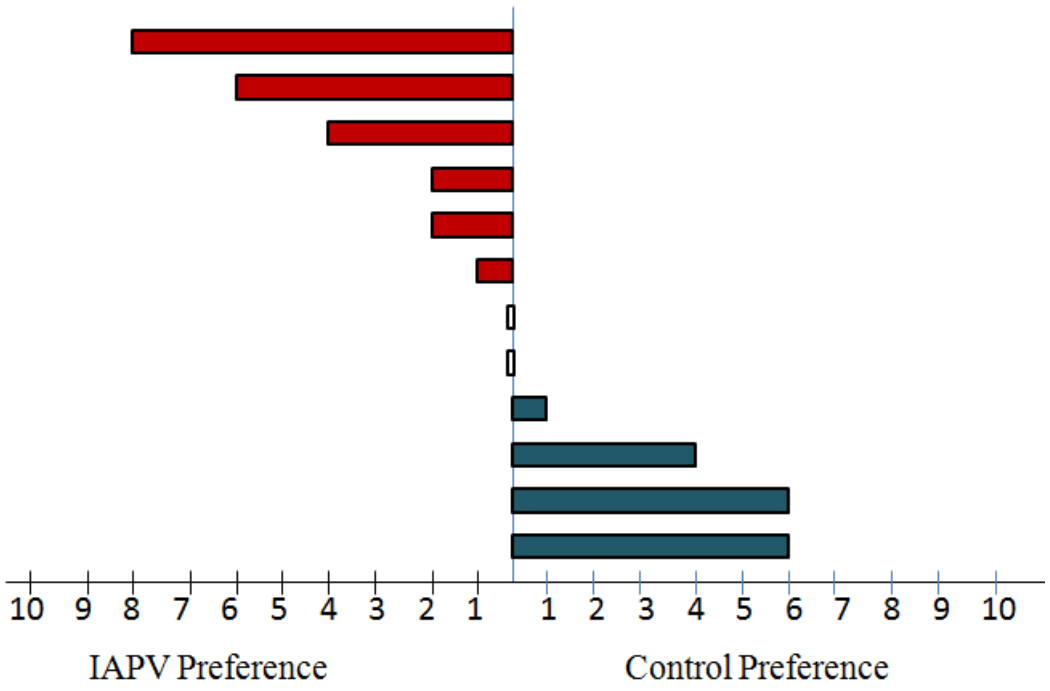

Figure 13. The Preference of Two to Four-days Old Workers Between 12 Pairs of Infected and Non-infected Queens. The red indicates the worker preference for an infected queen while the dark blue indicates worker preference for non-infected queen. There are two pairs of queens at the zero mark in which workers showed no preference for neither infected nor non-infected queen. Workers did not significantly prefer infected queens over control queens (p=0.645).

Experiment 2: Virus Transmission Routes and Immune Priming

Virus Transmission Routes

The samples from the 17 colonies were collected, but not evaluated for IAPV presence due to the results of the summer 2015 preliminary experiment. These surveys of experimental nucs for IAPV demonstrated that IAPV could even be found without IAPV inoculation. Therefore, analyzing these samples would not be meaningful.

Immune Priming

The progeny of eight IAPV treated and eight PBS treated queens was compared with regards to survival of an IAPV 10^{-2} application. The progeny of IAPV treated queens or primed progeny and the progeny of control queens or non-primed survival rate was monitored for a week and analyzed. Survival of the primed and non-primed progeny was not significantly different (Mantel-Cox log rank test, Chi-square = 0.005 p = 0.941; Figure 14).

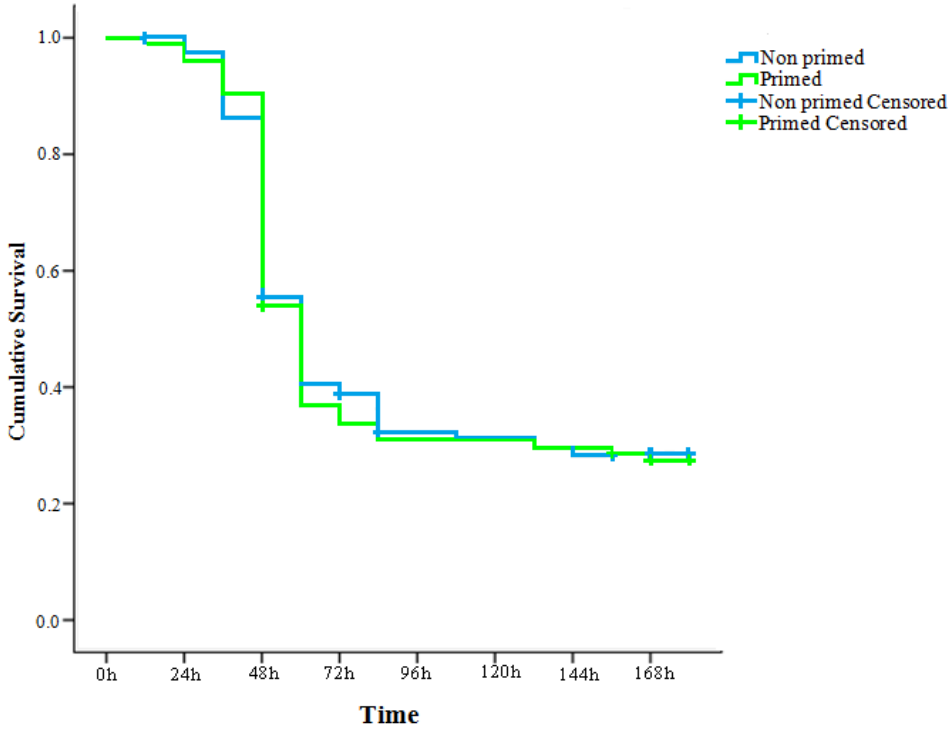

Figure 14. Cumulative Survival of the Immune Primed Individuals and Non-primed Individuals in 16 Colonies. No significant difference was observed in cumulative survivals (p=0.941).

IAPV Infection Status of Experimental Queens at the End of the Experiment

The IAPV status of the experimental queens was evaluated at the end of the second experiment since queens had to be sacrificed to extract the RNA for cDNA and qPCR. Two queens of the 22 tested queens had high IAPV infection in both head and abdomen (Figure 15). The other 20 queens had low IAPV levels similar to the natural levels identified in the 2015 preliminary survey experiment of natural IAPV infections. There were eight samples that had no IAPV presence detected in the abdomen, six of which had presence of IAPV in the head. The other two samples were not tested for IAPV in the head. The difference in IAPV infection levels of IAPV treated and PBS treated queens was not significant in neither the head (t-test, $\mathbf{t}(15)= 0.490$, $p= 0.631$) nor the thorax (t-test, $\mathbf{t}(17)= 1.535$, $p= 0.143$).

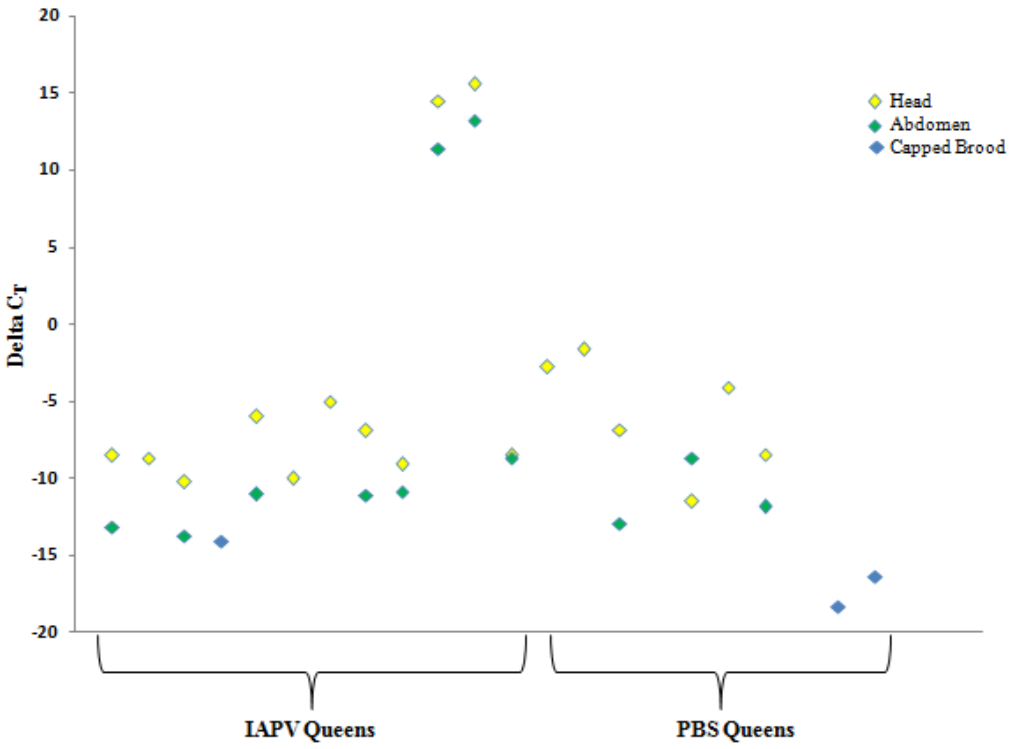

Figure 15. The Delta Ct of IAPV in the Main Experimental Queens' Segments. Delta C_T was calculated mean C_T of reference gene minus mean C_T of targeted gene. There were eight abdomen samples that amplified for the reference gene *A. mellifera* actin, but not for IAPV, so they were excluded from the graph.

CHAPTER IV

DISCUSSION

Few studies focus on queen-virus interactions due to the difficulty of rearing queens and purifying targeted virus. My study aimed at understanding IAPV's impact on the queens' attractiveness, transmission routes in the colony, and the potential of immune priming. The results of my preliminary experiments demonstrated that it is difficult to infect honey bees queens with IAPV without killing them. The best method to infect a queen with an effective dose of IAPV is by a topical application of IAPV onto the surface of the thorax from which the hairs have been shaved off. In experimental colonies, IAPV was found in all investigated life history stages: egg, capped brood, and adult. This suggests IAPV is vertically transmitted to the eggs and the infection may persist on to the developing capped brood. It addition, the presence of IAPV in adults suggests a horizontal route of transmission because the adults were already developed adults that were derived from IAPV-free source colonies. IAPV infection of queens did not affect their attractiveness and did not lead to immune priming of the offspring. However, these results have to be interpreted with caution because the confirmation of an established IAPV infection in inoculated queens was not determined at the end of the experiments.

The two most prevalent methods of introducing viruses into honey bee workers are injection and feeding, while topical application is less common. Due to the lack of success of the two most prevalent methods, topical application was considered as a viable

delivery method to be tested. My study evaluated all three methods. Injection methods have been previously employed with BQCV, DWV,KBV, and IAPV and are similar to the way *Varroa* mites vector diverse viruses by directly "injecting" virus particles into the honey bees' haemolymph (Genersch & Aubert, 2010; Leat et al., 2000; Maori et al., 2007a; Ryabov et al., 2014). Injection has been employed with IAPV by Maori et al. 2007 study, which observed an 80% mortality rate over a span of four days after injecting 100 ug of IAPV RNA into larvae. An 80% mortality rate was observed in my study's IAPV 10^{-5} dilution results over the span of a week. The inoculum used in my study was not quantified for viral genomes and contained a mixture of infective and non-infective particles, so it is impossible to evaluate the number of infective viral genomes injected. Previous studies have not focused on establishing an effective dose due to the difficulty of purifying the targeted virus (Chen et al., 2006b), but in my study a purified inoculum of IAPV with negligible presence of other viruses was used (Boncristiani et al., 2013).

In my 2015 preliminary experiments an injection method was used to establish an IAPV effective dose on workers and was subsequently evaluated on queens. Queens are the sole female reproductive members of the colony and in a previous study, workers were symptomatic to viral infections while queens remained asymptomatic (Francis et al., 2013). Therefore, it was assumed in my study that the queens would have a higher resistance to the IAPV injections. The outcome of the injection of an effective dose established in workers resulted in the death of all eight experimental queens, which suggests that queens are more susceptible than workers to either the injection procedure or the virus. Queen-worker interactions after the injected queens were placed back into

their original nucs may also have been an important factor. Workers could have removed the dead queen, especially since workers display necrophoric behavior (Evans & Spivak, 2010), or killed the compromised queen. The queen could have removed herself from the colony upon the "realization" that she was sick, which is an altruistic self-removal behavior that is displayed in workers (Rueppell et al., 2010). Since it was late in the season there were no control queens for the injections and direct observations were not possible in the nucleus colonies. It is therefore difficult to evaluate whether the queens died due to the injury from the injection, viral levels, or the workers removing them from the colony. Overall, the injection method was an unsuccessful invasive method attempted in my preliminary experiments of 2015 to infect queens that resulted in the death of all eight injected queens.

The second evaluated inoculation method in my preliminary experiments of 2015 was feeding or oral infection, which occurs naturally and is a way to introduce the virus into the individual via contaminated food. It has been successfully employed with BQCV, DWV, ABPV, and IAPV in honey bees and with IAPV and KBV in bumblebees (Chen et al., 2006a; Genersch & Aubert, 2010; Maori et al., 2007a; Meeus et al., 2014). Feeding IAPV to workers can induce a 70-80% mortality rate within a week and 100% mortality rate within 10 days (Maori et al., 2009; Maori et al., 2007a). My study's feeding experiment mortality rate was 15% for IAPV 10^{-3} within a week. This low mortality rate suggests IAPV was not being introduced into the workers at a sufficient level and may not lead to an established IAPV infection. The difference between mortality rate in previous studies and my study could be accounted for by lower

concentration of viral genomes present in my inoculum. In nature, feeding via trophallaxis is an effective route of virus transmission (Chen et al., 2006b), but our feeding method was an unsuccessful method of introducing viruses into workers. Therefore, it was not tested on queens.

The topical method has been previously employed in virus studies, such as with CBPV which causes "Hairless-Black Syndrome" (Rinderer & Rothenbuhler, 1975). Shaving the cuticular hairs off is assumed to open the cuticle. The open wounds act as access points for viruses to infect individuals (Amiri et al., 2014). However, my study is the first study in which shaving was used to remove hairs on the thorax for the topical application of IAPV. Shaving is a less-invasive method of introducing virus particles when compared to injection since it creates a superficial wound on the cuticle. Based on my preliminary experiments of 2016, the topical method yielded a higher success of IAPV introduction into queens than injection and feeding. However, the six queens' IAPV viral levels were analyzed 5-7 days after application and there was a high variability of viral levels within queens applied the same treatment. This could be accounted for by the introduction of different numbers of virus particles via topical application to all queens or the potential differences in queen's immune responses. A previous study of CBPV noted uneven topical application of the virus (Amiri et al., 2014). Quantities of CBPV ranged from 10^4 to 10^6 copies per treated queens' heads applied the same concentration (Amiri et al., 2014). Further studies need to be conducted to optimize the topical method and make it a more consistent method of viral introduction into the organism. Across the three treatments, half of the inoculated queens exhibited

viral levels below the previously recorded naturally-occurring IAPV levels found in my 2015 preliminary experiments. The other half of the inoculated queens viral levels were above the naturally-occurring IAPV levels, which were reached by successful introduction of IAPV via topical application. Due to the lack of success of the two previous methods, the topical method was selected from the three evaluated methods for my main experiment.

At the conclusion of my main experiment the experimental queens' head and abdomen were analyzed for IAPV titer levels. Only two of the analyzed experimental queens' head and abdomen had high IAPV levels comparable to the previously highly infected queens in my topical 2016 preliminary experiment. These two queens were the only two queens that were collected one week post IAPV application because they died. The remaining experimental shaved queens were collected one month post IAPV application at the conclusion of my main experiments. The inverse relationship of viral titer levels and collection time could suggest the possibility of the queens' immune system suppressing the viral infection thus allowing IAPV to be "cleared" from their system. There are no studies focused on queens' immune system response to IAPV or any other viruses, especially in regards to the main honey bee antiviral defense RNA interference pathway (Brutscher & Daughenbaugh, 2015). This limits the understanding of any potential mechanism to suppress IAPV infections.

It is vital to develop a non-invasive method to monitor viral infection status of queens over time. Overall, the evaluation of infection status of live queens is a hurdle in queen-virus studies. Testing the queen's fecal material represents one possible way to

evaluate infection status non-destructively (Chen et al. 2006b), which was attempted in my study. However, the method of collecting queen's fecal matter proved to be too challenging. Alternatives would be to test viral levels of eggs to indirectly test for IAPV infection of live queens or to develop a reporter assay with green fluorescent protein or firefly luciferase that would allow us to monitor viral replication of live queens (Schuster et al., 2014).

Most of the main experimental queens' head and abdomen tested positive for IAPV. Levels of IAPV in both queens' segments were similar to the naturally-occurring IAPV levels found in my 2015 preliminary experiment. The potential for control nucs to get infected from sharing the same foraging ground as infected nucs or from the natural levels of IAPV already present in the environment is supported by the results of my 2015 preliminary experiment and the fact that IAPV titers between infected and control queens in 2016 were not significantly different. The 2015 preliminary experiment included 12 colonies composed of workers from IAPV-free source colonies placed at the same experimental site used for my main experiments. After one to two months the colonies had IAPV in 50% of the eggs and capped brood items collected. A study has found IAPV in royal jelly, honey, and pollen (Chen et al., 2014), which suggests it can be transmitted via a common foraging ground or a shared apiary. Most of the experimental queens had a slightly higher IAPV viral level in the head than the abdomen. Previous IAPV studies of different worker tissues found the highest viral levels of IAPV in gut tissues and nerve tissues (Chen et al., 2014).

My 2015 colony survey detected IAPV in egg, capped brood, and adult developmental stages of honey bees. The presence of IAPV in queens and eggs suggest that IAPV is vertically transmitted to the eggs, which supports previous results (Chen et al., 2014). IAPV is similar to the iflavirus DWV, which is also vertically transmitted from queens to offspring (Yue et al., 2007). DWV was found in eggs, larvae, pupae, drones, and workers, which provides indirect experimental data suggesting DWV is vertically transmitted to the eggs (Chen et al., 2005a). Direct experimental data supporting DWV vertical transmission was provided by Yue et al. (2007), the study tested vertical transmission via drone semen into eggs. The Chen et al. (2014) study analyzed IAPV vertical transmission via drone semen. Unlike these previous studies, my study did not attempt to analyze vertical transmission via the drone semen into the eggs. Without dissecting the queen and testing the ovaries and the spermatheca, it is impossible to determine whether the IAPV infection came from the mother or the father (Chen et al., 2014; Chen et al., 2006a). Therefore, semen may have been the origin of the IAPV rather than the queens. Regardless of the origin of IAPV from mother or father, my study provides indirect experimental data suggesting IAPV is vertically transmitted.

In my study, IAPV was also detected in capped brood in the 2015 preliminary experiments. In other studies, IAPV was found in high concentrations in the hypopharyngeal glands of nurse bees, which produces the royal jelly used to feed queens and larvae (Chen et al., 2014). This increases the potential of horizontal transmission of IAPV from nurses to larvae while feeding. Other viruses, such as KBV and SBV, have also been found in brood food and royal jelly (Chen et al., 2006a). In my study the

detection of IAPV in the capped brood suggests the potential of a persistent infection of IAPV from egg to larva. Alternatively, the larvae could be infected by *Varroa* or contaminated brood food especially, since IAPV has been found in royal jelly (Chen et al., 2014). Workers in my experimental nucs were originally from IAPV-free source colonies. The presence of IAPV in adult workers from those experimental nucs, the homeostatic enclosed hive space (Evans et al., 2006), and the daily social interactions (Chen et al., 2006a) between workers and between workers and uncapped brood facilitates the horizontal transmission of IAPV via feeding and trophallaxis. My study provides indirect experimental data that IAPV is horizontally transmitted. The potential of horizontal transmission from worker to drones and from drones to queens as a venereal infection was unexplored in my study, and has only been documented in DWV (Amiri et al., 2016; De Miranda & Genersch, 2010).

In my immune priming experiment, the mortality data of immune primed and non-primed progeny was tested. The results from my experiments showed no significant difference in cumulative survival between immune primed and non-primed individuals suggesting that honey bee queens do not immune prime their offspring in response to IAPV exposure. An established IAPV infection in experimental queens was not detected, so further experiments need to be conducted to validate this conclusion. It has been demonstrated that honey bee queens can immune prime their offspring to other pathogens. An example is a study on the trans-generational immune priming of honey bee offspring against bacteria *Paenibacillus larvae*. The queens were exposed to heat killed *Paenibacillus larvae*, then the immune primed offspring was exposed to infectious

spores. This resulted in an increased survival rate of 26% in the immune primed offspring compared to non-primed offspring (Hernández López et al., 2014). Immune priming has been recorded in other insects, such as *Bombus terrestris*, in which a difference in cumulative survival has been noted within the same generation when exposed repeatedly to a bacterial pathogen (Sadd & Schmid-Hempel, 2006). In addition, trans-generational immune priming in other invertebrates from mother and father has been recorded (Moret, 2006; Roth et al., 2009).

An increasing number of studies show immune priming effects within generations and across generations against microbial pathogens, but the area of immune priming to viruses is understudied. The only study within the insect class was in the order Lepidoptera: *Plodia interpunctella* demonstrated within generation and trans-generational immune priming against a DNA virus (Tidbury et al., 2011). My results are not concurrent with the Tidbury et al. (2011) findings, but there are several key differences. One of the differences was the viral genome. IAPV is a positive ss-RNA virus while *Plodia interpunctella* granulosis virus is a DNA virus. The immune priming mechanisms against viruses are not well understood and, there is no established immune mechanism. There are 23 RNA honey bee viruses and only one double stranded DNA-genome virus (Gisder & Genersch, 2015), so most of the studies focus on RNA-genome viruses. Insects may have different immune mechanisms for ss-RNA viruses than DNA viruses. Another key methodological difference between the Tidbury et al. (2011) study and mine is that the immune primed offspring were applied a different viral concentration than their parents (Tidbury et al., 2011). In contrast, the dose applied to the honey bee queens

in my study was the effective dose established, which had a 26.1 % mortality rate and it was the same dose applied later to the offspring. Maternal immune priming in other species comes at a cost to the maternal fitness whether it be via a decrease in fecundity or an increase in immune system activity (Freitak et al., 2009; Linder & Promislow, 2009). Potentially my experimental queens were given such a high concentration of virus that it compromised their immune system and prevented immune priming of their offspring. Also, IAPV is an acute virus rather than a chronic virus. There may be less time for the queen to prime their offspring because once she crosses a certain viral threshold she will die unless her immune system is able to deal with the infection. This explanation is based on the premise that queens have rapid mortality once they have an overt infection as workers do when infected with IAPV (Genersch & Aubert, 2010). Correspondingly, high acute mortality was observed in queens in my 2015 injection preliminary experiments and in the first group of queens in my main experiment, which suggests that queens are more susceptible than workers.

Not all immune priming studies have found an immune priming effect. In the peach-potato aphid no significant maternal immune priming against parasitoids was identified (Vorburger et al., 2008). Overall, the results from my study suggest that honey bee queens do not have the ability to immune prime their offspring against IAPV and perhaps other viruses. The individual variation within treatment groups outweighed a potential treatment effect. One problem with my study is the inability to determine the infection status of queens while they were still alive. If there was no IAPV infection, then the lack of a maternal immune priming effect will not be a significant outcome. In

order to accurately evaluate an absence of an immune priming effect, the queens have to be tested and verified of their IAPV status throughout the experiment. As mentioned previously, perhaps the infection can be monitored by checking IAPV status of eggs.

A 2-choice olfactometer bioassay was used to evaluate infected and non-infected queens' attractiveness to workers. The analysis of the observations resulted in no significance difference between infected and non-infected queens' attractiveness. However as mentioned previously an established IAPV infection was not detected in the treated experimental queens, so further experiments need to be conducted. Queen attractiveness is a complex behavioral trait that is partly regulated by the queen mandibular pheromone (QMP) and four other components (Keeling et al., 2003; Zmarlicki & Morse, 1964). In contrast to the results of my study, the microsporidean disease *Nosema ceranae* increases one of the QMP components, 9-ODA (Alaux et al., 2011). However in QMP bioassay studies workers originating from different colonies attended the queen differently suggesting a genetic component for queen attendance (Pankiw et al., 1995). My study is the first to investigate whether queens' attractiveness is impacted by viruses, but the results indicated no significant effect. The genetic component for queen attendance may have outweighed any potential treatment effects. My results provide indirect evidence that does not support the Alaux et al. (2011) study because infected queens were not significantly selected by workers. Some queens were significantly preferred by workers in the choice tests, but the preference was not systematically related to infection status. This conclusion may not be valid if only some of the IAPV treated queens were effectively infected with IAPV during the behavioral

choice assay. In order to provide direct experimental data on the impact of IAPV on queens' attractiveness, QMP and four other identified compound (Keeling et al., 2003) levels have to be evaluated and a verification of IAPV status of infected queens throughout the experiment has to be performed.

In summary, results from my study provide further evidence for the vertical and horizontal transmission of IAPV. Also in my study an effective viral dose was established via a topical method, which is a method that has never been used for the delivery of IAPV. However, a simple and non-invasive method to detect viruses in queens is needed to monitor disease status throughout the experiments. Immune priming is leading to a paradigm shift in our understanding of immunity in invertebrates. My results regarding immune priming offer a starting point for future research regarding immune priming of viruses in honey bees. The immune priming mechanisms remain unknown for viral infection in any insect. Immune priming may offer the ability to target specific viruses for improving honey bee health. Specifically, immune priming may lead to the development of vaccines against these viruses that could be applied to honey bees by general beekeepers. Queens are the most important female reproductive members of the colony and queen failure is an important contributor to honey bee hive collapse (vanEngelsdorp et al., 2008; vanEngelsdorp et al., 2012). However, few studies address specifically queen health. Thus, my study yielded some insights that can help improve honey bee health by contributing to the understanding of IAPV transmission routes and exploring new potential anti-viral immune mechanism.

REFERENCES

Aizen, M. A., Garibaldi, L. A., Cunningham, S. A., & Klein, A. M. (2008). Long-Term Global Trends in Crop Yield and Production Reveal No Current Pollination Shortage but Increasing Pollinator Dependency. *Current Biology*, *18*(20), 1572–1575.

Aizen, M. A., Garibaldi, L. A., Cunningham, S. A., & Klein, A. M. (2009). How much does agriculture depend on pollinators? Lessons from long-term trends in crop production. *Annals of Botany*, *103*(9), 1579–1588.

Alaux, C., Folschweiller, M., McDonnell, C., Beslay, D., Cousin, M., Dussaubat, C., Brunet, J., & Conte, Y. L. (2011). Pathological effects of the microsporidium Nosema ceranae on honey bee queen physiology (Apis mellifera). *Journal of Invertebrate Pathology*, *106*(3), 380–385.

Allen, M. (1960). The honeybee queen and her attendants. *Animal Behaviour*, *8*(3–4), 201–208.

Amiri, E., Meixner, M., Büchler, R., & Kryger, P. (2014). Chronic bee paralysis virus in honeybee queens: Evaluating susceptibility and infection routes. *Viruses*, *6*(3), 1188–1201.

Amiri, E., Meixner, M. D., & Kryger, P. (2016). Deformed wing virus can be transmitted during natural mating in honey bees and infect the queens. *Scientific Reports*, *6*.

Bauer, D. M., & Wing, I. S. (2010). Economic Consequences of Pollinator Declines: A Synthesis. *Agricultural and Resource Economics Review*, *39*(3), 368–383.

Boncristiani, H. F., Evans, J. D., Chen, Y., Pettis, J., Murphy, C., Lopez, D. L., Simon-Finstrom, M., Strand M., Tarpy, D. R., and Rueppell, O. (2013). In Vitro Infection of Pupae with Israeli Acute Paralysis Virus Suggests Disturbance of Transcriptional Homeostasis in Honey Bees (Apis mellifera). *PLoS ONE*, *8*(9).

Bourke, A. (1988). Worker Reproduction in the Higher Eusocial Hymenoptera. *The Quarterly Review of Biology*, *63*(3), 291–311.

Brutscher, L., & Daughenbaugh, K. (2015). Antiviral Defense Mechanisms in Honey Bees. *Current Opinion in Insect Science*, *10*, 71–8

Büchler, R., Andonov, S., Bienefeld, K., Costa, C., Hatjina, F., Kezic, N., Kryger, P., Spivak, M., Uzunov, A., &Wilde, J. (2013). Standard methods for rearing and selection of Apis mellifera queens. *Journal of Apicultural Research*, *52*(1), 1–30.

Calderone, N. W. (2012). Insect pollinated crops, insect pollinators and US agriculture: Trend analysis of aggregate data for the period 1992-2009. *PLoS ONE*, *7*(5).

Carter, J. B., & Saunders, V. A. (2007). *Virology : principles and applications*. John Wiley & Sons, NJ.

Champetier, A., Sumner, D. A., & Wilen, J. E. (2015). The Bioeconomics of Honey Bees and Pollination. *Environmental and Resource Economics*, *60*(1), 143–164.

Chen, Y. P., Higgins, J. A., & Feldlaufer, M. F. (2005a). Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (Apis mellifera L.). *Appl Environ Microbiol*, *71*(1), 436–441.

Chen, Y., Pettis, J. S., & Feldlaufer, M. F. (2005b). Detection of multiple viruses in queens of the honey bee Apis mellifera L. *Journal of Invertebrate Pathology*, *90*(2), 118–121.

Chen, Y., Evans, J., & Feldlaufer, M. (2006a). Horizontal and vertical transmission of viruses in the honey bee, Apis mellifera. *Journal of Invertebrate Pathology*, *92*(3), 152–159.

Chen, Y., Pettis, J., & Collins, A. (2006b). Prevalence and Transmission of Honeybee Viruses. *Applied and Environmental Microbiology*, *72*(1), 606–611.

Chen, Y. P., & Siede, R. (2007). Honey Bee Viruses. *Advances in Virus Research*, *70*, 33–80.

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., Huang, S. K., Zheng, H. Q., Li, J. L., Zhang, X., Zhou, A. F., Wu, L. Y., Z., J. Z., Lee, M. L., Teixeira, E. W., Li, Z. G., and Evans, J. D. (2014). Israeli Acute Paralysis Virus: Epidemiology, Pathogenesis and Implications for Honey Bee Health. *PLoS ONE*, *10*(7).

Corona, M., Velarde, R. A., Remolina, S., Moran-Lauter, A., Wang, Y., Hughes, K. A., & Robinson, G. E. (2007). Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *PNAS*, *104*(17), 7128–7133.

Cox-Foster, D. L., Conlan, S., Holmes, E. C., Palacios, G., Evans, J. D., Moran, N. A, Quan, P., Briese, T., Hornig, M., Geiser, D. M., Martinson, V., vanEngelsdorp, D. Kalkstein, A. L., Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchison, S. K., Simons, J. F., Egholm, M., Pettis, J. S., and Lipkin, W. I. (2007). A Metagenomic Survey of Microbes in Honey Bee Colony Collapse Disorder. *Science*, *318*(5848), 283–287.

Dainat, B., Evans, J., Chen, Y., Gauthier, L., & Neumann, P. (2012). Dead or alive: deformed wing virus and Varroa destructor reduce the life span of winter honeybees. *Applied and Environmental Microbiology*, *78*(4), 981–987.

De Miranda, J. R., & Fries, I. (2008). Venereal and vertical transmission of deformed wing virus in honeybees (Apis mellifera L.). *Journal of Invertebrate Pathology*, *98*(2), 184–189.

De Miranda, J. R., Cordoni, G., & Budge, G. (2010). The Acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex. *Journal of Invertebrate Pathology*, *103*(SUPPL. 1), S30–S47.

De Miranda, J. R., & Genersch, E. (2010). Deformed wing virus. *Journal of Invertebrate Pathology*, *103*(SUPPL. 1), S48–S61.

Desneux, N., Decourtye, A., & Delpuech, J.-M. (2007). The Sublethal Effects of Pesticides on Beneficial Arthropods. *Annual Review of Entomology*, *52*, 81–106.

Di Prisco, G., Pennacchio, F., Caprio, E., Boncristiani, H. F., Evans, J. D., & Chen, Y. (2011). Varroa destructor is an effective vector of Israeli acute paralysis virus in the honeybee, Apis mellifera. *Journal of General Virology*, *92*(1), 151–155.

Dietemann, V., Pflugfelder, J., & Anderson, D. (2012). Varroa destructor: research avenues towards sustainable control. *Journal of Apicultural*, *51*(1), 125–132.

Efem, S. E. E., & Iwara, C. I. (1992). The antimicrobial spectrum of honey and its clinical significance. *Infection*, *20*(4), 227–229.

Ellis, J. D., Evans, J. D., & Pettis, J. (2010). Colony losses, managed colony population decline, and Colony Collapse Disorder in the United States. *Journal of Apicultural Research*, *49*(1), 134–136.

Ellis, J. D., & Munn, P. A. (2005). The worldwide health status of honey bees. *Bee World*, *86*(4), 88–101.

Evans, J., & Pettis, J. (2005). Colony-level impacts of immune responsiveness in honey bees, Apis mellifera. *Evolution*, *59*(10), 2270–2274.

Evans, J., Aronstein, K., Chen, Y., & Hetru, C. (2006). Immune pathways and defence mechanisms in honey bees Apis mellifera. *Insect Molecular Biology*, *15*(5), 645–656.

Evans, J. D., & Spivak, M. (2010). Socialized medicine: Individual and communal disease barriers in honey bees. *Journal of Invertebrate Pathology*, *103*, S62–S72.

Evans, J. D., & Schwarz, R. S. (2011). Bees brought to their knees: microbes affecting honey bee health. *Trends in Microbiology*, *19*(12), 614–620.

Farina, W. (1996). Food-exchange by foragers in the hive–a means of communication among honey bees? *Behavioral Ecology and Sociobiology*, *38*(1), 59–64.

Forsgren, E., De Miranda, J. R., Isaksson, M., Wei, S., & Fries, I. (2009). Deformed wing virus associated with Tropilaelaps mercedesae infesting European honey bees (Apis mellifera). *Exp Appl Acarol, 47*, 87-97.

Fouks, B., Lattorff, H., & Marion-Poll, F. (2011). Recognition and Avoidance of Contaminated Flowers by Foraging Bumblebees (*Bombus terrestris*). *PLoS ONE*, *6*(10).

Francis, R. M., Nielsen, S. L., & Kryger, P. (2013). Patterns of viral infection in honey bee queens. *Journal of General Virology*, *94*, 668–676.

Freitak, D., Heckel, D. G., & Vogel, H. (2009). Dietary-dependent trans-generational immune priming in an insect herbivore. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1667), 2617–2624.

Fries, I., & Camazine, S. (2001). Implications of horizontal and vertical pathogen transmission for honey bee epidemiology. *Apidologie*, *32*(3), 199–214.

Galbraith, D., Yang, X., Niño, E., Yi, S., & Grozinger, C. (2015). Parallel epigenomic and transcriptomic responses to viral infection in honey bees (Apis mellifera). *PLoS Pathogens*, *11*(3).

Gary, N. (1961). Queen honey bee attractiveness as related to mandibular gland secretion. *Science*, *133*(3463), 1479–1480.

Gätschenberger, H., Azzami, K., Tautz, J., & Beier, H. (2013). Antibacterial immune competence of honey bees (Apis mellifera) is adapted to different life stages and environmental risks. *PloS One*, *8*(6).

Genersch, E. (2010). Honey bee pathology: current threats to honey bees and beekeeping. *Applied Microbiology and Biotechnology*, *87*(1), 87–97.

Genersch, E., & Aubert, M. (2010). Emerging and re-emerging viruses of the honey bee (Apis mellifera L.). *Veterinary Research*, *41*(6).

Gisder, S., & Genersch, E. (2015). Special Issue: Honey Bee Viruses. *Viruses*, *7*, 5603–5608.

Gregorc, A., Evans, J. D., Scharf, M., & Ellis, J. D. (2012). Gene expression in honey bee (*Apis mellifera*) larvae exposed to pesticides and Varroa mites (*Varroa destructor*). *Journal of Insect Physiology 58,* 1042-1049.

Griffin, D. R., von Frisch, K., & Chadwick, L. E. (1968). The Dance Language & Orientation of Bees. *American Scientist, 56*(3), 289A-291A.

Guidugli, K., Nascimento, A., & Amdam, G. (2005). Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. *FEBS*.

Hernández López, J., Schuehly, W., Crailsheim, K., & Riessberger-Gallé, U. (2014). Trans-generational immune priming in honeybees. *Proceedings of the Royal Society B, 281*.

Hölldobler, B., & Wilson, E. O. (2009). *The Superorganism: The Beauty, Elegance, and Strangeness of Insect Societies*. W. W. Norton & Company, New York.

Keeling, C., Slessor, K., & Higo, H. (2003). New components of the honey bee (Apis mellifera L.) queen retinue pheromone. *PNAS*, *100*(8), 4486–4491.

Kevan, P. G., & Viana, B. F. (2003). The global decline of pollination services. *Biodiversity*, *4*(4), 3–8.

Klatt, B. K., Holzschuh, A., Westphal, C., Clough, Y., Smit, I., Pawelzik, E., Tscharntke, T. (2014). Bee pollination improves crop quality, shelf life and commercial value. *Proceedings of the Royal Society B, 281*.

Kurtz, J. (2005). Specific memory whithin innate immune systems. *Trends in Immunology*, *26*(4), 186–192.

Laidlaw , H. H., & Page, R. E. (1996). *Queen rearing and bee breeding*. Wicwas Press, Kalamazoo.

Leat, N., Ball, B., Govan, V., & Davison, S. (2000). Analysis of the complete genome sequence of black queen-cell virus, a picorna-like virus of honey bees. *Journal of General Virology*, *81*, 2111–2119.

Lee, K. V., Steinhauer, N., Rennich, K., Wilson, M. E., Tarpy, D. R., Caron, D. M., Rose, R., Delaplane, K. S., Baylis, K., Lengerich, E. J., Pettis, J., Skinner, J. A., Wilkes, J. T., Sagili, R., vanEngelsdorp, D. (2015). A national survey of managed honey bee 2013-2014 annual colony losses in the USA. *Apidologie*, *46*(3), 292–305.

Levitt, A. L., Singh, R., Cox-Foster, D. L., Rajotte, E., Hoover, K., Ostiguy, N., & Holmes, E. C. (2013). Cross-species transmission of honey bee viruses in associated arthropods. *Virus Research*.

Linder, J. E., & Promislow, D. E. L. (2009). Cross-generational fitness effects of infection in Drosophila melanogaster. *Fly*, *3*(2), 143–150.

Lindström, A., Korpela, S., & Fries, I. (2008). Horizontal transmission of Paenibacillus larvae spores between honey bee (Apis mellifera) colonies through robbing. *Apidologie*, *39*, 515–522.

Maori, E., Lavi, S., Mozes-Koch, R., Gantman, Y., Peretz, Y., Edelbaum, O., Tanne, E., & Sela, I. (2007a). Isolation and characterization of Israeli acute paralysis virus, a dicistrovirus affecting honeybees in Israel: Evidence for diversity due to intra- and inter-species recombination. *Journal of General Virology*, *88*(12), 3428–3438.

Maori, E., Tanne, E., & Sela, I. (2007b). Reciprocal sequence exchange between non-retro viruses and hosts leading to the appearance of new host phenotypes. *Virology*, *362*(2), 342–349.

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.

Martin, S. J. (2001). The role of Varroa and viral pathogens in the collapse of honeybee colonies: A modelling approach. *Journal of Applied Ecology*, *38*, 1082–1093.

Martin, S. J., Highfield, A. C., Brettell, L., Villalobos, E. M., Budge, G. E., Powell, M., Nikaido, S., & Schroeder, D. C. (2012). Global Honey Bee Viral Landscape Altered by a Parasitic Mite. *Science*, *336*(428), 1304–1306.

Meeus, I., de Miranda, J. R., de Graaf, D. C., Wäckers, F., & Smagghe, G. (2014). Effect of oral infection with Kashmir bee virus and Israeli acute paralysis virus on bumblebee (Bombus terrestris) reproductive success. *Journal of Invertebrate Pathology*, *121*, 64–69.

Möckel, N., Gisder, S., & Genersch, E. (2011). Horizontal transmission of deformed wing virus: Pathological consequences in adult bees (Apis mellifera) depend on the transmission route. *Journal of General Virology*, *92*(2), 370–377.

Moore, P. A., Wilson, M. E., & Skinner, J. A. (2015). Honey Bee Viruses , the Deadly Varroa Mite Associates. *Bee Health*.

Moret, Y. (2006). "Trans-generational immune priming": specific enhancement of the antimicrobial immune response in the mealworm beetle, Tenebrio molitor. *Proceedings of the Royal Society B*, *273*(1592), 1399–1405.

Motten, A., Miller, H., Campbell, D., & Alexander, D. (2009). Pollination effectiveness of specialist and generalist visitors to a North Carolina population of Claytonia viginica. *Ecology*, *1*(2), 196–206.

Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., VanEngelsdorp, D., & Pettis, J. S. (2010). High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. *PLoS ONE*, *5*(3), e9754.

National Academy of Sciences. (2007, April 13). Status of Pollinators in North America.

Neumann, P., & Carreck, N. L. (2010). Honey bee colony losses. *Journal of Apicultural Research*, *49*(1), 1–6.

Oldroyd, B. (2007). What's Killing American Honey Bees. *PLoS Biology*, *5*(6).

Oliveira, M. de. (2013). Polietismo e detecção de vírus deformador das asas em abelha Apis mellifera scutellata (Africanizada) e Apis mellifera ligustica (Europeia). *Universidade de São Paulo*.

Page, R. E., & Peng, C. Y. S. (2001). Aging and development in social insects with emphasis on the honey bee, Apis mellifera L. In *Experimental Gerontology*, *36*, pp. 695–711.

Palacios, G., Hui, J., Quan, P. L., Kalkstein, A., Honkavuori, K. S., Bussetti, A. V., Conlan, S., Evans, J., Chen, Y. P., vanEngelsdorp, D., Efrat, H., Pettis, J., Cox-Foster, D., Holmes, E. C., Briese, T., & Lipkin, W. I. (2008). Genetic analysis of Israel acute paralysis virus: distinct clusters are circulating in the United States. *Journal of Virology*, *82*(13), 6209–6217.

Pankiw, T., Winston, M. L., & Slessor, K. N. (1995). Queen attendance behavior of worker honey bees (Apis mellifera L.) that are high and low responding to queen mandibular pheromone. *Insectes Sociaux*, *42*(4), 371–378.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology and Evolution*, *25*(6), 345–353.

Ravoet, J., De Smet, L., Wenseleers, T., & de Graaf, D. C. (2015). Vertical transmission of honey bee viruses in a Belgian queen breeding program. *BMC Veterinary Research*, *11*(1), 61.

Rinderer, T., & Rothenbuhler, W. (1975). The fate and effect of hairs removed from honeybees with hairless-black syndrome. *Journal of Invertebrate Pathology*, *26*, 305–308.

Rosenkranz, P., Aumeier, P., & Ziegelmann, B. (2010). Biology and control of Varroa destructor. *Journal of Invertebrate Pathology*, *103 Suppl*, S96-119.

Roth, O., Sadd, B. M., Schmid-Hempel, P., & Kurtz, J. (2009). Strain-specific priming of resistance in the red flour beetle, Tribolium castaneum. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1654), 145–151.

Rucker, R. R., Thurman, W. N., & Burgett, M. (2012). Honey bee pollination markets and the internalization of reciprocal benefits. *American Journal of Agricultural Economics*, *94*(4), 956–977.

Rueppell, O., Hayworth, M. K., & Ross, N. P. (2010). Altruistic self-removal of health-compromised honey bee workers from their hive. *Journal of Evolutionary Biology*, *23*(7), 1538–1546.

Ryabov, E. V., Wood, G. R., Fannon, J. M., Moore, J. D., Bull, J. C., Chandler, D., Mead, A., Burroughs, N., & Evans, J. (2014). A Virulent Strain of Deformed Wing Virus (DWV) of Honeybees (Apis mellifera) Prevails after Varroa destructor-Mediated, or In Vitro, Transmission. *PLoS Pathogens*, *10*(6).

Sadd, B. M., & Schmid-Hempel, P. (2006). Insect Immunity Shows Specificity in Protection upon Secondary Pathogen Exposure. *Current Biology*, *16*(12), 1206–1210.

Schuster, S., Zirkel, F., Kurth, A., van Cleef, K. W. R., Drosten, C., van Rij, R. P., & Junglen, S. (2014). A Unique Nodavirus with Novel Features: Mosinovirus Expresses Two Subgenomic RNAs, a Capsid Gene of Unkown Origin, and a Suppressor of the Antiviral RNA Interference Pathway. *Journal of Virology*, *88*(22).

Seeley, T. (1995). *The wisdom of the hive: the social physiology of honey bee colonies*. Harvard University Press, Cambridge.

Seeley, T. D. (1985). *Honeybee ecology*. *Monographs in behavior and ecology*. Princeton University Press, Princeton.

Simone, M., Evans, J. D., & Spivak, M. (2009). Resin collection and social immunity in honey bees. *Evolution*, *63*(11), 3016–3022.

Singh, R., Levitt, A. L., Rajotte, E. G., Holmes, E. C., Ostiguy, N., VanEngelsdorp, D., Lipkin, W. I., dePamphilis, C. W., Toth, A. L., & Cox-Foster, D. L. (2010). RNA Viruses in Hymenopteran Pollinators: Evidence of Inter-Taxa Virus Transmission via Pollen and Potential Impact on Non-Apis Hymenopteran Species. *PLoS ONE*, *5*(12), e14357.

Tidbury, H. J., Pedersen, A. B., & Boots, M. (2011). Within and transgenerational immune priming in an insect to a DNA virus. *Proceedings of the Royal Society B*, *278*(1707), 871–6.

USDA. (2016). *USDA Releases Results of New Survey on Honey Bee Colony Health*. Washington.

Vanbergen, A. J., & Insect Pollinators Initiative (2013). Threats to an ecosystem service: pressures on pollinators. *Frontiers in Ecology and the Environment*, *11*(5), 251–259.

vanEngelsdorp, D., Hayes, J., Underwood, R., & Pettis, J. (2008). A Survey of Honey Bee Colony Losses in the US, Fall 2007 to Spring 2008. *PLoS ONE*, *3*(12).

vanEngelsdorp, D., Evans, J. D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B. K., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y., Underwood, R., Tarpy, D. R., and Pettis, J. S. (2009). Colony collapse disorder: A descriptive study. *PLoS ONE*, *4*(8).

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.

vanEngelsdorp, D., Caron, D., Hayes, J., Underwood, R., Henson, M., Rennich, K., Spleen, A., Andree, M., Snyder, R., Lee, K., Roccasecca, K., Wilson, M., Wilkes, J., Lengerich, E., Pettis, J., & for the Bee Informed Partnership. (2012). A national survey of managed honey bee 2010-11 winter colony losses in the USA: results from the Bee Informed Partnership. *Journal of Apicultural Research*, *51*(1), 115–124.

Vorburger, C., Gegenschatz, S. E., Ranieri, G., & Rodriguez, P. (2008). Limited scope for maternal effects in aphid defence against parasitoids. *Ecological Entomology*, *33*(2), 189–196.

Wilson, E. (1971). *The insect societies*. Belknap Press of Harvard University Press, Massachusetts.

Wilson, E. O., & Hölldobler, B. (2005). Eusociality: Origin and consequences. *PNAS*, *102*(38), 13367–133371.

Wilson-Rich, N. (2014). *The Bee: A Natural History*. Princeton University Press, Princeton.

Winston, M. L. (1987). *The biology of the honey bee*. Harvard University Press, Cambridge.

Wyatt, G. (1961). The biochemistry of insect hemolymph. *Annual Review of Entomology*, *6*, 75–102.

Yañez, O., Jaffé, R., Jarosch, A., Fries, I., Robin, F. A. M., Robert, J. P., & De Miranda, J. R. (2012a). Deformed wing virus and drone mating flights in the honey bee (Apis mellifera): Implications for sexual transmission of a major honey bee virus. *Apidologie*, *43*(1), 17–30.

Yañez, O., Zheng, H. Q., Hu, F. L., Neumann, P., & Dietemann, V. (2012b). A scientific note on Israeli acute paralysis virus infection of Eastern honeybee Apis cerana and vespine predator Vespa velutina. *Apidologie*, *43*(5), 587–589.

Yue, C., & Genersch, E. (2005). RT-PCR analysis of Deformed wing virus in honeybees (Apis mellifera) and mites (Varroa destructor). *Journal of General Virology*, *86*(12), 3419–3424.

Yue, C., Schroder, M., Gisder, S., & Genersch, E. (2007). Vertical-transmission routes for deformed wing virus of honeybees (Apis mellifera). *Journal of General Virology, 88*, 2329–2336.

Zhang, X., He, S. Y., Evans, J. D., Pettis, J. S., Yin, G. F., & Chen Y. P. (2012). New evidence that deformed wing virus and black queen cell virus are multi-host pathogens. *Journal of Invertebrate Pathology, 109*, 156-159.

Zmarlicki, C., Morse, R. A. (1964). The effect of tmandibular gland extirpation on the longevity and attractivenees to workers of queen honeybee Apis Mellifera. *Ann. Ent. Soc. America, 57*(1), 73–73.