Infiltrating the hive mind: Immune and viral effects on behavior of the honey bee (Apis mellifera)

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Infiltrating the hive mind: Immune and viral effects on behavior of the honey bee (*Apis mellifera*)

by

Amy Christine Geffre

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Ecology and Evolutionary Biology

Program of Study Committee:
Amy Toth, Major Professor
Bryony Bonning
James Adelman

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2018

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DEDICATION

This thesis is dedicated to several loving humans, two loving cats, and many paper wasps and honey bees who were on occasion rather irritable, but also very loving [in their way] as well.
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Pathogens are important to the ecology of all organisms. This thesis describes host-pathogen interactions between *Apis mellifera* and Israeli acute paralysis virus (Dicistroviridae). Viral pathogens are critical factors in honey bee health, but effects of viral infection on honey bee behavior are difficult to study and can manifest differently across contexts. Collectively, this work seeks to better describe these host-pathogen interactions between honey bees and their viruses.

Firstly, we fed bees with viral sequence-based dsRNA, as a sham-virus to elicit RNA interference-based anti-viral immune response. We paired these bees with untreated bees and observed their interactions. Notably, we found that dsRNA-treated bees trophallaxed less than control bees, but found no differences in other social interactions. We hypothesize that anti-viral immune response likely induces sickness signals to conspecifics in the affected bee, reducing trophallaxis, but this signal might only be received through other social interaction (e.g. antennation, etc).

Secondly, we fed bees virus or sham-virus, paired them with unmodified partner bees, and observed their interactions. Both virus-infection and sham-infection elicited reduced trophallaxis; virus-infection led to reduced physical contact and antennation. Virus-infected bees were also more active than other bee types, suggesting infection could induce early onset foraging, previously described as altruistic self-removal, from a colony. We found support for adaptive sickness behavior, as virus-infected bees were more socially isolated, potentially preventing infection spread within the hive.

Finally, we explore how drifting in apiary settings is affected by honey bee-virus interactions. We paired bees with virus-infected, sham-infected and control bees from a different colony and observed their interactions. Virus-infected bees experienced more non-agonistic interactions and less aggression than either normal or sham-infected bees. We also found subtle shifts in cuticular hydrocarbon profiles among treatments. These results indicate drifting behavior has likely been co-opted by viral pathogens to enhance transmission between colonies, especially in apiary settings, and that virus-infection likely modifies chemical signals in the host to make them more acceptable.
Collectively, these chapters present the first description of potential adaptive honey bee behavioral manipulation by viruses, and highlight the need to reduce pathogen spread in apiaries by improving honey bee management practices.
CHAPTER 1: INTRODUCTION

**Pathogens are critical players in the lives of hosts.**

There are essentially no organisms that do not play host to pathogens (including exo- and endoparasites, pathogenic fungi, bacteria and viruses; reviewed in Poulin and Morand, 2000). Given their preponderance, pathogens play critical roles in structuring host communities and the communities that hosts exist within (reviewed in Poulin, 1999 and Lefevre et al, 2008; Seabloom et al, 2015) and even create novel host phenotypes and ecological niches (Thomas et al, 1999). Additionally, pathogens are key players driving the evolution of hosts and their communities (Lefevre et al., 2008; Kerstes and Martin, 2014; Lagrue et al., 2015). Given the far-reaching impacts of pathogens, it is important to consider them whenever exploring the biology of free-living organisms (Poulin, 1999; Lafferty et al 2008). This includes understanding the subtle mechanics of how pathology manifests and how pathogens have coevolved to take advantage of host resources. Especially relevant to this is pathogen manipulation of the host, or how pathogen infection affects host behavior and the host molecular machinery it co-opts to induce this behavioral alteration (Adamo, 2013).

Most well-known examples of host-pathogen manipulation include those charismatic interactions in which the pathogen radically alters normal host behavior in conspicuous ways to enhance the well-being of the pathogen. *Toxoplasma gondii* induces cat-seeking behavior in infected host mice, to increase the host's risk of predation by cats (Ingram et al, 2013). A baculovirus induces abnormal tree-climbing, death and liquification of infected host caterpillars (Hoover et al, 2013). A helminth induces light-seeking behavior in traditionally light-avoidant gammarids (Helluy, 2013). Hairworms infesting field crickets cause their hosts to leap into water when they come upon it (Thomas et al, 2002). These examples all describe instances wherein a pathogen induces conspicuous abnormal behavior for the host to benefit the pathogen. However, host-pathogen interactions need not be particularly dramatic, nor particularly divergent from normal host behaviors to enhance transmission. Indeed, sophisticated examples of host-pathogen interactions
merely draw on normal behavioral modules that exist in the host. This can be seen in the manipulation of typical brood care in green crabs by parasitic *Sacculina* larvae that infest the crab’s brood patch (Phillips and Cannon, 1973; Høeg, 1995). Additionally, enhanced pre-mating aggression and mating rate is seen in Chrysomelid beetles infested with a sexually-transmitted mite (Abbot and Dill, 2001). cuckoo chicks trick host magpie parents to feed them over their own nestlings with visual cues (Redondo and Zuñiga, 2002). In these examples, we see that highly routine behaviors (e.g. parental care and mating behaviors) are subtly twisted to the benefit of the parasites or pathogens involved.

**Host sociality presents novel pay-offs and challenges for pathogens.**

Survival in pathogens goes beyond the need for replication, requiring not only the pathogen survive with or in the host, but also that the pathogen can spread to new hosts. In solitary hosts, interhost transmission can be difficult, as hosts tend to be sparse, with limited interaction between each other. Transmission between social hosts, however, may be relatively easy for pathogens. Indeed, social animals are excellent targets in several ways. Not only do they tend to live in close contact with other potential hosts, but they also tend to regularly engage in intraspecies interactions (i.e. allo-grooming, cohabitation, food sharing, etc.), which may be potentially modulated to increase transmission. In several different social animal taxa, infection has been linked to social structure (Otterstatter and Thomson, 2007) and sociobehavioral modulation, both to prevent transmission (Schmid-Hempel, 1998; Goblirsch et al, 2013) as well as enhance it (Klein, 2003). As such, social behaviors and physiological mechanisms controlling them are prime targets for pathogen manipulation.

Eusociality is a sophisticated form of social organization requiring the following conditions in the colony: 1) reproductive division of labor, 2) overlapping generations and 3) cooperative broodcare (Crespi and Yanega, 1995). Insects have perfected this system, with at least four orders being notably, if not entirely, eusocial – this includes Thysanoptera (thrips), Hemiptera (aphids), Isoptera (termites) and Hymenoptera (ants, bees and wasps) (Wilson, 1971; Hölldobler and Wilson, 2008).
Like other eusocial organisms, these insect colonies are typically dense collections of closely-related individuals that necessarily contact each other with extraordinary frequency. The close-quarters of eusocial insect colonies dictate that individuals will passively contact each other with high frequency (e.g. walking near to or on top of other colony members), for one. Additionally, because of their social lifestyle, no individual in the colony can persist without direct contact; indeed, the good of the colony often depends on mutual inspection, allo-grooming (the grooming of one individual by another) and food-sharing – all of which require purposeful, direct contact between two or more individuals.

Because these interactions involve such close associations of two closely related individuals of the same species, often with the exchange of body fluids, they are particularly excellent opportunities for pathogen transmission and, as such, targets for pathogen manipulation. Moreover, individuals in eusocial societies tend to use castes elicited from “genetic toolkits” (Toth et al, 2009; Berens et al, 2014), or suites of behavior elicited by differential expression of an identical genome – this can also be useful for invading pathogens, as it means that there are sets of phenotypes they may work with to facilitate their lifecycle, likely with even greater ease than soliciting novel phenotypes in their hosts (as is common with more disturbing instances of host-pathogen manipulation. In the context of host manipulation, thus, pathogens of social insects have the distinct advantage in that they can increase their own transmission via social interactions – however, eusocial insects are not without unique adaptations to combat such an onslaught of pathogens, often evolving excellent collective immune responses, based in altruistic behavior (Schimd-Hempel, 1998; Cremer et al, 2007; Evans and Spivak, 2010). This leads to the fascinating question—can pathogens of social insects, especially highly eusocial insects such as honey bees, cause them to become more social, in order to increase their own transmission? And how do these pathogens deal with the novel hive-mind immune responses implemented by their eusocial hosts?
Understanding host-pathogen interactions is important for honey bee health

In this thesis, I explore pathogen manipulation of a eusocial species, the honey bee (*Apis mellifera*). Honey bees are simultaneously charismatic, ecologically important and economically relevant. Not only are they one of the most recognizable insects to the public, but they also provide key pollination services and are one of a sparse number of domesticated insects. Recently, the stressors put upon these key pollinators have been studied with fervor, to address the growing pollinator conservation crisis. A number of extraneous factors affect honey bee survival, including habitat loss, pesticide exposure and the many pathogens they host (Vanbergen and The Pollinator Initiative, 2013). As discussed in the previous section, their eusociality makes them prime hosts for numerous pathogens, including some 23 viruses (Chen and Siede, 2007; Chen et al., 2014; Gisder and Genersch, 2015).

Israeli acute paralysis virus (IAPV) is a prominent such pathogen of concern in apiculture (Chen et al., 2014). IAPV is one of a common complex of dicistroviruses, nonenveloped, positive single-stranded RNA viruses characterized by a dicistronic genome, with a 5’ proximal ORF encoding nonstructural proteins, and 3’ encoding structural proteins, (including acute bee paralysis virus (ABPV) and Kashmir bee virus (KBV), (deMiranda et al., 2010)). These viruses are found in apiaries worldwide (Ellis and Munn, 2005, reviewed in deMiranda et al., 2010). Of these viruses, IAPV is a particular concern, as it can infect all life stages of the honey bee, has far reaching physiological consequences in the host (Chen et al., 2014), and is notably associated with devastating colony loss (Cox-Foster et al., 2007). Acute infection by IAPV induces progressively worsening paralysis, including trembling, spasms and inability to fly (Maori et al., 2007). However, IAPV is usually present in relatively low titers in colonies (deMiranda et al., 2010), though related dicistroviruses may be exacerbated by the ever more prevalent *Varroa destructor* mite (Ribiére et al., 2008; Shen et al., 2005).

While diagnostic characteristics and pathology of IAPV infection are well-characterized (Maori et al., 2007; reviewed in deMiranda et al., 2010), knowledge concerning host behavior during infection is decidedly less described. This lack of
understanding is of special concern, as IAPV particles tend to replicate and concentrate in the salivary glands, hypopharyngeal glands, nervous system, and gut of host bees and can be vectored by saliva (Chen et al., 2014; other transmission pathways also exist). The majority of these organs are linked to the mouth, which is the site of numerous social interactions between honey bees (e.g. allo-grooming, food storing, and notably food-sharing, or “trophallaxis”). Because of this, it could be useful to the pathogen to enhance social interactions between honey bees, as this route could serve as a potential mechanism to increase IAPV transmission. Revealing the routes of transmission via host behavioral changes is useful for understanding how this virus, and potentially others, may spread within and between colonies.

**Thesis questions and organization**

To explore honey bee behavioral modification during virus infection, I performed a series of experiments that create a framework to better understand host-parasite interactions and transmission in honey bee-virus systems. Chapter 2 describes the first use of a novel control for virus-infection in honey bees – a virus-based double-stranded RNA (dsRNA) which functions to stimulate a key component of anti-viral immune response (RNA interference, or RNAi) in the host, in the absence of replicating virus. In this chapter, my findings support the notion that merely comparing virus-infected bees to untreated bees is not a complete exploration of the effects of virus-infection. Indeed, “sham-infection” by dsRNA-treatment causes behavioral changes in honey bee hosts. While previous works seeks to understand how dsRNAs can be used as vaccines against viruses in bees (Maori et al., 2009), Chapter 3 details one of the first experiments to observe individual bee-bee interactions between virus-infected bees and normal sisters (including the dsRNA-based sham-infection). We see that in the context of interactions between related individuals, social isolation of virus-infected individuals occurs. We suggest this may be adaptive for individual honey bee colonies to reduce virus transmission within colonies.
Chapter 4 describes how virus-infection affects interactions between non-nestmate bees from different colonies, thus addressing intercolony transmission. Honey bees regularly enter non-nestmate colonies (known as “drifting”) due to navigational mistakes. This is particularly relevant to domestic honey bees, as they are kept in close quarters, and drifting of individuals to non-natal colonies is extremely common. In this experiment, I found that virus-infected honey bees are more likely to be accepted by bees from foreign colonies. This indicates that, though they may perform altruistic isolation from their natal colony, virus-infected bees may still vector the virus between colonies through drifting. This may represent the first known example of adaptive host manipulation of social behavior by a virus in honey bees. Together, these studies describe useful new techniques in exploring host-virus interactions, and further the argument that honey bee management should better consider these complex host-pathogen interactions.
CHAPTER 2: ANTI-VIRAL IMMUNE-RESPONSE DECREASES KEY SOCIAL BEHAVIOR IN HONEY BEES

This chapter in preparation for: Insectes Sociaux or Journal of Insect Behavior

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Abstract

Viral pathogens are important factors in honey bee health, but the effects of viral infection on honey bee behavior are difficult to study. In this study, we discuss the use of double-stranded RNAs (dsRNAs) designed from virus templates to simulate viral infection by soliciting the RNAi response, without viral replication. We experimentally fed honey bees with Kashmir bee virus-based dsRNA, and observed their behavioral interactions with normal honey bees. Notably, we found that pairs with dsRNA-treated focal bees trophallaxed less than those with sugar-treated controls, but found no other differences in social interactions. This indicates that dsRNA-treatment likely induces some social signal to conspecifics that the treated bee is undergoing an immune response, but that this signal is only noticeable as the result of social interaction (e.g. antennation, grooming or other contact).

Introduction

Viruses are key players in the pathogen landscape of honey bees and major contributors to colony loss (reviewed in Gisder and Genersch, 2015). There is substantial knowledge available concerning viral pathology for several honey bee viruses, but a comparatively small body of work detailing sublethal effects of viruses on honey bee physiology and behavior (Chen and Siede 2007). Many honey bee viruses are commonly harbored by bees at sublethal levels (Runckel et al., 2011;
Carrillo-Tripp et al., 2016), thus it is important to better understand how these pervasive viruses may affect honey bee phenotypes.

Many host-pathogen associations involve subtle alterations to host behavior (called “pathogen manipulation”), such that the pathogen life cycle is facilitated. However, before we can understand potentially subtle viral effects on honey bee phenotypes, it is important to understand how anti-viral immune stimulation in the absence of viral infection affects bees. Like other eusocial insects, honey bees live in dense colonies of conspecifics, and repeatedly interact, providing ample opportunity for pathogens to spread amongst multiple hosts with comparative ease. Because of this, honey bees have evolved various forms of “social immunity” (e.g. detection of and aggression towards infected individuals, altruistic self-removal from colonies, early transition from within-hive tasks to out-of-hive tasks by infected individuals (Schmid-Hempel, 1998; Cremer et al, 2007; Evans and Spivak, 2010, Rueppell et al, 2010; Alaux et al, 2012)). Clearly, such behaviors are beneficial to the honey bee hosts.

To date, it is not yet known whether pathogens, including viruses, manipulate the social behavior of honey bees. Importantly, pathogens need not elicit novel behaviors from honey bee hosts, but rather just rescue sickness-associated behaviors or cues, so that either detection by conspecifics, or altruistic sacrifice is diminished. Thus, pathogen manipulation of the host may not take the appearance of highly modified behavior (e.g. treetop syndrome in baculovirus-infected caterpillars (Hoover et al, 2013)), but could simply modify an existing behavior (e.g. enhanced mating and aggression in chrysomelobid beetles infested by a sexually-transmitted mite (Abbot and Dill, 2001)). In honey bees, such manipulation may inhibit social immune responses and/or return the host’s level of social interactions to “normal”.

However, host immune response has long been noted as a potential confounding factor in understanding host-manipulation (Adamo, 2002). Therefore, it is necessary to develop methods to document the effects of anti-viral immune stimulation, so that we may then isolate viral effects on host systems. Insects
possess a suite of anti-viral immune pathways, including the Toll, JAK-STAT, Imd and other pathways (Reviewed in Brutscher et al., 2015; however, it is generally accepted that the RNA-interference (RNAi) pathway plays a central role in defense against viral pathogens in insects (and other taxa; Mussabekova et al., 2017). RNAi is an innate anti-viral defense system that relies on recognition of double-stranded RNAs (dsRNAs) that signify the presence of actively replicating viruses, resulting in targeted degradation.

Previous work has expanded on the potential of dsRNAs to be used as vaccines against viruses in honey bee colonies: dsRNAs are relatively stable (Amdam et al., 2003; Nunes and Simões, 2009), can be delivered non-destructively through feeding (Nunes and Simões, 2009), and appear to knock down virus titers in infected bees (Maori et al., 2009; Flenniken and Andino, 2013). However, these studies have not specifically addressed the effects of dsRNA treatment on normal honey bee behavior. Because synthetic dsRNAs resemble the dsRNA intermediate produced by replicating single-stranded RNA viruses, and elicit this molecular anti-viral immune response, it is possible to utilize dsRNA as a “sham-virus”, to study the effects of virus infection, divorced from actual pathology. This indicates we can use dsRNA studies to separate viral-effects on host behavior from immune-response effects of the RNAi anti-viral pathway on host-behavior.

This study explores whether “sham-virus” dsRNAs can affect honey bee social behavior. Previous studies have demonstrated “off-target” effects of dsRNA, used as an experimental tool for gene silencing, on honey bee gene expression (Nunes et al., 2013; Jarosch and Moritz, 2015). Here, we extend this research by stimulating anti-viral immune response to a virus-based dsRNA in honey bees, and carefully documenting subtle effects on honey bee social interactions. To do this, we fed healthy bees with dsRNA mimicking a Kashmir bee virus (KBV) sequence ((deMiranda et al, 2010); see Figure 1) to stimulate RNAi response. The goal was to treat bees that lacked acute viral infection, thus creating “sham virus infected” bees with an activated RNAi system, without any actual change in viral replication. We examined pairwise social interactions between treated bees and untreated partner
**Figure 1.** Map of dsRNA sequence in relation to the KBV genome. A) simplified KBV genome (informed by deMiranda et al., 2004 and 2010); B) BLAST results of full KBV genome compared to IAPV and ABPV (red indicates sequence discrepancy; numbers: (Coverage% / Identity%) with placement of dsRNA sequence (Green) and KBV primers (dark and light Blue); C) expanded view of dsRNA sequence with comparison to IAPV and ABPV genomes.
bees in cages, to examine both immune stimulated individuals, as well as the response of normal untreated bees to the immune stimulated bees. We hypothesized that dsRNA-treatment would induce immune response-related behavioral symptoms, such as adaptive behavioral changes dampening performance of social behaviors, like trophallaxis, allo-grooming and other behaviors that require close contact between conspecifics.

**Methods**

**Bee Collection and Treatment assignment**

In summer of 2016, we collected frames of capped brood containing a high number of late-stage pupae and transported them to an indoor rearing chamber (kept dark, with high (80%) humidity and temperature (~32°C) until eclosion. For each round of observations, we collected such brood frames from at least 3 different colonies. Bees emerging from these frames were gently brushed from the frame into a common bin for temporary containment. On the same day, we randomly selected bees for treatment – bees were paint-marked with an oil-paint marker, to denote treatment type.

**dsRNA Preparation**

The dsRNA is a 250 bp sequence that mimics the KBV genome (from 6168 to 6418 bp; see Figure 1); notably, we ensured that primers for KBV did not overlap this sequence, to avoid quantifying dsRNA during quantification of viral titers. dsRNA was mass-produced by proprietary methods (care of Merav Gleit-Kielmanowicz, Monsanto).

**Treatment of Bees and Set-up of Observation Dishes**

For each treatment, we made three cages of 30 bees each. Treatment identity of each paint mark type was kept secret from the future observer. Each cage received a dish with 600 µL treatment exclusively for 24hr (either 0.5µG/µL dsRNA in 30% sucrose solution; approximately 1µg per bee as described in Maori et al, 2009); or unadulterated 30% sucrose-solution). The next day, the bees were given ad lib 30% sucrose-solution. This dish-feeding method was selected over direct-feeding or injection of treatment because 1) it requires substantially less time to apply and
therefore there is less variety in temporal exposure to treatment, 2) it is far less invasive to the bees in question (i.e. will not solicit stress response, which would potentially modify the behavior or survival potential of the bees) and 3) bees are known to constantly share food via trophallaxis, and should therefore spread the treatment relatively equally throughout the cage population. Because this method is intended to be used alongside virus treatment (which typically requires a 2 day incubation period) we incorporated a 2 day “incubation” period post feeding. This time frame is reasonable as dsRNA is known to persist in treated individuals for a relatively long period of time (up to 15 days post injection, Amdam et al., 2003) and has been shown to have prolonged effects post-feeding (Nunes and Simões, 2009).

On the third day after emergence (two days post-treatment (dpt)), focal bees were paired with 30% sucrose-fed partner bees, which were raised in separate cages from and marked to differentiate them from sugar-treated control bees, in experimental arenas. The arenas consisted of petri dishes prepared with) wax foundation pressed in, and mounted in an upright fashion, to mimic in-colony environment (similar to arenas described in Shpigler and Robinson, 2015). Subsequently, each pair was observed for 10 instances of 10 seconds each, tallying a number of behaviors (see Table 1) by indicating how often each behavior was seen during a single 10sec interval. Bees were then fed ad lib sucrose for an additional day, allowed to interact with each other in their pairs, before being collected on dry ice for downstream molecular work. We observed 79 pairs with a dsRNA-treated focal bee, and 81 pairs with a sugar-treated focal bee.
**Table 1.** List of all behaviors monitored in the pairwise-interaction experiments and onus for observing them.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
<th>Purpose</th>
<th>Expected Effects (Focal Bees)</th>
<th>Expected Effects (Partner Bees)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggression</td>
<td>A bee lunges at, mandibulates, or otherwise attacks her dishmate</td>
<td>Bee-Bee interaction; associated with detection of pathogens</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Allo-Grooming</td>
<td>A bee grooms her dishmate</td>
<td>Bee-Bee interaction; exchange of bodily fluids</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Antennation</td>
<td>A bee inspects her dishmate with her antennae</td>
<td>Bee-Bee interaction; inspection behavior</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Begging</td>
<td>A bee extends her proboscis toward her dishmate, to solicit trophallaxis</td>
<td>Bee-Bee interaction; exchange of bodily fluids</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Contact</td>
<td>A bee touches her dishmate for several seconds</td>
<td>Closeness of bees to one another; Bee-Bee interaction</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Self-grooming</td>
<td>A bee grooms herself</td>
<td>Hygenic behavior</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Still</td>
<td>A bee is standing without movement</td>
<td>Prolonged stillness may indicate immune stress</td>
<td>↑</td>
<td><strong>No effect</strong></td>
</tr>
<tr>
<td>Trophallaxis</td>
<td>A bee and her dishmate share food via contact between their tongues</td>
<td>Bee-Bee interaction; exchange of bodily fluids</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Walking</td>
<td>A bee moves around the observation arena.</td>
<td>Increased activity; premature changing of tasks, assoc. with pathogen stress</td>
<td>↑</td>
<td><strong>No effect</strong></td>
</tr>
</tbody>
</table>
Quantification of Viral Titers

To quantify baseline virus levels in our focal bees, and to examine whether dsRNA-treatment elicited antiviral immune response, we quantified viral genome presence (using methodology and qPCR primers described in Carrillo-Tripp et al, 2015) of four different viruses (KBV, Israeli acute paralysis virus (IAPV), Deformed wing virus (DWV) and Sacbrood virus (SBV)) in focal bees. We did this by extracting the RNA from entire focal bees, via Trizol (with DNase treatment) and quantification of each virus type via RT-qPCR. qPCR was run using a one-step system (iTaq, Life Technologies), on the BioRad-384 LightCycler platform. Titers were quantified using a viral genome standard curve, produced from PEG viral extracts of IAPV-infected honey bee pupae and, as such, is measured in “viral-genome equivalents” (vge). We measured viral levels for 15 whole bees from each treatment.

Data Analysis

We maintained original behavior count data (analyzed using Poisson regression) and created a new data series calculating the percentage of observations a bee spent doing a particular active behavior. This serves to explore if dsRNA-treated bees spent a greater fraction of their time performing or not performing a behavior, compared to normal bees, and was calculated as such: percentage of observations spent for behavior A = 100*(number of instances of behavior A)/(total number of instances of recorded active behaviors), where active behaviors are all behaviors except “Stillness”). These data were analyzed using Gaussian regression. We recorded treatment and round of observations, keeping the prior as a fixed effect, and round as a random effect. To compare viral titers between treatments, we compared log(mean VGE) between each treatment (we checked normality of log(mean VGE) data using the function qqp, from the car package (Fox and Weisberg, 2011)), using a standard GLM. All analyses were performed in R (R Core Team, 2017), using packages lme4 (for GLM, Bates et al, 2015) and stats (R Core Team, 2017). Graphics were created using ggplot2 (Wickham, 2008).
**Results**

*Pairwise-interactions*

In our count data, we found that partner- and focal-bees from the sugar-treatment trophallaxed twice as much as dsRNA-treated bees and their partners ($p = 0.002$, $X^2 = 0.691358$, $Z = 3.05$, $\exp(\beta) = 2.01$ for Sugar-treated bees and their partners; Fig 2A). Additionally, they spent more of their total active observations trophallaxing than dsRNA-treated bees ($p = 0.017$, $X^2 = 3.762605$, $t = 2.368$, $\exp(\beta) = 1.08$ for focal bees, $p = 0.042$, $X^2 = 4.046071$, $t = 2.032$, $\exp(\beta) = 6.43$ for partner bees; note there is difference between the two, as these percentages are derived from the total count of active behaviors for either bee, which varied between bees; Fig. 2B).

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**Figure 2.** Effects of dsRNA-treatment on trophallaxis ($n_{\text{dsRNA}} = 79$ pairs, $n_{\text{sugar}} = 81$ pairs). A) Counts of trophallaxis (the same for both focal and partner bees), B) Percentage of observations spent trophallaxing for Partner bees (top), and Focal bees (bottom). Error bars indicate standard error.

We note also, that there was no apparent effect on other social behaviors, such as begging, allo-grooming, antennation or contact, nor did we see enhanced
aggression towards dsRNA-treated focal bees. We also did not see differences in self-grooming, a presumed self-hygienic behavior, or walking.

**Virus Titers**

Overall, background levels of viruses in our focal bees were low (<0.2 log(VGE) of SBV, <2 log(VGE) for KBV) or below chronic infection (<7 log(VGE) of DWV, <6 log(VGE) of IAPV), as seen in Carrillo-Tripp et al, 2015. As intended, the dsRNA treatment did not appear to have a significant effect in virus titers between sugar- and dsRNA-treated focal bees (Figure 3).

![Graph showing virus titers](image)

**Figure 3.** Viral titers (in log(mean viral genome equivalents)); n_{dsRNA} = 15 for each virus quantified; n_{sugar} = 15 for each virus. There were no significant differences (NS) found for the levels of any of the viruses. p = 0.188 for DWV; p = 0.223 for IAPV; p = 0.582 for KBV; p = 0.336 for SBV. Error bars indicate standard error.

**Discussion**

We sought to explore the potential effects of RNAi-based anti-viral immune response on the behavior of honey bees. This is crucial to understanding how actual pathogen-induced immune response affects behavior in this organism. We have addressed this question, showing that dsRNA anti-viral immune stimulation affects an important and ubiquitous social behavior in honey bee colonies, trophallaxis. However, our data also show there is no difference in a number of other notable
social behaviors, as found in studies on effects of the microsporidian pathogen *Nosema* on honey bees (McDonnell et al, 2013; though increased contact with treated bees were found in bacterial-sham studies, Richard et al, 2008). This indicates that young bees may be reticent to engage in trophallaxis, perhaps because they detect sickness in their partner from a number of other behaviors, such as contact, antennation or allo-grooming. This suggests that these other social interactions perhaps serve as means for partner bees to detect sickness cues emitted by focal bees, leading to trophallaxis avoidance.

It is likely that this behavioral effect is exclusively due to the dsRNA treatment, as viral titers were overall low compared to levels seen in acutely infected bees (Carrillo-Tripp et al. 2016). Additionally, we saw no significant difference in virus titers for any of the viruses assayed. Overall, we take this to indicate that the effects we saw in the dsRNA-treatment were likely due to the bees’ response to the dsRNA itself, rather than other causes (e.g. the bees selected for this experiment did not have high viral load, and therefore the presence of pathogens is not likely to be the cause of behavioral effects).

This decreased likelihood of trophallaxis in dsRNA-treated bees is in keeping with the notion that virally immune-challenged bees are more socially isolated than their “normal” sisters. We note this phenomenon contradicts previous findings (Richard et al, 2008), which describe increased social attention received by sham bacteria [LPS] -treated bees, suggesting that anti-viral immune effects on behavior are different than that of anti-bacterial response. We also found that dsRNA-treated bees did not experience increased aggression, as bees infected with virus (DWV) did in Barrachi et al., 2012, suggesting anti-viral immune response effects on behavior are different than those of actual virus infection. Our data show a difference in only one key social behavior – other common social interactions, such as antennation, allo-grooming and contact were more or less the same across both treatments. This suggests there is not an outward change in the behavior of the “sick” bee, but perhaps an external signal that their partners perhaps perceive upon contact with the focal bee. Moreover, these data merely describe the behavioral
effect, not the onus behind it – it could be that dsRNA-related immune-response induces this external signal of “sickness”, but one that requires close contact to sense. This type of signal could be communicated via contact through cuticular hydrocarbons of the treated bee, which may vary in response to that bee’s immune status. Additionally, while these results give us cues into normal honey bees’ response to foreign dsRNAs in certain contexts, we will not completely understand immune-response effects in natural settings until we can successfully observe sham virus-induced anti-viral immune-stimulation (with viral knockdown) in colony environments as well.

Our data suggest the potential of anti-viral immune response effects on behavior, especially reductions in social interactions with other bees. This reduction in social interactions, while it may merely be an unavoidable consequence of immune response, could be an adaptive “social immune” response that could act to reduce the spread of pathogens (in a real-world setting in which the immune response was accompanied by an actual pathogen), as saliva has historically been shown to spread pathogens between individuals of a colony (Chen et al, 2014). Overall, this study provides novel information about the effects of anti-viral immune stimulation that can be useful in future studies involving the use of dsRNA, and importantly, to assess actual effects of viral infection (independent of anti-viral immune response) on honey bee behavioral phenotypes.

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CHAPTER 3: VIRAL INFECTION IS ASSOCIATED WITH WORKER HONEY BEE SOCIAL ISOLATION AND HYPERACTIVITY

This chapter currently in preparation for: Science (with Chapter 4)

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Abstract

Viral pathogens are key players in considerations of honey bee health; we understand much about pathology during viral infections, but little is known about how viral infection affects honey bee behavior. This study examined how infection by Israeli acute paralysis virus (IAPV, Dicistroviridae) affects honey bee behavior, considering two alternative hypotheses. 1) Adaptive sickness response: To prevent the spread of infection, virus infected bees avoid contact with other bees and/or vice versa. 2) Virus manipulation of host behavior: virus infected individuals show increased contact with other bees, which may increase virus transmission within a hive. To investigate this, focal individual bees inoculated with virus in their food were paired with unmodified partner bees in observation arenas, and their behavioral interactions compared to untreated and sham-virus (double stranded RNA, dsRNA) controls. Both viral infection and sham-infection led to reduced trophallaxis between pairs of bees. Virus infected bees were treated differently than controls, with partner bees showing less physical contact and lower levels of antennation with virus infected bees. Virus infected bees were also more active than controls, spending less of their time sitting still. The hyperactivity we observed in association with IAPV-infection suggests the infection could induce behaviors associated with early onset of foraging, which has previously been suggested to be a form of altruistic self-removal from a colony. We find support for adaptive sickness behavior, in that IAPV-
infected bees are more socially isolated than healthy bees, which may prevent them from readily spreading infection within the hive. However, further research is necessary to explore the possibility for cryptic transmission benefits for IAPV as result of this altruistic behavior (e.g. transmission via food stores, or transmission between hives as result of drifting).

**Introduction**

Viral pathogens are key players in the ecology and health of honey bees (Cornman et al, 2012; Gisder and Genersch, 2015). There are some 23 characterized viruses found in honey bee colonies worldwide, several of which have been implicated in recently observed honey bee colony losses (Chen and Siede, 2007; Cox-Foster et al, 2007; Gisder and Genersch, 2015). In this study, we discuss the effects of a widespread discistrovirus pathogen, Israeli acute paralysis virus (IAPV); this virus is found in apiaries on several continents and has been implicated as a potential factor in “colony collapse disorder”, a dramatic colony loss characterized by a noticeable absence of bees in and around the hive (e.g. infected bees would leave the hive, become paralyzed in the field, and become unable to return back) (Cox-Foster et al, 2007; Cornman et al, 2012; Hou et al, 2014). Although many honey bee pathogens have been studied in depth, with reference to their phenotypic and behavioral effects (reviewed in Schmid-Hempel, 1998 and Gisder and Genersch, 2015), most of our understanding of viral pathogens is limited to pathology, especially of visually observable symptoms. While there exists a small body of work concerning behavioral effects in infected honey bees (Mallon et al, 2003; Iqbal and Mueller, 2007, for example), there is still much to learn about how viruses spread within colonies, especially the ways they might co-opt their hosts' social behavior to do so.

Honey bee colonies are both excellent targets for pathogens and extremely difficult to infiltrate because of their sociality. As potential hosts, they exist in high density, are closely related to each other, and have extremely frequent and intimate contact with one another (Schmid-Hempel, 1998). It is impossible for honey bees to exist alone; they are perpetually in contact with other honey bees, as long as they
are present in the hive. As eusocial animals, this contact includes intimate behaviors such as allo-grooming (grooming of one bee by another bee) and trophallaxis (food sharing from proboscis to proboscis). Many of these behaviors involve direct contact and exchange of bodily fluids between individuals, presenting prime opportunities to transmit disease between individuals. Moreover, these behaviors could be excellent targets for manipulation by pathogens to enhance transmission.

Because of their sociality, though, honey bees possess “social immunity”, collective or socially-mediated defense mechanisms that promote the health of the colony, as opposed to individual health. This includes the detection and social isolation of infected individuals (Richard et al, 2008), concerted social “fever” in colonies (Starks et al, 2000) and accelerated behavioral maturation (e.g. the premature transition of an infected individual to out-of-nest tasks) (Rueppell et al, 2010, Alaux et al, 2012), among others (reviewed in Evans and Spivak, 2010). We’ve just begun to explore the ways viral infection affects honey bee behavior, and whether such changes are consistent with two hypotheses. 1) Adaptive sickness behavior: behavioral changes elicited by virus infection are beneficial to the honey bee, reducing transmission of the virus and/or improving individual bee health (the bee is “winning” against the virus) and 2) Pathogen manipulation: behavioral changes elicited by viral infect benefit the virus, increasing transmission of the virus and harming individual and/or colony health (the virus is “winning” against the bee).

In this experiment, we test whether and how virus infection affects worker bee social behaviors. Our approach to this problem advances upon previous research in that it includes a control accounting for previously characterized behavioral changes due to anti-viral immune stress (Geffre et al, in prep), a problem that has been well-documented, especially in response to stimulation by non-infective bacterial antigens and other immunostimulants (Mallon et al, 2003; briefly reviewed in Schedlowski, 2006; Goblirsch et al, 2013). Here, we use a dsRNA treatment, designed to match a shared sequence of discistroviruses (e.g. Kashmir bee virus, IAPV, Acute Bee Paralysis Virus), to induce anti-viral immune response in the absence of noticeable virus load; notably, previous work found anti-viral immune stimulation reduces
trophallaxis, a key social behavior in honey bee colonies (Geffre et al., in prep). Given that immune-stimulated bees elicit different behavioral responses in related conspecifics, invading viruses may not need to manipulate host honey bees to do highly atypical or novel behavior, merely they must overcome these immune stimulation-related signals to colony mates, or perhaps even take advantage of these altruistic responses for their own benefit.

In this study, we artificially infect honey bees with IAPV, a prevalent apiary virus associated with colony collapse disorder (CCD; Cox-Foster et al., 2007; deMiranda et al., 2010), and observe their behavior when paired with untreated conspecifics. With IAPV treatment and a virus-based dsRNA control, we endeavor to tease apart potential viral manipulation from immune stimulation-related changes to behavior in the bees. The adaptive sickness behavior hypothesis predicts that virus-infected bees would be less likely to trophallax, have contact, and attract less attention from partner bees. The pathogen manipulation hypothesis predicts that virus-infected bees would be more likely to trophallax, beg for food, and receive contact and attract attention in the form of antennation from partner bees. With these experiments, we aimed to provide a window into the bee-bee interactions within honey bee colonies during viral infections.

**Methods**

**Producing Viral Inoculants**

We produced large quantities of general viral extracts by processing adult honey bees (from colonies with mild IAPV infection), which are filtered and concentrated by ultracentrifugation, and subsequently cultured in white-eyed honey bee pupae [to amplify particles (methods derived from Boncristiani et al., 2013; Carrillo-Tripp et al., 2016)]. The infected pupae were then homogenized and the viral particles purified by polyethylene glycol (PEG) extraction and quantified by species using RT-qPCR (method and primers described in Carrillo-Tripp et al., 2015). We selected homogenates with a high proportion (>90%) of IAPV to be used for artificial infection later; previous work by Carrillo-Tripp et al. (2015) has shown that mixed viral inoculants with a high concentration of IAPV will induce IAPV infection, despite
the presence of other viral particles. To determine a dosage to induce chronic infection, as opposed to acute infection, we treated newly-emerged bees with a series of dilutions (in bee-palatable 30% sucrose) of the original virus stock, tracked their mortality over 7 days, and eventually collected them on dry ice to quantify viral titers via RT-qPCR; we opted to use a -4-fold dilution (~10000 particles/uL) of the stock as this treatment 1) produced only moderate (~40%) mortality over the week of observation, and 2) produced significantly higher viral titers in treated-bees than in untreated controls (data not shown).

**Sham-infection Treatment via dsRNA**

Sham-infection treatment implemented dsRNA designed from a shared genomic sequence of Kashmir bee virus (KBV), IAPV and Acute bee paralysis virus (ABPV) (nt 6168 to 6418 of KBV genome). Sham-infection by dsRNA was intended to induce anti-viral immune response in the absence of viral replication. This control accounts for effects of immune challenge on behavior, which has previously been observed (Geffre et al., *in prep*; Nunes et al., 2013) With this dsRNA control and IAPV treatment together, we were better able to tease apart potential viral manipulation from immune stimulation-related changes to behavior in the bees.

**Experimental Treatments and Behavioral observations**

We collected frames of emerging bees from at least four different colonies kept at the Toth Apiary at ISU Horticulture Research Station, in mid-summer, 2016. These frames were kept in incubation boxes overnight, in a controlled rearing room kept at approximately 80% humidity and 90°F. The following day, newly emerged bees were brushed into a common tub for treatment assignment.

For each treatment (i.e. virus, dsRNA or sugar (control)), three cages of 30 newly-emerged bees each were prepared; these “focal” bees were marked with an oil-paint marker to indicate treatment group. The identity of each treatment was kept secret from the future observer. Each cage received a dish with 600 uL treatment (either a sublethal viral dose in 30% sucrose (~6x10^5 IAPV genome equivalents per cage (ideally ~2x10^5 IAPV genome equivalents per bee)), 0.05uG/uL dsRNA in 30% sucrose solution (approximately 1µg per bee; Maori et al, 2009) or unmixed 30%
sucrose-solution) and given nothing else for an entire day. One day after this first feeding, the cages were given ad lib 30% sucrose-solution. From the same batch of newly-emerged bees, 6 cages of 30 bees were designated to be untreated “partner” bees; these bees were given 30% sucrose solution on the first day, then ad lib 30% sucrose solution on the second day. Partner bees were differentiated from control focal bees by paint-mark.

On the third day after emergence (2 dpt), behavioral observations were conducted. In each observation, a focal bee of a randomly selected treatment was paired with a partner bee in an experimental arena comprised of a petri dish with wax foundation pressed in, mounted in an upright fashion to mimic the in-colony environment. Each pair was observed for 10 instances of 10 seconds each, with behaviors noted (Table 1) for each 10 sec interval. Bees were then left together in their dishes to incubate with ad lib 30% sucrose solution for 2 additional days (to allow potential viral infections to manifest). All bees were then collected on dry ice, for downstream processing. This process was performed for 80 pairs with a dsRNA-treated focal, 81 with a control focal, and 77 with a virus-treated focal bee.

To determine whether viral inoculation was successful, we extracted RNA from individual focal bees of each treatment, via Trizol (with DNase treatment and marginal tailoring for use with whole honey bees) and then quantified IAPV titers via RT-qPCR (primer sequences from Carrillo-Tripp et al, 2015). qPCR was run using a one-step system (iTaq, Life Technologies), on the BioRad-384 LightCycler platform, with a no template control for each primer group. To ensure the effects we saw were the result of IAPV infection, we also quantified viral titers for three other common apiary viruses (Kashmir bee virus (KBV), deformed wing virus (DWV) and sacbrood virus (SBV), using primers described in Carrillo-Tripp et al, 2015). Titers were absolutely quantified in “viral-genome equivalents” (VGE) by use of a viral genome standard curve, produced from PEG viral extracts of honey bee pupae (Carrillo-Tripp et al, 2015).
Table 1. List of all behaviors monitored in the pairwise-interaction experiments, onus for observing them and predicted effect direction for focal bees given either 1) Adaptive sickness hypothesis or 2) Pathogen Manipulation hypothesis.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
<th>Purpose</th>
<th>Adaptive Sickness Effect Direction</th>
<th>Pathogen Manipulation Effect Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggression</td>
<td>A bee lunges at, mandibulates, or otherwise attacks her dishmate</td>
<td>Bee-Bee interaction; associated with detection of pathogens</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Allo-Grooming</td>
<td>A bee grooms her dishmate</td>
<td>Bee-Bee interaction; exchange of bodily fluids</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Antennation</td>
<td>A bee inspects her dishmate with her antennae</td>
<td>Bee-Bee interaction; inspection behavior</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Begging</td>
<td>A bee extends her proboscis toward her dishmate, to solicit trophallaxis</td>
<td>Bee-Bee interaction; exchange of bodily fluids</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Contact</td>
<td>A bee touches her dishmate for a prolonged period of time</td>
<td>Closeness of bees to one another; Bee-Bee interaction</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Self-grooming</td>
<td>A bee grooms herself</td>
<td>Hygienic behavior</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Still</td>
<td>A bee is doing no behavior of note (e.g. standing without movement)</td>
<td>Prolonged stillness may indicate immune stress</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Trophallaxis</td>
<td>A bee and her dishmate share food via contact between their proboscises</td>
<td>Bee-Bee interaction; exchange of bodily fluids</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Walking</td>
<td>A bee moves around the observation arena at a noticeable rate.</td>
<td>Increased activity; premature changing of tasks, assoc. with pathogen stress</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>
Data Analysis

Behavioral data were collected for both the focal bee and her partner in each pair. Counts of behavior were compared by bee type (i.e. partner or focal) and by treatment, using Poisson regression. To compare viral titers between treatments, we compared log(mean VGE) between each treatment (we checked normality of log(mean VGE) data using the function qqp, from the car package (Fox and Weisberg, 2011)), using a standard GLM. All analyses included random effects for observation round and were put through Tukey-HSD post-hoc tests. All analyses were performed in R (R Core Team, 2017), using packages lme4 (for GLM, Bates et al, 2015) and stats (R Core Team, 2017). Graphics were assembled in R using ggplot2 (Wickham, 2008).

Results
Quantification of viral titers

Viral titers for IAPV in the virus-treated bees were significantly higher than in dsRNA-treated bees ($p = 0.048$, $t = 2.441$, $\beta = 1.3895$) and sugar-control bees ($p = 0.00547$, $t = 3.311$, $\beta = 1.8852$); we note also there is a difference between dsRNA- and virus-treated bees in SBV ($p = 0.033$, $t = 2.064$, $\beta = 0.7849$), but SBV titers among all treatments were lower than the threshold of infection (minimum limit of infection for IAPV was $4.92E+02$ VGE and for KBV, DWV and SBV $4.92E+03$, as determined with a Universal Standard Reference by Carrillo-Tripp et al, 2015, and implemented in Dolezal et al, 2016) (see Figure 1). There were no significant differences in viral titers for KBV or DWV. DWV titers are relatively high for all treatments; this is unavoidable, as most bee colonies have DWV present to some degree; however, as there is no difference in DWV between treatments, we still assume that behavioral effects are primarily driven by IAPV.
Figure 1. Viral titers for 2016 Comb-Dish assays; letters represent the connecting letters report of a Tukey-HSD test. N=5 per round, per treatment (total n(treatment)=15) for all except the virus treatment in round 2 (n(round 2)=4; total n(treatment)=14). Note that KBV and SBV are not shown, as they were found to be below the limit of detection (Carrillo-Tripp et al, 2015).

These results indicate that the viral treatment was successful in causing a reasonable IAPV infection in the virus-treated bees, and that we can assume behavior changes for this treatment were the result of IAPV, and not other viruses.

Behavioral Observations

Honey bees infected with IAPV or sham-infected with KBV-based dsRNA trophallaxed about half as often as untreated focal bees (Control-dsRNA: p=0.006, exp(β)=2.005; Virus-Control: p=0.003, exp(β)=0.4576); see Figure 2A. Notably, there is no significant difference in begging behavior (Figure 2E,F). IAPV-infected bees were 70% as likely to be observed still (Virus-dsRNA: p=0.005, exp(β)=0.7028); control bees non-significantly trended towards being observed still less often than sham-infected bees as well (p=0.08; exp(β)=0.7925) - see Figure 2B.

Partners are about 60% as likely to antennate an IAPV-infected partner, compared to both controls (Virus-dsRNA: p=0.023, exp(β)=0.6394; Virus-Control: p=0.0471, exp(β)=0.6678); see Figure 2C. However, partners were 70% as likely to be observed in contact with an IAPV-infected focal as they were with a sham-

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**Figure 1:** Bar graphs showing viral titers for 2016 Comb-Dish assays. Letters (A, B) represent the results of a Tukey-HSD test. N=5 per round, per treatment for all except the virus treatment in round 2 (n(round 2)=4). KBV and SBV were not shown as they were below the limit of detection (Carrillo-Tripp et al, 2015).
infected focal ($p=0.0442$, $\exp(\beta)=0.7238$), but contacted IAPV-infected focals essentially as often as they would a control focal; see Figure 2D.

**Figure 2.** Counts of select behaviors and results from Poisson GLM with Tukey-HSD post-hoc test. A) Trophallaxis in focals (and, because trophallaxis can only occur through interaction, also for partners); B) Stillness in focals; C) Antennation by partners; D) Contact initiated by partners; E) Begging by focals; F) Begging by partners.

**Discussion**

This study provides novel information for understanding the effects of viral infection on bee behavior in that we observe individual-level changes to behavior as a result of infection or sham virus-induced immune response. These data provide a key component to the current knowledge of this topic in that they are the first of their kind that can be tracked to a single treated individual, rather than a colony. Such detailed behavioral observations of how individuals respond to infection provides an
added precision when extrapolating how a colony might look under infection, and offer insights into how infections may start with a few or even a single bee.

Overall, the main finding of our study is consistent with the hypothesis that IAPV infection results in adaptive sickness behavior in honey bee hosts. In general, IAPV infection leads to reduced social interaction, particularly of a key social behavior in honey bee colonies. Uninfected conspecifics trophallaxed with IAPV-infected bees less than half as often as uninfected controls, though there was no difference in begging behavior (Figure 2E,F). This suggests partner bees may cue into an externally perceivable signal elicited by immune response emitted from the infected focal. This is not a strange phenomenon – several bee pathogens solicit antiseptic response in conspecifics, such as the removal of chalkbrood-infected brood (Swanson et al, 2009) or of DWV-infected adults (Barrachi et al, 2012). Since we see this diminished trophallaxis in sham-infected dsRNA-treated focals as well, it is probable that such a signal is produced by the honey bee as part of immune response to pathogen or pathogen-like stimuli; indeed we see that other studies looking at external signals of quality, the cuticular hydrocarbon profiles of bees, are modified during fungal infections (Nosema, Swanson et al, 2009), virus infections (DWV, Barrachi et al, 2012) or even exposure to sham-pathogens, such as LPS (Richard et al, 2008).

Interestingly, virus-infected bees appear to receive less overall attention from partners than sham-infected bees did. Virus-infected bees experienced less contact and antennation with partners than sham-infected dsRNA treated controls. This finding is in contrast to other studies, in which bees experiencing immune- or pathogen stress receive heightened attention from their normal sisters. For example, honey bees experiencing antibacterial immune response from LPS treatment received significantly higher rates of “non-agonistic” contacts, such as allo-grooming and antennation (Richard et al, 2008), suggesting a hygienic response on the part of the untreated conspecifics. Additionally, other studies observing interactions with DWV-infected bees showed that nestmates aggress at and remove DWV-infected focals at a greater rate than normal bees (Barrachi et al, 2012). In contrast, we see
neither enhanced social interaction nor aggression, rather a seeming reduction in social contact. These results are consistent with the adaptive sickness behavior hypothesis, and could potentially serve to reduce intracolony transmission of the virus. However, we did not directly quantify virus transmission in this study; it is not yet known whether these behavioral effects effectively reduce virus transmission.

Why are IAPV-infected bees more likely to be “ignored” by other bees? Several alternative explanations are possible. 1) IAPV-infected bees may not provoke response of other bees around them, perhaps in such a way that an IAPV-infected bee may move throughout the colony with little resistance, potentially spreading virus as she does. 2) IAPV-infected bees may in fact be the ones refraining from contact, and could be altruistically isolating themselves. However, this does not address the fact that virus-infected focals are still contacted generally as often as untreated controls, and are more active than other focals.

It is reasonable that an infected bee roaming the colony without resistance, as alternative explanation 1 describes, could still be an effective vector for a virus. IAPV can be spread, for example, through saliva – even though she may not trophallax as often, if an infected bee were capable of roaming a colony unimpeded, she could still perhaps partake of multiple food stores. This is notable in the fact that honey bee food stores have already been shown to harbor both SBV particles and those of KBV (Shen et al, 2005), a sister virus of IAPV. Potentially, as an infected bee feeds at a food store, she will leave virus particles in her wake, to infect other bees that feed there after her. While we know a relatively moderate number of IAPV particles (<10^{11} particles; deMiranda et al., 2010) can cause mortality, we see in this study that even a small quantity of virus particles (e.g. \sim 2 \times 10^5 per bee) given orally is enough to induce a reasonably high IAPV viral titer – it’s possible that even such trace amounts of virus in food stores may be infective. Given the fact that we see bees infected with other pathogens elicit increased attention from their nestmates, this study may indicate that IAPV-infection causes some physiological alteration that makes an IAPV-infected bee less noticeable to a normal bee, likely through some external signal, such as cuticular hydrocarbon profiles. With this in mind, a future trajectory
for this research should include: A) describing how virus-infected bees interact with food sources, B) understanding how infected food stores play into the persistence of virus infections in colony and C) exploring physiological alterations happening inside IAPV-infected honey bees which could potentially cause this decreased interaction with conspecifics.

Alternative explanation 2 details that virus-infected bees may altruistically isolate themselves; this is supported somewhat by the fact that virus-infected focals were not observed in stillness as often as other focal bees. This increased activity may suggest that virus-infected bees may be transitioning to "out-of-hive" tasks, such as foraging, a documented aspect of altruistic immune response in honey bees. However, this does not necessarily mean that a virus-infected bee becomes a dead-end for the virus. Foragers, for example, are not isolated – they routinely trophallax with colony members, who take food from these foragers inside the colony. In bees that precociously forage because of immune stress, this interaction may be a site for exchange of viral propagules via saliva, both to the bees IAPV-infected individuals interact with and foodstores elsewhere in the colony.

Additionally, previous work has observed detrimental effects on cognitive function in insects; for example, immunocompromised bumblebees show poorer ability for adaptive learning (Ridell and Mallon, 2006) and that infection by DWV reduces learning ability in honey bee foragers (Iqbal and Mueller, 2007). If these effects are common with different types of pathogens, this may be associated with increased drifting behavior in pathogen-infected bees, as infected bees may have depressed ability to relocate their natal colony and could end up in a completely different one. Sickness-associated transition to foraging tasks may traditionally function as an adaptive immune response, but on what level? It may serve to protect the natal colony, but increase drifting of infected bees to new colonies, potentially increasing cross-colony infection. Considering this, we would imagine that virus-infected bees are likely drift to new colonies with greater frequency, potentially vectoring the virus between colonies. Although DWV and IAPV are different pathogens, this remains an important hypothesis to explore, especially in the context
of apiaries, where drifting is already extremely common, and found to be associated with the spread of pathogens (V. destector (Nolan and Delaplane, 2016), Nosema (Bordier et al., 2017) and bacteria (Lindström et al., 2008)). Future research concerning this hypothesis should include A) the rate at which virus-infected bees drift from their natal colony and potential physiological mechanisms that would induce increased drifting (juvenile hormone titers, for one), B) how infected bees are received by bees in the colonies they have drifted into and C) if subsequent infection occurs in bees contacting these infected drifters.

In this study, we see evidence that honey bees experience what appears to be altruistic semi-isolation when infected with IAPV. However, while this study provides a new level on which host-virus interactions are described in honey bees, it does not cover other potential ways in which viruses may co-opt this behavior to enhance intra- or intercolonial transmission. Honey bee colonies are sophisticated superorganisms with complex solutions to the problems of pathogen invasion, such as this altruistic isolation. However, there is still much left to explore to well understand the myriad ways pathogens can be transmitted in this charismatic eusocial species.

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**References**


Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E and Sela I. (2009). IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. Insect Molecular Biology. 18(1); pp55-60. doi:10.111/j.1365-2583.2009.00847.x


CHAPTER 4: INVADERS FROM ANOTHER HIVE: VIRAL INFECTION IN HONEY BEES (APIS MELLIFERA) INCREASES ACCEPTANCE BY NON-NESTMATES

This paper in preparation for: Science (with Chapter 3)

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Abstract

Pathogens are known to modify the behavior of their hosts to enhance their own transmission, and may twist previously adaptive host-pathogen responses to their own means, especially in novel environments. Viral pathogens are important players in considerations of honey bee health, especially the ways in which viruses may readily adapt to the novel environment of the apiary. It is likely that common apiary viruses, such as Israeli acute paralysis virus (IAPV), have been able to adapt to and repurpose the typical altruistic isolation and premature task transition in hosts such that they may enhance their transmission, not between individuals in a single hive, but between hives in dense apiaries. This is likely achieved through “drifting”, wherein a bee will “drift” into a neighboring non-natal colony, as this appears to be increased during infection. This study describes the response of guard bees to IAPV-infected non-nestmate bees, as well as sham-infected controls, to understand if increased acceptance could be a viable mechanism for intercolonial virus transmission. We found that virus-infected bees experience more non-agonistic interactions, notably trophallaxis, and less aggression than either normal or sham-infected bees from non-sister conspecifics. We also found subtle shifts in cuticular hydrocarbon (CHC) profiles among treatments, suggesting the response could be mediated through chemical signals. This suggests virus infection may modify
chemical signals in the host, making them more acceptable to non-nestmates from other hives. This suggests that drifting behavior may have been co-opted by viral pathogens to enhance transmission between colonies, especially in dense apiary settings.

**Introduction**

Pathogens are well known to alter the behavior of their hosts in ways that enhance their transmission. Often this type of manipulation takes on strange and novel manifestations (e.g. caterpillars climbing trees when infected with baculoviruses (Hoover et al., 2011), or mice becoming attracted to the scent of cat urine (Ingram et al., 2013)). While these manifestations can be some of the most fascinating to observe, host manipulation more often is subtle, adapting normal host behaviors to the needs of the pathogen – this is especially the case in pathogens that require their host survive to enhance pathogen survival (e.g. sacculina larvae require the innate parental care of their host crabs (Phillips and Cannon, 1973), strepsipteran endoparasites rely on the aggregation behavior of host wasps to facilitate parasite mating (Geffre et al., 2015). This subtle behavioral modification strategy is perhaps the best option in cases of pathogens infecting social animals, wherein it is extremely easy for a pathogen to find new hosts within colonies. In these instances, there is intense selection in hosts for altruistic immune response to prevent the spread of pathogens inside colonies. However, when population density increases, some of these highly advantageous social responses to pathogen threat may become less adaptive to the host as a species, and more beneficial to the pathogen. A charismatic demonstration of this can be seen in the honey bee, *Apis mellifera*.

The honey bee colony has finely-honed adaptive responses to pathogen threat, including social isolation of infected individuals (reviewed in Schmid-Hempel, 1998; Evans and Spivak, 2010; Gisder and Genersch, 2015; Geffre et al., *in prep* A), and altruistic self-isolation through premature transition to out-of-hive tasks by infected individuals (Rueppell et al., 2010; Alaux et al., 2012; Goblirsch et al., 2013). However, in apiary and commercial bee migration settings, where honey bee
colonies can be tightly clustered together, some of the adaptive responses may no longer be beneficial to the host, but benefit honey bee pathogens instead (reviewed briefly in Fries and Camazine, 2001). This is because of the peculiar phenomenon of drifting, wherein a foraging bee leaves her natal colony, but returns to a non-natal one through navigational mistake. Drifting has been documented at an extremely high rate in apiaries (some colonies may be comprised of approximately 40% drifted bees (Pfeiffer and Crailsheim K, 1998)). There is also an increasing body of evidence that immune-compromised bees are more likely than normal bees to drift: 1) pathogen-infected bees are more likely to leave the hive, as a result of premature caste-transition (Rueppell et al., 2010; Alaux et al., 2012; Goblirsch et al., 2013), 2) bees prematurely transitioning to out-of-hive tasks, such as foraging, are worse at orienting back to their natal colonies (Mallon et al., 2003; Kralj and Fuchs, 2006), and 3) virus-infected bees have reduced learning capacity, making it harder to learn to orient back to their natal colony (Deformed wing virus; Iqbal and Mueller, 2007). With these factors combined, it is likely that pathogens have quickly adapted to take advantage of this pathway to new colonies enabled by modern high density apiary conditions.

It is known that intercolonial interactions in apiaries, notably drifting, are key components of the intercolonial read of *Nosema* (Bordier et al., 2017), *Varroa destructor* (Nolan and Delaplane, 2017), and of other pathogens (Lindström et al., 2008). How is it that honey bee colonies, bristling with invader defenses, allow these sick invaders to enter into their homes? To better understand how viral pathogens spread among highly social hosts with specialized pathogen defenses, such as the honey bee, it is important to understand how normal hosts, especially of guard castes, respond to infected conspecifics. This study considers this problem using the honey bee-Israeli acute paralysis virus (IAPV) system. Previous work describes that IAPV-infected bees tend to be socially isolated in their own colonies, but also suggests increased activity (Geffre et al., *in prep* B)). In addition, preliminary data from the Toth lab (Dolezal and Narvaez, unpublished) suggest increased hive exiting by IAPV-infected bees, suggesting that they, like many other pathogen-infected bees, may prematurely transition to out-of-hive behaviors as part of altruistic immune
response. While this is adaptive behavior for bees in the wild, where non-natal colonies are often very far away, the modern apiary is far more densely packed. In the modern apiary, virus-infected bees may drift between hives more, spreading the virus as they do. For successful virus-transmission via drifting, though, the infected bee must be accepted into a non-natal colony. Here, we address this question experimentally through behavioral and chemical assays.

Honey bees identify their sisters based on a variety of different chemical cues, including cuticular hydrocarbon (CHC) signatures (reviewed in Breed et al., 2015). Typically, if a honey bee attempts to enter a foreign colony, she will be recognized as an outsider and attacked by guard bees, who patrol the colony entrance to protect their colony from invaders – this is an adaptive response to prevent robbing of the receiving colony by the foreign bee. There is some evidence that pathogens (Barrachi et al., 2012; McDonnell et al., 2013), and even inactive components of pathogens (Richard et al., 2008) can alter the CHCs of their host. We suggest that IAPV-infection could alter how receiving bees interpret the host, specifically that infection may modify host CHC signature, increasing its chances of invading the receiving colony.

To address this hypothesis, we observed the behavior of receiver bees to IAPV-infected, sham-infected and untreated focal bees from a different colony. If viruses adaptively manipulate their host in ways that enhance transmission between colonies via drifting, we predicted 1) IAPV-infected bees are less likely to receive aggressive reactions from receiving normal-bees and may even receive more non-agonistic social behaviors than other bees, and 2) IAPV-infected bees have a CHC profile different from that of similar normal- or sham-infected bees, which may help them, and their viruses, invade new colonies.

**Methods**

**Treatment preparation**

Control treatment consisted of 30% sucrose (commercial cane sugar) in boiled, nano-filtered water.
Virus-infection treatment consisted of IAPV particles prepared from infected pupae and purified to a concentrated stock as described in Carrillo-Tripp et al., 2015. We used a virus stock containing approximately 99.99% IAPV, at a concentration of approximately 15x10^{10} viral genome equivalents (VGE) per uL of RNA. Acute bee paralysis virus (ABPV), deformed wing virus (DWV) and Kashmir bee virus (KBV) were present as 5.12x10^{-7}, 4.14x10^{-5} and 1x10^{-7}% of the stock respectively; this small amount was unlikely to interfere with IAPV infection efficiency as IAPV can out-compete other viruses in vivo (Carrillo-Tripp et al., 2015, though see that KBV may outcompete IAPV). We performed a 10-day mortality trial with a serial dilution series of this stock. We found that a 2.5x dilution caused reasonable infection, but not high mortality over time (e.g. this dose constituted a “chronic infection”, rather than an acute, lethal one). We inoculated this 2.5x dilution into 30% sucrose solution, 500uL (containing approximately 7.83x10^9 viral particles) of which was given to each cage of 25 bees destined to be virus-infected focals. Because bees are known to trophallax often, we assume that this dosage was shared relatively evenly, and as such each bee received approximately 3.13x10^8 viral genome equivalents through food sharing; exact inoculation of each bee was not quantified however.

Sham-infection treatment implemented dsRNA (produced by Merav Gleit-Kielmanowicz, using a proprietary method at Monsanto) designed from a shared genomic sequence of both KBV and IAPV (nt 6168 to 6418 of KBV genome). dsRNA-treatment was prepared according to Maori et al. (2009), at a concentration of 0.05uG/uL dsRNA in 30% sucrose solution, or approximately 1µg per bee. Sham-infection by dsRNA was intended to induce anti-viral immune response in the absence of viral replication. This control accounts for effects of RNAi-related immune response on behavior, which has previously been observed (Geffre et al., in prep A, B; Nelson et al., 2007) With this dsRNA control and IAPV treatment together, we were better able to tease apart potential viral manipulation from immune stimulation-related changes to behavior in the bees.
Honey bee Collection and Behavioral Observations

To prepare focal bees, we collected brood frames from 3 different colonies, from our research apiary (ISU Horticulture Research Station; Ames, IA) in summer 2017. These frames were housed in emergence boxes kept in an indoor rearing room kept at approximately 32°C and 80-90% humidity. Newly-emerged honey bees from these frames were brushed into a shared receptacle. From this bin, bees were mixed and randomly selected for a treatment group and painted with oil paint markers on the thorax to denote this (color of treatment was randomly chosen for each experimental round). For each treatment, we created 3 cages of 25 bees each. On the first day post-emergence, bees were given 500 μL of treatment (virus, sham or control); 24hr post-treatment, ad lib feeders filled with 30% sucrose were given to all cages, and refilled as necessary for the next two days.

Two days after focal bees received treatment, we transported the focals to our research apiary lab. To obtain receiver bees, we blocked the entrance of normal hives (located several meters away from the home colonies of all focal bees), and waited 15min; the hives were gently jostled and then the block removed. Using a bee-vac (BioQuip), we collected bees rushing out of the hives and those returning (selecting for those returning with pollen (e.g. older foragers)). These bees thus represented a pool of older, more aggressive bees comprised of both guards and foragers. Receivers were sedated on wet ice briefly, and then transferred to observation arenas (10 receivers/arena). Receivers were given 15min to revive and acclimate. We then introduced a treated bee and recorded the responses of the receivers (Table 1) to the focal bee for 10min. After observations, all bees were humanely euthanized on dry ice and collected in glass vials with PTFE-lined screw caps. Over the course of five experimental rounds, we observed and collected samples for 164 arena matches (n(sham-infection)=55, n(control)=55, and n(virus-infection)=54).
Table 1. Behaviors recorded during focal introduction to receivers.

<table>
<thead>
<tr>
<th>Aggressive Behaviors</th>
<th>Description</th>
<th>Predictions if Host-Manipulation Occurs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stinging</td>
<td>A bee attacks another bee with her stinger</td>
<td>Receiver bees will try to sting or will sting virus-infected focals less than others.</td>
</tr>
<tr>
<td>Biting</td>
<td>A bee attacks another bee with her mandibles</td>
<td>Receiver bees will try to bite or will bite virus-infected focals less than others.</td>
</tr>
<tr>
<td>Chasing</td>
<td>A bee aggressively follows another bee, perhaps biting or attempting to drag her around</td>
<td>Receiver bees will not chase virus-infected focals as much as other focals.</td>
</tr>
<tr>
<td>Death Of Focal</td>
<td>A focal bee dies as the result of an aggressive interaction</td>
<td>Virus-infected bees will be killed less often than other bees.</td>
</tr>
<tr>
<td>Total Counts of Aggression</td>
<td>The sum of counts of all the above behaviors</td>
<td>Virus-infected focals will receive less aggression than other focals.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-agonistic behaviors</th>
<th>Description</th>
<th>Predictions if Host-Manipulation Occurs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allo-Grooming of Focal</td>
<td>A bee grooms the focal bee</td>
<td>Virus-infected bees will be allo-groomed more often than others.</td>
</tr>
<tr>
<td>Trophallaxis with Focal</td>
<td>A bee shares food via proboscis with the focal bee</td>
<td>Virus-infected bees will trophallax with receivers more often than others.</td>
</tr>
<tr>
<td>Antennation of Focal</td>
<td>A bee inspects the focal with her antennae</td>
<td>Virus-infected bees will be antennated more often by receivers.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Neutral Behaviors</th>
<th>Description</th>
<th>Predictions if Host-Manipulation Occurs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self-Grooming</td>
<td>A bee grooms herself</td>
<td>Receiver bees paired with a virus-infected focal will groom less often than those paired with other bees</td>
</tr>
<tr>
<td>Trophallaxis with sisters</td>
<td>A bee trophallaxes with her sisters (not the focal)</td>
<td>Receivers paired with virus-infected focals will not modify how often they trophallax with their sisters.</td>
</tr>
</tbody>
</table>
**Quantification of Viral Titers**

We extracted total RNA from a randomly-selected subset of focal bees of each treatment (n=9/treatment), via Trizol (with DNase treatment) and then quantified IAPV titers via RT-qPCR (primer sequences from Carrillo-Tripp et al., 2016). qPCR was run using a one-step system (iTaq, Life Technologies), on the BioRad-384 LightCycler platform, with a no template control for each primer group. To ensure the effects we saw were the result of IAPV infection, we also quantified viral titers for three other common apiary viruses (Kashmir bee virurs (KBV), deformed wing virus (DWV) and sacbrood virus (SBV), using primers described in Carrillo-Tripp et al., 2016). Titers were absolutely quantified in “viral-genome equivalents” (VGE) by use of a viral genome standard curve, produced from PEG viral extracts of honey bee pupae (Carrillo-Tripp et al., 2016).

**Characterization of Cuticular Hydrocarbons (CHCs)**

A randomly selected subset of focal bees was selected for CHC extraction and characterization by GC/MS (n=15/treatment) on a J&W Scientific DB-5 (30mm x 0.25mm) non-polar capillary column, with an HP 5890 Series II gas chromatograph paired with an HP 5872 Series mass selective detector. Each bee was submersed in HPLC-grade hexanes for 5min in her original collection vial. This extract was dried completely with a N2 stream, and resuspended in 20uL HPLC-grade hexanes. 4uL of this concentrate was singly injected, held at 60°C for 1min, and exposed to incrementally higher temperatures (up to 230°C) at a rate of 10°C/minute, then held at 340°C for 15min. Peaks were manually identified according to both GC retention time and MS spectra and subsequently checked against compounds identified in honey bees in several studies: a nestmate and caste-recognition study (Kather et al., 2011), an LPS-inoculation study (Richard et al., 2008), and two studies observing pathogen-induced CHC changes (Barrachi et al., 2012; McDonnell et al., 2013).

**Statistical Analyses**

Behavioral data were compared between treatments in raw count form using a Poisson regression, with a Tukey-HSD post-hoc test, wherein both experimental round and home colony of receiver bees were included as random effects. To
compare viral titers between treatments, we compared log(mean VGE) between each treatment (we checked normality of log(mean VGE) data using the function qqp, from the car package (Fox and Weisberg, 2011)), using a standard GLM. All analyses included random effects for observation round and were put through Tukey-HSD post-hoc tests. All analyses were performed in R (R Core Team, 2017), using packages lme4 (for GLM, Bates et al., 2015), multcomp (Hothorn et al., 2008), plyr (Wickham, 2011) and stats (R Core Team, 2017). Graphics were assembled in R using baseline graphics and ggplot2 (Wickham, 2008).

All CHC peaks were normalized as the percent of total peak areas within the individual sample (percent peak area), and ranked within compound; ranking compounds allows us to better compare subtle differences in CHCs and been used in other (Guillem et al., 2016) and similar studies (Richard et al., 2008). Entire CHC profiles of individuals were compared between treatments by linear discriminant analysis (LDA; using the MASS (Venables and Ripley, 2002) and car packages (Fox and Weisberg, 2011) in R.

**Results**

**Viral Titers**

IAPV-infection was verified to be successful as the virus-infected focals showed significantly higher titers of IAPV than either the control or sham-infected focals (Virus v. sham-infected: $p = 1 \times 10^{-4}$, $\beta = 1.56$, $z = 7.525$; Virus v. control: $p = 1 \times 10^{-4}$, $\beta = 1.41$, $z = 6.791$; control v. sham-infected: $p = 0.743$, $\beta = 0.152$, $z = 0.734$; see Figure 1). We saw no significant differences in viral titers of either DWV (virus-infected v. sham-infected: $p = 0.525$, $\beta = -0.14$, $z = -0.36$; virus-infected v. control: $p = 0.75$, $\beta = -0.28$, $z = -0.73$; control v. sham-infected: $p = 0.93$, $\beta = -0.14$, $z = -0.36$) or KBV (virus-infected v. sham-infected: $p = 0.86$, $\beta = 0.07$, $z = 0.521$; virus-infected v. control: $p = 0.99$, $\beta = -0.006$, $z = -0.04$; control v. sham-infected: $p = 0.836$, $\beta = 0.078$, $z = 0.57$) between any of the treatments.
Figure 1. Viral titers derived via qRT-PCR for DWV, IAPV and KBV for each treatment (n = 9/treatment). Connecting letters describe output of a GLM and Tukey-HSD post hoc test.

**Behavioral Observations**

Virus-infected focals were the significantly less likely to receive aggression than control or sham-infected focals (virus-infected v. sham-infected: $p = <0.001$, $\beta = 0.17$, $z = -5.212$; virus-infected v. control: $p = 0.007$, $\exp(\beta) = 0.33$, $z = -2.980$); this effect may be driven in particular by the counts of biting by receivers. Significant difference exists in biting (virus-infected v. sham-infected: $p = 0.001$, $\exp(\beta) = 0.10$, $z = -3.837$; virus-infected v. control: $p = 0.03$, $\exp(\beta) = 0.20$, $z = -2.507$). Counts of stinging (virus-infected v. sham-infected: $p = 0.06$, $\exp(\beta) = 0.18$, $z = -2.24$; virus-infected v. control: $p = 0.48$, $\exp(\beta) = 0.38$, $z = -1.14$; control v. sham-infected: $p = 0.31$, $\exp(\beta) = 0.46$, $z = -1.42$) and chasing (virus-infected v. sham-infected: $p = 0.07$, $\exp(\beta) = 0.33$, $z = -2.16$; virus-infected v. control: $p = 0.79$, $\exp(\beta) = 0.69$, $z = -0.64$; control v. sham-infected: $p = 0.23$, $\exp(\beta) = 0.47$, $z = -1.63$) shared similar trends, but were not significantly different
between treatments. Conversely, sham-infected focals were most likely to receive general aggression (control v. sham-infected: \( p = 0.009, \exp(\beta) = 0.50, z = -2.190 \)), and the most likely to be bitten by receivers (Figure 2).

![Graphs showing aggression by receivers to focals, by treatment.](image)

**Figure 2**: Aggression by receivers to focals, by treatment; A) Total counts of aggression; B) Counts of receivers stinging the focal; C) Counts of receivers chasing the focal; D) Counts of receivers biting the focal; connecting letters describe results from Poisson GLM, with Tukey-HSD post-hoc test. \( N_{\text{sham}} = 55; N_{\text{control}} = 55; N_{\text{virus}} = 54 \). (Note the changes to scale between graphs)

Virus-infected focals were the most likely to receive allo-grooming from receivers (virus-infected v. sham-infected: \( p = 1 \times 10^{-4}, \exp(\beta) = 2.05, z = 4.35 \); virus-infected v. control: \( p = 0.006, \exp(\beta) = 1.6, z = 3.03 \)). Total trophallaxis (i.e. between receivers and their sisters, as well as with focals) did not differ between treatments. However, virus-infected focals were the most likely to be seen engaging in trophallaxis with receivers (virus-infected v. sham-infected: \( p = 1 \times 10^{-4}, \exp(\beta) = 2.64, z = 7.85 \); virus-infected v. control: \( p = 0.0002, \exp(\beta) = 1.53, z = 3.9 \)). Sham-infected individuals were the least likely to be observed engaging in trophallaxis with receivers (sham-infected v. control: \( p = 0.0002, \exp(\beta) = 1.73, z = 3.94 \)), and conversely, receivers presented with sham-infected focals were more likely to be
seen trophallaxing with sisters (virus-infected v. sham-infected: p = 0.009, exp(β) = 0.82, z = -2.92; control v. sham-infected: p = 0.02, exp(β) = 0.84, z = -2.65). Receiver bees were less likely to be seen self-grooming when presented with a control focal than if presented with either of the other treatments (control v. sham-infected: p= 0.001, exp(β) = 0.24, z = -3.85; virus-infected v. control: p = 0.02, exp(β) = 2.61, z = 2.53); interestingly, receivers were as likely to be seen self-grooming when in the presence of a virus-infected focal, as with a sham-infected focal (virus-infected v. sham-infected: p= 0.18, exp(β) = 0.65, z = -1.73) – see Figure 3. Notably, receiver bees antennated all focal types equally.

![Figure 3: Non-agonistic responses by receivers to focals, by treatment; A) Counts of receiver trophallaxis, (total (L), with sisters (Center) and with a focal (R)); B) Counts of receivers allo-grooming; C) counts of receivers self-grooming; D) counts of receiver antennating the focal. Connecting letters report the results of Poisson GLM with Tukey-HSD post-hoc test. N_sham and N_control = 55; N_virus = 54. (Note the changes to scale between graphs)](image_url)
CHC Characterization

We identified 21 different compounds, 11 of which were found in only a few samples (none of these compounds were found exclusively in any one treatment) and 10 of which were found in all samples (see Table 2).

**Table 2.** Summary of CHCs found on focal bees.

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Present in All</th>
<th>Present in &lt;2 samples</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Henicosane</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; McDonnell et al., 2013</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>X</td>
<td></td>
<td>Kather et al., 2011</td>
</tr>
<tr>
<td>Tricosene 1</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Kather et al., 2011; Barrachi et al., 2012; McDonnell et al., 2013</td>
</tr>
<tr>
<td>Tricosene 2</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Kather et al., 2011; Barrachi et al., 2012; McDonnell et al., 2013</td>
</tr>
<tr>
<td>Tricosane</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Kather et al., 2011; Barrachi et al., 2012; McDonnell et al., 2013</td>
</tr>
<tr>
<td>Pentacosane</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Kather et al., 2011; Barrachi et al., 2012; McDonnell et al., 2013</td>
</tr>
<tr>
<td>Methylpentacosane</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Barrachi et al., 2012</td>
</tr>
<tr>
<td>Heptacosane</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Kather et al., 2011; Barrachi et al., 2012; McDonnell et al., 2013</td>
</tr>
<tr>
<td>Methylheptacosane</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Barrachi et al., 2012; McDonnell et al., 2013</td>
</tr>
<tr>
<td>Methyloctacosane</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Kather et al., 2011; Barrachi et al., 2012; McDonnell et al., 2013</td>
</tr>
<tr>
<td>Nonacosane</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Kather et al., 2011; Barrachi et al., 2012</td>
</tr>
<tr>
<td>Methylnonacosane 1</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Barrachi et al., 2012; McDonnell et al., 2013</td>
</tr>
<tr>
<td>Methylnonacosane 2</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Barrachi et al., 2012; McDonnell et al., 2013</td>
</tr>
<tr>
<td>Hentriacosene 1</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Kather et al., 2011; Barrachi et al., 2012</td>
</tr>
<tr>
<td>Hentriacosene 2</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Kather et al., 2011; Barrachi et al., 2012</td>
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<tr>
<td>Hentriacosane</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; Kather et al., 2011; Barrachi et al., 2012</td>
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<tr>
<td>Methylhentriacosane</td>
<td>X</td>
<td></td>
<td>Richard et al., 2008; McDonnell et al., 2013</td>
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</tbody>
</table>
There were no significant differences when comparing the percent peak area of each compound across treatments. However, when comparing ranked percentages of those CHCs detected in all samples with LDA, we do see that whole CHC profiles have some subtle differences by treatment (see Figure 4), with the profiles of sham-infected focals tending to cluster separately from those of virus-infected and control focals along LD1 (primarily loaded by heptacosane, methylhentriacosane, tricosane, methylnonacosane and hentriacosane – see supplement for LDA details). We also see that virus-infected focals tend to cluster with controls, but were marginally separated along LD2 (primarily loaded by methylnonacoasne, tricosane, methylhentriacosane, methylheptacosane, and heptacosane).

Discussion

This study is the first to describe potential adaptive virus-manipulation of honey bee behavior. Specifically, we found that virus-infected bees are typically greeted with reduced aggression and increased acceptance behaviors. Notably, we saw not only that virus-infected focals are less likely to receive aggressive contacts, such as biting (Figure 2), but receivers were more likely to allo-groom and trophallax with a virus-infected drifter than other focals (Figure 3). When exploring overall CHC profiles of focals, we saw subtle shifts that differentiate treatments (Figure 4), indicating virus-infection causes CHC shifts. Presumably, this altered CHC profile induces behavioral shifts in receiver bees towards the focal, enhancing the
probability of transmitting virus to these receivers. Additionally, this modified receiver behavior may potentially increase chances of infecting the rest of the new colony.

Figure 4. LDA showing best separation of treatments based on relative peak areas of all shared CHCs; $N_{\text{Control}} = 6$, $N_{\text{Sham}}$ and $N_{\text{Virus}} = 5$.

Our results indicate that, when an IAPV-infected bee arrives at this foreign colony, she is less likely to receive aggression compared to other drifters (Figure 2), especially more than bees experiencing anti-viral immune response (as in the sham-infected focals). We suggest this could facilitate her entrance into a new colony. Additionally, she receives more trophallaxis and allo-grooming than other drifters (Figure 3), indicating she engages in more bee-bee interactions, which are well-known sites of IAPV transmission between individuals (deMiranda et al., 2010; Chen et al., 2014). These all occur despite the fact that receiver bees antennate and inspect IAPV-infected bees with similar frequency as other drifters (Figure 3). This suggests that IAPV-infection masks signals of anti-viral immune response, or makes the IAPV-infected bee either 1) less threatening or 2) more familiar-seeming.
Our explorations of CHC profiles during IAPV-infection and sham-infection show noticeable but subtle changes in CHCs depending on infection status. Notably, we see that profiles of sham-infected focals tended to cluster distantly from those of control focals, whereas IAPV-infected individuals tended to cluster closer to controls. As immune stimulation can modify CHC profiles (Richard et al., 2008), this could indicate that virus-infection masks poor health indicators embedded in the CHCs of the host, making them appear “normal”, even though they harbor active virus. This supports the idea that IAPV infection masks CHC changes correlated with immune-stimulation.

However, such altered CHC profiles do not explain the difference in behavioral responses of receivers when only comparing control- and IAPV-infected focals. We observed that receivers allo-groomed and trophallaxed with virus-infected bees more than control focals. Additionally, receivers self-groomed more often in the presence of a virus-infected focal. This indicates that receivers may be able to differentiate between the two types of focal. CHC profiles of control- and virus-infected focals are reasonably similar (Figure 4), but other studies describe that other types of pathogens not only affect the CHCs, but also volatile chemical agents (e.g. those of sacbrood virus-infected larvae, Swanson et al., 2009). It is possible that virus infection may induce other chemical changes beyond CHCs (e.g. volatiles or the rate of emission of these). As such, future research is needed to understand the importance of these additional chemical communication agents.

Viruses and other pathogens are scourges of apiaries, as it is difficult to control their spread between colonies once they invade an apiary. Traditional methods for controlling outbreaks usually rely on active management by beekeepers, such as isolating or killing infected hives. These methods do not sufficiently consider bee biology. As such, they may fail to address how bees may spread pathogens in the course of normal behavior, like drifting. Natural honey bee colonies can exist miles apart from each other, rather than within inches, as is convenient for modern apiarists. This close proximity dramatically increases the likelihood that even healthy bees will accidentally drift to non-natal colonies (Jay,
A virus-infected bee altruistically transitioned to out-of-hive tasks has an even lesser probability of successfully navigating home. Previous work indicates that such infected drifters are in fact a cause of intercolonial pathogen transmission (V. destructor (Nolan and Delaplane, 2016), Nosema sp. (Bordier et al., 2017) and of other pathogens (Lindström et al., 2008)), but do not detail the exact reasons why.

Our work expands the ever-growing body of literature suggesting beekeepers should rethink their management practices for infected colonies and the organization of apiaries. Viral pathogens may have adapted rapidly to take advantage of both altruistic task-transitioning of hosts and the close-proximity of colonies in apiaries to enhance intercolonial transmission by increasing the acceptability of virus-infected drifters. It is possible that rising incidences of colony loss due to disease could be related to pathogens adapting to take advantage of the practices humans have designed to rear honey bees. Indeed, crowding in populations of many species, including eusocial insects, elicits increased both pathogen transmission and virulence (reviewed in Fries and Camazine, 2001). It is possible that other rapidly evolving pathogens (bacteria, unicellular fungi, etc.) may have similarly adapted to the novel conditions of the apiary and large scale migratory beekeeping and are, in turn, pathogens causing havoc they would never accomplish in the natural habitat of the honey bee. With the enhanced pathogen pressures present in these tightly-packed apiaries, we suggest developing management strategies which minimize drift and the high density conditions that provide viruses a novel opportunity for transmission.

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CHAPTER 5: SUMMARY AND CONCLUSIONS

Summary

In this thesis, I describe three experiments by which I explore the complex interactions between honey bees and an important apiary pathogen. Notably, this research has produced three critical results:

In the Chapter 2, I describe the first instance in which dsRNA designed from a common discistrovirus genome sequence was used as a control for behavioral effects resulting from anti-viral immune response. This dsRNA control was implemented in this body of work because dsRNAs are traditionally used to solicit RNA interference, or anti-viral immune response, to destroy target RNAs; this means that exposure to dsRNA solicits a key form of anti-viral immune response (RNAi), despite the absence of replicating virus. This previously unexplored control for host-virus interactions serves a critical role in helping tease apart the effects of immune response from virus-manipulation. In this experiment, we fed honey bees with dsRNA, and observed their behavioral interactions with normal honey bees. Notably, we found that dsRNA-treated focal bees trophallaxed less than sugar-treated controls, but found no other differences in social interactions. This indicates that dsRNA-treatment may induce changes in social cues resulting in altered social interactions. The fact that we observed behavioral modification as result of dsRNA treatment indicates that anti-viral immune response does in fact have an effect on host behavior. As such we suggest that future studies of honey bee-virus behavioral manipulation should include such controls to ensure adequate description of virus effects.

In Chapter 3, we describe how IAPV induces host adaptive response, including the social isolation of infected individuals, in bee-bee interactions within hives. By infecting bees with IAPV, we examined fine scale interactions between these virus-infected focals and untreated sisters. This setup helped us describe the individual-level interactions by which large scale social immune response could manifest in honey bee colonies. Additionally, we included the anti-viral immune response control, sham-infected focal bees exposed to virus sequence-based dsRNA, to tease apart response to virus infection from immune stimulation in hosts.
Both viral infection and sham-infection led to reduced trophallaxis between pairs of bees. Virus infection also reduced physical contact with and antennation of foci. Virus infected bees were also more active than controls, spending less of their time sitting still. The hyperactivity we observed in association with IAPV-infection suggests infection could induce behaviors associated with early onset of foraging, which has previously been suggested to be a form of altruistic self-removal from a colony. We found support for adaptive sickness behavior, in that IAPV-infected bees are more socially isolated than healthy bees, which may prevent them from readily spreading infection within the hive.

Lastly, in Chapter 4, I show virus infection may lead to maladaptive shifts in honey bee behavior in dense apiary settings, wherein infected bees are likely to drift to new colonies. In Chapter 3, we found evidence suggesting that IAPV infection induces altruistic self-isolation in hosts and we speculate it could also relate to an early transition to foraging behavior. Early transition to foraging and virus infection have the potential to increase worker drifting between colonies, which could spread pathogens from one colony to another. For intercolonial virus transmission via drifting to be successful, the virus-infected bee needs to be accepted at foreign colonies. To explore this, we infected honey bees with IAPV and observed how bees from foreign hives responded to their presence in mock drifting assays. Infected honey bees are accepted more than others, likely because of modified cuticular hydrocarbon signatures. It is possible that common apiary viruses, such as IAPV have been able to adapt to and repurpose the typical altruistic isolation and premature task transition in hosts such that they may enhance their transmission, not between individuals in a single hive, but between hives in dense apiaries.

Conclusions

These studies are the first to describe individual level interactions between virus-infected bees and conspecifics, with the added specificity of an anti-viral immune response control. Additionally, these results present the first observed instance of potential adaptive virus-manipulation of honey bee behavior. Both of these are important to considerations of honey bee health and the ecology of honey
bee viruses, as we know a plethora of pathogens play key roles in the lives of their hosts (including exo- and endoparasites, pathogenic fungi, bacteria and viruses; reviewed in Thomas et al., 1999, Poulin and Morand, 2000, and Lefevre et al, 2008; Seabloom et al, 2015). It is crucial to consider pathogens whenever exploring the biology of any free-living organism (Thomas et al., 1999; Lafferty et al 2008), including pathology, host-pathogen coevolution and the intricacies of host-pathogen interactions. Thus it is important to explore interactions between the honey bee and their viral pathogens, not only to further our knowledge of honey bee ecology, but also better understand the threats pathogens present to this ecologically and economically crucial insect.

The research discussed in this thesis describes several explorations of honey bee-pathogen interactions and the insights they provide to the problem of honey bee health. Below, I discuss three primary conclusions of this work: 1) the need for anti-viral immune response controls, 2) the social context and adaptive nature of social immune response to viruses in honey bees and 3) the potential implications for honey bee management presented by my research.

The need for anti-viral immune response controls

Honey bees present a novel frontier for understanding host-pathogen interactions. Because of these close associations between honey bees they are excellent targets for pathogen manipulation, as pathogens can readily hijack social behavior to enhance their own transmission. To combat this intense pathogen threat, eusocial insects have evolved unique adaptations: social immune responses, based, in part, in altruistic behavior (Schimd-Hempel, 1998; Cremer et al, 2007; Evans and Spivak, 2009). We see verification of this in bee-virus interactions with the results of Chapters 1 and 2, wherein we found that honey bees isolate virus-infected bees, and even those experiencing anti-viral immune stress. Notably, we saw a decrease in a key social behavior, trophallaxis, with infected or sham-infected bees. Evidence from Chapter 3, though, indicates that sham-infection is different than actual infection (virus-infected bees received less general contact and were more active than sham-infected bees). In trials with live IAPV, IAPV infection also lead to
reduced social interaction, particularly reduced trophallaxis and general contact. However, the most notable evidence for the need of an anti-viral immune control stems from the drifting assays in Chapter 4, where we saw dramatic rejection of dsRNA-treated bees, and support for host chemical signal modification during such immune response. Virus-infected bees, on the other hand, in many ways resembled the normal, untreated focals. These studies together indicate that virus-infection does affect focal behavior, but may not do so dramatically, perhaps just restoring them to a “normal” phenotype. Without the sham-infection control, the results we found in Chapter 4 could have been completely missed. To better understand the effects of immune response on behavior, we advocate that future work consider other anti-viral pathways beyond RNAi.

Our research with a dsRNA sham-virus found that an anti-viral immune response (RNAi) has the potential to affect interactions between bees. Recent work describes use of dsRNAs as vaccines against viral pathogens (Maori et al., 2009; Hunter et al., 2010; Flenniken and Andino, 2013) with success. Because we see that dsRNA treatment solicits a hygienic defense in honey bees (decrease in trophallaxis with and rejection of dsRNA-treated bees by conspecifics), we suggest that these dsRNA vaccines may not only tap into RNAi anti-viral machinery in treated bees, but also normal social defenses of honey bee colonies to achieve rescue from pathogen infection. We suggest further observation of honey bee behavior during dsRNA treatment, especially in colony settings (as in Hunter et al., 2010), will be important to take into account when designing dsRNA mediated virus control strategies.

**Social context affects adaptive nature of immune response**

The conclusions from these experiments primarily focus on the response of partner bees, but the behavior of the focal bee may provide insight as well. Two future directions should be considered from these results. Firstly, in Chapter 3, we found that IAPV-infected bees are comparatively isolated by other bees. This may still be adaptive for the virus, as the host bee does not appear to draw as much attention from conspecifics as a bee merely experiencing immune response. This may mean the host bee is able to move throughout the colony with little resistance,
vectoring the virus as she does. IAPV can be spread through saliva; an infected bee, despite her isolation from other bees, may still spread virus through contact with food stores. Food stores in honey bee colonies have already been shown to harbor both SBV particles and those of KBV (Shen et al, 2005), a sister virus of IAPV. To better understand the potential ways infected, but socially-isolated, bees may still vector pathogens, future research should include exploring how virus-infected bees interact with resources in colonies, and how such contaminated resources could affect viral spread therein.

Secondly, IAPV-infected bees may in fact be the ones refraining from contact, rather than partners. Virus-infected bees appeared more active than others, suggesting hyperactivity associated with premature transition to out-of-hive tasks (as seen in Lecocq et al., 2016 and Natsopoulou et al., 2016). This behavioral response to sickness is a common component to social immunity in social insects. The more time an infected colony member spends out of the colony, presumably the less likely she is to vector disease therein. However, while this is adaptive in the ancestral context of the honey bee, it may take on a maladaptive twist in modern apiaries. Natural honey bee colonies exist far apart from each other. Modern beekeeping practices densely pack colonies together, though, introducing the problem of drifting. In apiaries, drifting is already extremely common (Jay, 1966 and 1968). A virus-infected bee altruistically transitioned to out-of-hive tasks has an even greater propensity to drift (Mallon et al., 2003; Kralj and Fuchs, 2006; Iqbal and Mueller, 2007; Rueppell et al., 2010; Alaux et al., 2012; Goblirsch et al., 2013).

Such infected drifters are in fact a cause of intercolonial pathogen transmission (Lindström et al., 2008; Nolan and Delaplane, 2016; Bordier et al., 2017), but the exact reasons why are unclear. Chapter 4 addresses some of these reasons, finding that virus-infected bees are accepted more often, perhaps because of a shift of CHCs. Sham-infected bees showed reasonably different CHC profiles, while those of virus-infected bees seemed closer, but not exactly like normal bees. It is possible even that the small shift from normal bee CHC profiles in virus-infected bees could be responsible for such increased acceptance. This small shift could even include the reduction of total CHCs, as this may make the bee appear more
like a newly emerged bee which tend to be more accepted by non-nestmates. Regardless of the details of this shift, however, it appears that social immune response to infected bees differs on the social context of their interactions with normal bees: among sisters, there seems to be reduced contact, but among unrelated conspecifics, there is enhanced social interaction between infected and uninfected individuals.

Management implications of honey bee-virus interactions

Since we have begun to consider honey bee health with greater fervor, a number of extraneous factors have been found to affect honey bee survival (Vanbergen and The Pollinator Initiative, 2013). In particular, some 23 viruses, including IAPV, have been implicated in declining honey bee health (Chen and Siede, 2007; Cox-Foster et al., 2007; Chen et al., 2014; Gisder and Genersch, 2015). While diagnostic characteristics and pathology of IAPV infection are well-characterized (Maori et al., 2007; reviewed in deMiranda et al., 2010), knowledge concerning host behavior during infection is decidedly less described. This body of work has sought to flesh out this gap in knowledge, so that we can better inform honey bee management practices.

As discussed in the previous section, honey bees tend to socially isolate virus-infected sisters, but tend to accept virus-infected drifters. This suggests that, while honey bees themselves may not have quite adapted to the apiary, their pathogens, including viruses, have. In the interaction between the honey bee and IAPV, it appears that virus infection may cause altruistic transition to out-of-hive tasks (e.g. induced hyperactivity); however, if these IAPV-infected bees happen to drift in the process of doing such tasks, they will be more likely to infiltrate new colonies. We see here that the virus may in fact be twisting an ordinarily beneficial host response to one obviously beneficial to the virus in the context of drifting between colonies. In fact, it is possible that rising incidences of colony loss due to disease could be related to pathogens adapting to take advantage of the practices humans have designed to rear honey bees. Indeed, crowding in populations of many species, including eusocial insects, elicits increased both pathogen transmission and
virulence (reviewed in Fries and Camazine, 2001). It is possible that other rapidly evolving pathogens (such as unicellular fungi, (Z. Huang, personal communication)) may have similarly adapted to the novel conditions of the apiary and large scale migratory beekeeping and are, in turn, pathogens causing havoc they would never accomplish in the natural habitat of the honey bee.

With the enhanced pathogen pressures present in these tightly-packed apiaries, and the threat of transmission via infected drifters, we suggest at least three directions for further research. 1) The rate at which infected bees drift from their natal colony should be described. Anecdotal evidence exists for higher rates of drifting in virus-infected bees (Dolezal and Narvaez, unpublished), but not quantified. 2) If infected bees are accepted more while drifting, they may have access to the interiors of these colonies. Understanding how the behave inside foreign colonies will help us understand the ways that virus transmission can occur through drifting. Lastly, 3) future work should consider if pathogen transmission in apiaries can be mitigated by altering management practices. Since it’s already understood that pathogens are transmitted through drifting, an excellent prevention method could be merely ensuring that bees have limited opportunity to do so. The current model of beekeeping is highly unnatural, and is often more oriented to the ease of beekeepers rather than the biology of honey bees. There already exists a growing body of work suggesting that the modern apiary is not a beneficial environment for honey bees (Jay 1966 and 1968; Lindström et al., 2008; Nolan and Delaplane, 2016; Bordier et al., 2017 and others), which my work only serves to support.

Pathogens are important players in the lives of all organisms, including the honey bee. Continuing to explore host-pathogen interactions in honey bees is therefore important to understand the threats to pollinator health posed by pathogens. We must also learn to implement the knowledge we glean from honey bee-pathogen ecology in how we keep bees. Developing management strategies, especially in large scale apiculture, which minimize drift and address the high density conditions that provide viruses a novel opportunity for transmission will be critical to quell the ever growing threats to honey bee health.
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