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ECOLOGY OF BEE VIRUSES

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by

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ABSTRACT

Throughout much of human history honey bees have provided pollination services crucial to our economy and human survival. Thus, in yet more recent history humans have developed a keen interest in the health of honey bees and how it relates, directly and indirectly, to their effectiveness as pollinators. Furthermore, other pollinator species have also become of interest to humans due to their own contributions to pollination services. Here we seek to add to a global understanding of bee health by taking a closer look at viruses known to negatively impact honey bee health and suspected to impact the health of other bees, as well. In Chapter 1, we review the literature incriminating viruses as a cause of honey bee colony losses. This includes a breakdown of the different kinds of evidence collected, including correlative evidence, experimental evidence verifying viral pathology, experimental evidence of therapeutic techniques, and the impact of a vector. In Chapter 2 we take a look at the seasonal variance of viruses in Kenyan honey bee colonies and the effect of hive type (Langstroth, Log or Kenyan Top Bar) on colony health as well as swarm preference for 3 different hive types. Briefly, we found that viral infection is higher in the dry season, when the bees are nutritionally stressed, though there seems to be no effect by hive type. Furthermore, swarms preferred the Langstroth hive, and secondly the Log hives. After giving considerations to costs and hive productivity, we recommend a modified Kenyan Top Bar hive may present the best course of action for beekeepers in the Mwingi District of Kenya. Lastly, in Chapter 3, we take a preliminary look at the role that a *Bombus impatiens* plays in the ecology of a honey bee virus (Deformed Wing virus, DWV). Our results suggest that individuals of this species may not be effective or competent transmitters of this virus. Further studies that improve upon our study design would need to be done to confirm or deny our result. It is our hope that this thesis provides an impetus for other scientists to look more closely at

current apicultural practices used in the West, with a particular focus on how these practices impact the epidemiology of known honey bee pathogens.

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Chapter 1

Honey bee colony losses and associated viruses

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Abstract

Recent large-scale colony losses among managed Western honey bees (Apis mellifera) have alarmed researchers and apiculturists alike. Here, the existing correlative evidence provided by monitoring studies is reviewed which (i) identified members of the deformed wing virus and acute bee paralysis virus clades as lethal pathogens for entire colonies, and (ii) identified novel viruses whose impact on honey bee health remains elusive. Also discussed in this review is related evidence obtained via controlled experimental infection assays and RNAi approaches underscoring the damage inflicted by some of these viruses on individuals and colonies. The relevance of the ectoparasitic mite Varroa destructor acting as mechanical and biological virus vector for the enhanced virulence of certain viruses or mite selected virus strains is carefully considered.

Introduction

Industrial management of the Western honey bee (Apis mellifera) has a long history with honey as a source of nutrition and trade for humans [1]. In addition to income from products directly harvested from honey bees, managed colonies contributed an average of s147 million to annual crop productivity from 2002 to 2007, as they have become an important supplement to pollination services provided by native pollinators across the globe [2●●]. Indeed, managed honey bees are the single most important global commercial insect pollinator [2●●,3–5]. Despite some decreasing trends in parts of Europe and North America, the global number of managed colonies has risen by about 45% over the last 60 years [6,7]. However, this increase in the number of managed honey bee colonies does not meet the steadily increasing need for managed pollinators in agriculture [6,8–10]. Hence, large scale managed colony losses, as experienced in the recent past in some parts of the world, exacerbate the shortage of pollinators and threaten human food security especially because wild pollinators are on the decline [11,12●●,13].

Winter colony losses in general and CCD in particular

It is worth noting that the death of a honey bee colony due to natural causes, including disease, is within the scope of reasonable expectations for a living organism [14,15]. However, reoccurring unusually high winter colony losses at or above 30% in the recent past startled beekeepers and scientists alike [16–18], in part because simple, causal relationships remained elusive. The picture that emerged over the last decade is that such losses are multifactorial with weak Fall condition, starvation, queen failure, pathogens, parasites, pesticides, and climate all playing a role. The impact of each of these factors may differ on a case-by-case basis and even regionally [15,19,20].

Often when talking about colony losses, this general phenomenon is conflated with CCD [20]. However, CCD has a very specific case definition and is mainly characterized by the presence of a live queen and a lot of capped brood, indicative of rapid loss of adult bees. Additionally, the absence of dead bees in collapsed colonies and increased pathogen incidence are diagnostic of CCD [17]. CCD sensu stricto has so far only been reported from North America where it has been identified as one of many causes of winter mortality [21–23] and likely arises from multiple etiological agents. However, it has not been cited as a significant cause of winter losses since the winter of 2006/2007 [19]. In this review we refer to winter losses of managed A. mellifera colonies as the problem of interest, though summer losses may also be substantial. Primarily, we will concisely review the role that recently discovered and emerging viruses play in observed winter colony losses.

Viruses associated with honey bees

Till date 23 viruses have been reported to infect honey bees worldwide, primarily positive-strand RNA viruses in the families Dicistroviridae and Iflaviridae [24,25 ●,26 ●●]. In the absence of the ectoparasitic mite *Varroa destructor* (hereafter referred to as *Varroa*) many honey bee pathogenic viruses only cause covert infections, which show no clinical signs and have no detectable impact on infected bees or colonies (for a definition of covert/overt virus infections see [27]). However, for acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), and sacbrood virus (SBV) historical reports from the pre-Varroa era on overt disease outbreaks exist indicative of clinically significant infections in the absence of Varroa (Table 1; for historical references see [28]). However, we know from molecular data that even these four viruses can frequently be detected in seemingly healthy colonies [16,29–31], hence, also for these

viruses covert infections are common. Most of the honey bee viruses might not even be honey bee specific because they infect a wide range of arthropod species [32●,33–35]. In the wake of Varroa, prevalence of virus infections has increased dramatically. This mite very quickly became established as a mechanical and biological vector of honey bee viruses as it spread though European honey bee populations since the 1980s (for a recent review see [36]). Augmented prevalence and virulence due to mite transmission was marked for members of the DWV/VDV-1 (V. destructor virus-1) and ABPV/ KBV/IAPV (Kashmir bee virus/Israeli acute bee paralysis virus) clades [37–39]). The remainder of the review is divided into the following sections: correlative evidence (prevalence studies in symptomatic, asymptomatic, and not otherwise specified colonies), positive experimental evidence (infection assays), and negative experimental evidence (RNAi approaches) for associations between viruses and colony losses with a final section on the role of Varroa in virus amplification and dissemination.

Correlative evidence for virus infections associated with colony losses

Recently, numerous prevalence studies have underscored the role of viral infections in honey bee colony losses [16,17,25 ●,26 ●●,29–31,40–45,46 ●,47]. However, the design of many of these studies was rather weak. In most cases, the study period was one year or less, colonies were sampled once or twice, and whole bee extracts of pooled bee samples were analyzed by RT-PCR for the presence of six to eight known bee viruses (ABPV, IAPV, KBV, CBPV, SBV, DWV, VDV-1, and BQCV). Diagnostic results were then related either to the health status of the sampled colonies or to incidences of large scale colony losses in the sampled region. These studies substantiated the observation that covert virus infections are wide-spread and common in asymptomatic colonies [29–31]. At the same time they provided correlative evidence for

members of the ABPV/KBV/IAPV and the DWV/VDV-1 clade being involved in colony losses, an interpretation that was later validated by studies which have improved on these previous designs by increasing the length of the sampling period [16,40], improving screening precision by analyzing specific tissues [16,47], or even using advanced genomic techniques like next generation sequencing (NGS) or microarrays for detection of novel honey bee pathogens [25●,26●●,40,46●,47].

At least two long term studies provided strong correlative evidence for IAPV and ABPV (both are members of the ABPV/KBV/IAPV clade) being involved in winter colony losses in the U.S. and Germany, respectively, [16,40] and DWV being a key factor for overwintering colony losses in Germany [16]. A recent survey of a large-scale migratory beekeeping operation in the U.S. identified for the first time a strain of aphid lethal paralysis virus (ALPV strain Brookings, *Dicistroviridae*) as a putative honey bee pathogenic virus (Table 1). However, its relevance to colony losses needs further investigation. [25●]. In addition, three novel viruses, Big Sioux river virus (BSRV), Lake Sinai virus 1 (LSV1), and Lake Sinai virus 2 (LSV2) were identified (Table 1). BSRV is a novel dicistrovirus, similar to the aphid-pathogenic virus *Rhopalosiphum padi* virus (RhPV) but sufficiently divergent to justify a new species. LSV1 and LSV2 are two novel RNA viruses showing some similarity to CBPV and presumably belonging to the *Nodavirales* superfamily. Based on the ability of these viruses to replicate in adult honey bees and their abundance compared to other significant honey bee viruses, the authors suggested that they may play a significant role in colony health (Figure 1). A comparative study on pathogen loads of CCD and non-CCD colonies revealed additional LSV variants and suggested a potential association between LSV strain and CCD status [47]. However, the role of LSV or certain LSV variants in colony losses still needs to be established (Figure 1).

The occurrence of ALPV, LSV1, and LSV2 in the honey bee population is obviously not restricted to the U.S., as similar viruses were also identified in a Spanish honey bee colony [46●]. Most recently, two independent studies reported Tobacco ring spot virus (TRSV) and Turnip ring spot virus (TuRSV), two plant pathogenic viruses of the family *Secoviridae*, to be associated with honey bees [26●●,46●]. TuRSV was proposed to be passively present in bees due to the ingestion of contaminated pollen [46●].

However, TRSV was shown to replicate in nearly every bee tissue and elevated virus levels were found in weak colonies [26 ●] (Table 1). This novel and surprising finding of TRSV replicating in honey bees caused some debate [48,49] although this is not the first example of a plant virus replicating in both plants and insects. So far, such viruses have been described from the RNA virus families *Bunyaviridae*, *Nodaviridae*, and *Rhabdoviridae* which are vectored by insects via persistent propagative transmission mode [50,51,52●,53]. However, TRSV is a member of the genus *Nepovirus* which is not transmitted through insects but through pollen [54]. It is long since known that honey bees mediate transmission of pollen-borne plant pathogenic viruses between infected and non-infected plants [55,56]. Hence, pollen collecting honey bees are exposed to plant viruses and it is conceivable that especially honey bee larvae fed with plant virus contaminated pollen might get infected and might then show infection in several tissues of the adult bee. The recent discovery of such an infection is likely for lack of looking for it. Hence, further investigations are necessary to unravel whether the TRSV–honey bee interaction is indeed another example of viral host range spanning the plant and animal kingdom and if so, it would be worthwhile to search for other similar relationships.

Experimental evidence for virus infections causing death of bees by infection assays (positive evidence)

Surveillance studies are at a disadvantage compared to controlled exposure bioassays because the former can only generate correlative evidence, as opposed to the causal relationship demonstrated by the latter. During the infancy of honey bee virology, around 50 years ago, it was via experimental infection that scientists identified ABPV and CBPV as etiological agents of bee paralysis [57]. Additionally, the authors describe not only plasticity of symptomology depending on route of transmission (injection versus ingestion), but they also noted the existence of covert infections caused by these commonly lethal viruses [57,58]. While years following led to discovery of novel viruses, it was not until Varroa began infesting the European honey bee that we began to see their potential lethality when contributing to Parasitic Mite Syndrome (PMS) [59–65]. In fact, it was in Varroa infested colonies in the 1980s that symptomatic DWV infections were noticed for the first time (i.e. crippled wings), driving the identification of this typically asymptomatic virus. However, as a factor within PMS, DWV has quickly become tightly associated with colony collapse (Figure 1), and is therefore one of the most well-characterized honey bee viruses [28,66].

This unique relationship has led to quite a few controlled experimental infections of bees of varying ages with DWV in order to elucidate its mechanism of virulence and whether transmission route affects virulence. Indeed, it has been shown that mimicking mite-vectored trans-mission by injecting isolated DWV particles into virus-free white-eyed pupae, results in dose-dependent mortality of pupae and emergence of adults with deformed wings [67] which are also not viable [68]. Furthermore, it has been demonstrated that when a feeding Varroa mite is actively infected with DWV, and therefore acting as a biological vector, a bee is more likely to acquire a clinically significant DWV infection from the transmitting mite [69].

Transmitting DWV to adult bees via injection, thus mimicking vectoral DWV transmission by phoretic mites, resulted in a systemic infection which included the nervous system when at least 10E+08 virus particles were injected [67] which may lead to cognitive impairment and learning deficits [70]. It is not clear, however, whether this latter effect was due to the virus or other components that may have been present in the whole bee extract used in this study. Therefore, this link between covert DWV infection and cognitive malfunctioning has yet to be adequately demonstrated. Recent studies have demonstrated the role of Varroa and the route of transmission in the evolution of virulence in DWV.

In 2011, Moore and colleagues began to clear up just how Varroa was augmenting the clinical severity of DWV infections in honey bees when they demonstrated that only certain variants of the DWV sequence space pre-dominated in mite-infested colonies. Specifically, variants of the DWV quasi-species that had VDV-1 capsid proteins and DWV non-capsid proteins prevailed [71]. In 2014, Ryabov along with Moore and colleagues demonstrated that augmentation of DWV titers by Varroa mites is clearly due to facilitation of this specific virulent variant. A follow-up injection assay showed that it was the injection of the virus directly into the hemolymph, thus mimicking virus inoculation by the mite, that selected for this recombinant [72●●]. It is conceivable, then, that these virulent DWV variants may be the precipitating factor for PMS.

It is possible that even oral transmission of DWV plays a significant role in increased mortality of larvae/pupae and the emergence of deformed adults, though the study did not evaluate this as no dose information was provided [73●]. However, the role of oral transmission seems to be substantiated by the infection of mid-gut epithelium, particularly in mite infested colonies suffering from PMS, which is conceivably DWV/VDV-induced rather than mite-induced [37,67]. In fact, highly infected brood are preferentially cannibalized by workers, who then are likely to

become infected given that the pupae being cannibalized have even higher viral titers than deformed adults [74 ●●,75]. Despite the restriction of infection in the gut to the mid-gut epithelium [67], the threat that cannibalism of infected pupae poses to the adult must be further evaluated.

For members of the ABPV clade, especially for IAPV and ABPV, good correlative evidence exist that they are involved in large scale colony losses [40] or winter losses [16] (Figure 1). Like DWV, these viruses are also effectively transmitted by Varroa [59,60,76] (Figure 1). ABPV was first isolated and described 50 years ago after adult bees were observed darkening in pigment following infection with some unknown agent [57]. We now know that IAPV, first described in 2007, causes similar symptoms. However, it was also shown that IAPV-injected adult bees die within 4 days, while oral transmission resulted in death after only 10 days [77]. Two recent studies [78 ●●,79 ●] aimed at a better and more detailed understanding of how IAPV infections damage honey bees.

Pupae injected with 10E+04 genome equivalents of IAPV showed a heterogeneous pattern of symptoms. Compared to controls (PBS (phosphate buffered saline) injected), IAPV injected pupae either stopped developing without any further symptoms or showed darkening of body parts [78●●]. It is uncertain however whether the darkening is due to virus induced tissue damage and necrosis, or melanization of tissue as a result of host immune response. Remarkably, studies suggest that heterogeneous pathology is dependent not just on IAPV strain diversity, but also on colony- dependent or patriline-dependent differences in susceptibility of the bees to IAPV infection [77,80●●]. As with DWV, IAPV has been found in healthy colonies despite being associated also with collapsing colonies [40].

Honey bee colonies might not only suffer from collapse when mortality rates of brood and adult bees increase due to virus infection but also when virus infections negatively affect bee behavior and, thus, colony performance. This aspect has been studied in adult forager bees injected with as little as 44 copies of IAPV and equipped with radio frequency identification (RFID) tags. While no mortality was observed, homing ability was significantly reduced at 2 and 3 days post-infection. Indeed, only between 2.3% and 0% of IAPV infected foragers found their way back, compared to 50% of those injected with PBS [79 ●]. It is reasonable to suspect that a considerable proportion of foragers not returning to the hive may have detrimental effects on the colony and contribute to weakening and eventually collapse of the entire colony.

Experimental evidence for the impact of viruses on honey bees by treatment with dsRNA (negative evidence)

RNA interference (RNAi) and Dicer, a multi-domain enzyme with RNase III activity, are key regulators in invertebrate antiviral immunity and are crucial for sup-pressing viruses producing double-stranded RNA (dsRNA) intermediates. Dicer acts as a sensor of viral dsRNA. After binding and cleaving of virus-derived dsRNA molecules into siRNAs, it delivers these siRNAs to the host's RISC complex where they then can target viral RNA for inhibition [81]. Therefore, in honey bee virology RNAi approaches with experimentally delivered virus-specific dsRNAs can be used to investigate the relation between certain viruses and symptoms observed in individual bees and bee colonies.

There is only one study applying RNAi approaches to DWV infected bees. Honey bee larvae orally infected in laboratory experiments with high enough DWV titers either died during larval

development or developed into adult bees exhibiting wing deformities [73●]. Preventive feeding of DWV-dsRNA revealed a more than 300-fold reduction in DWV levels in adult bees (21 days post egg hatching and infection) relative to bees developed from larvae that were fed with virus or with virus and GFP-dsRNA. In addition, significantly reduced frequencies of wing deformities relative to other virus-fed treatments were observed [73 ●] confirming that DWV is the etiological agent of the deformed wing syndrome.

For IAPV infections, data obtained through RNAi approaches at colony level are available. Maori and co-workers [82] used queen-right mini colonies with approximately 200 worker bees and experimentally infected these colonies by feeding IAPV in sucrose solution. By day 8 post infection, bee mortality in the non-infected control group was approximately 25% while around 80% of IAPV-infected bees had died. Preventive feeding of IAPV-specific dsRNA preparations (calculated as approximately 1 mg per bee) for three days prior to virus infection significantly reduced bee mortality in IAPV-infected bees to a level not significantly different from control mortality. Hence, ingesting IAPV-dsRNA obviously triggered an antiviral immune response in honey bees and prevented the bees from succumbing to a subsequently initiated IAPV-infection under laboratory conditions. This promising laboratory effect was then evaluated under natural bee keeping conditions using a total of 160 bee hives in Florida (USA) and Pennsylvania (USA) [83]. However, no significant correlation between in-creased colony or individual mortality and IAPV infection was observed in this study and, hence, it was difficult to conclusively assess the effect of dsRNA treatment on IAPV infection and colony survival. However, indirect measures suggested that IAPV-dsRNA treatment had some positive effect at the colony level.

A more recent study [80●●] suggested the existence of an IAPV-encoded viral suppressor of RNAi (VSR). In laboratory experiments with IAPV-infected caged bees it was demonstrated that

silencing this putative viral interference protein via RNAi through feeding of VSR-specific siRNA resulted in a remarkable reduction in IAPV replication within 24 hours post treatment. The authors concluded that the studies on IAPV inhibition through RNAi rein- force the therapeutic potential of carefully designed siR- NAs for treatment of viral infections in honey bees.

Hence, the laboratory studies so far implied that there is a curative effect of virus-specific dsRNA on the severity and outcome of infections with the corresponding virus. However, a more recent study demonstrated that any dsRNA might serve as a viral pathogen associated molecular pattern (PAMP) in honey bees triggering an antiviral response that controls virus infection in general [84 ●●]. Therefore, approaches using virus-specific dsRNAs might not be suitable to prove the role of a specific virus or viral protein in a certain context but rather the involvement of viruses in a more general sense. Furthermore, the use of dsRNA as a management strategy for viruses in the field might rather not help to control a specific virus but may activate a nonspecific antiviral immunity resulting in a general positive effect at the colony level as observed by Hunter and co-workers [83].

Impact of V. destructor

It is without question that Varroa infestation poses the most serious threat to the Western honey bee colonies and that this is related to the mite's ability to vector virus infections or to exacerbate preexisting infections [39]. Virus infections of honey bees became a serious health problem for entire colonies only after Varroa started to infest honey bee colonies. Varroa theoretically can take up any virus present in bee hemolymph and, hence, can mechanically vector any virus back

into the hemolymph when feeding on the next bee or pupa. Therefore, it is not surprising that since the introduction of Varroa the prevalence of honey bee viruses and infections in-creased. However, for most viruses, conclusive evidence linking them to colony losses is still lacking despite the nearly ubiquitous presence of the virus vector Varroa in the honey bee population. Only members of the ABPV clade and the DWV clade are thought to play a major role in colony losses in the presence of mite infestation.

Overt DWV infections, the development of the deformed wing syndrome, and colony collapse due to the PMS are closely associated with the vectoral transmission of DWV by Varroa (Figure 1). It is widely accepted by now that Varroa serves as a biological vector of DWV, and thereby plays a crucial role in the virulence of DWV. The selection of an especially virulent variant of DWV through Varroa has been anticipated and discussed for some time. Primarily, because it became obvious that DWV replication in the mite is necessary and sufficient for enhanced virulence of DWV as well as the development of de-formed wings [69,85]. A recent study in Hawaii found that the introduction of Varroa to a naive population greatly reduced the strain diversity of DWV while increasing the prevalence of infection [86]. These results supported the hypothesis that Varroa facilitates the dominance of certain strains which might be associated with increased viral virulence. Some hallmarks of such a virulent variant may have been identified recently [71,72●●]. The described variants were characterized by the capsid protein coding region of VDV-1 (a member of the DWV clade originally isolated as virus replicating in Varroa [87]) and the coding region for the non-structural proteins from DWV. How-ever, the question of whether this variant was selected by amplification in the mite or by transmission route was not fully solved. Both of these studies further our understanding of the molecular mechanisms that underlie the in-creased virulence of DWV in the presence of Varroa.

Although ABPV and its close relatives are also associated with Varroa and colony losses, a similarly fatal triangular relationship between virus, Varroa, and honey bees could not develop. Members of the ABPV clade, when injected into pupae by mites, rapidly kill the infected pupa there-by preventing the developing bee from emerging and interrupting the reproduction of the mite which is trapped in the capped cell. Therefore, although mite transmitted ABPV/IAPV/KBV infections are lethal and contribute to colony losses (Figure 1), mite transmission of this virus clade is a dead-end hampering any further virulence evolution [38].

Conclusion

This review is not an exhaustive review on colony losses but rather collects on different levels recent evidence for the involvement of viruses in colony losses (Figure 1). Hence, we did not include all factors involved in the collapse of honey bee colonies such as non-viral patho-gens, metazoan parasites other than Varroa, pesticides, malnutrition, climate, and beekeeping practice although we are aware of the relevance of these factors. It is important to point out, that THE colony loss or THE honey bee decline does not exist. Instead, the phenome- non of colony losses, although globally observed and reported, has different dimensions, reasons, and key players varying by region and time. It is also important to note that large scale colony losses are not happening for the first time but rather similar losses have been described in the historical past. This recurrence far from detracts from the importance of studying honey bee health, but rather increases the interest from the perspective of not only agriculture, but also basic science and ecology. Indeed, this is the first time in history that honey bee colony losses have attracted so much attention which has in turn boosted scientific interest and research in the field of honey bee

diseases. Much of this research is interesting in its broader applicability in terms of potential control strategies for viral infections.

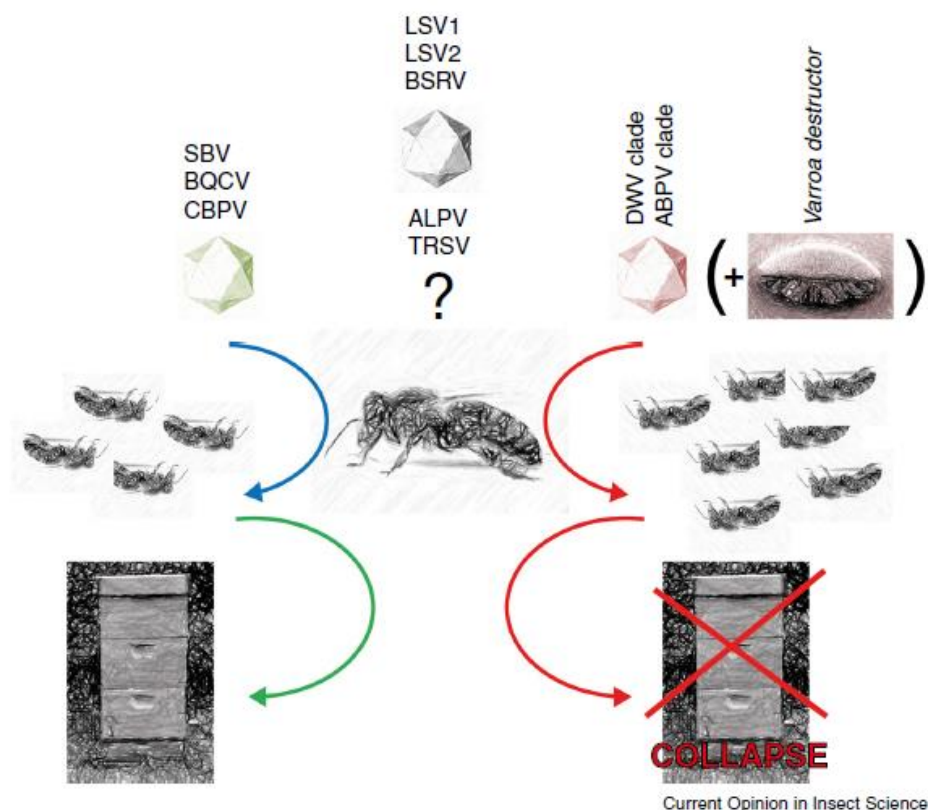

Figure 1-1. Honey Bees and Their Associated Viruses

Viruses affecting honey bees. Till date, 23 viruses have been found to infect honey bees. Although most bee viruses cause covert infections, some viruses do cause visible symptoms and death of individual bees (e.g. SBV, BQCV, and DWV). However, colony losses due to these viruses are rather rare. Members of the DWV and ABPV clade not only kill individual bees but have been related to the collapse of entire colonies, especially in the presence of the ectoparasitic mite Varroa. Recently, three novel bee viruses were discovered (LSV1, LSV2, and BSRV) and two known viruses were described for the first time to infect honey bees (ALPV, TRSV). The exact impact of these five viruses on individuals and colonies is still elusive (illustrated by the question mark) and awaits final experimental proof or disproof.

Table 1-1 Common and emerging viruses of the honey bee.
This list gives a summary of the most common (designated with *) and recently emerging (designated with ») honey bee viruses, whether they are vectored by Varroa, and their symptoms. If Varroa has been shown to be a significant biological vector, the virus was designated with (+++). If the virus is frequently associated with Varroa but the mite has not been determined to be a biological vector, the vector status was designated with (++). Finally, if the virus is sporadically associated with tissues of the mite, or the vectoring status is in question but possible, it was designated with (+). The final column is a reference where more details can be found about the virus, including symptomology, the evidence of its association with Varroa, prevalence data, association with colony collapse, among other things.

Virus	Family	*Varroa* Vector Status	Symptoms of Overt Infection	Reference
*ABPV clade	*Dicistroviridae*	++	Paralysis Darkened cuticle pigment Impaired cognition and homing ability Mortality (adult and immature bees) Colony collapse	[77,82]
» ALPV	*Dicistroviridae*	Unknown	Unknown	[25]
*BQCV	*Dicistroviridae*	+	Pale-yellowish, leathery cuticle of capped larva Failure of larva to pupate Sac-like appearance Mortality (of larvae) Deceased larvae and walls of cell turn black	[24]
» BRSV	*Dicistroviridae*	Unknown	Unknown	[25]
*DWV clade	*Iflaviridae*	+++	Deformed wings Learning deficits Discoloring Shortened and bloated abdomens Mortality (adult and immature bees) Colony collapse	[37]
» LSV1/2	*Nodaviridae*	Unknown	Unknown	[25]
*SBV	*Picornavirales* (super family)	++	Pale-yellowish, leathery cuticle of capped larva Failure of larva to pupate Sac-like appearance Mortality (potentially of adults, certainly capped larvae) Dead larva becomes dark, brittle scale	[24]
» TRSV	*Secoviridae*	+	Winter colony collapse?? (correlative only!!)	[26]

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Chapter 2
The impact of hive type on the behavior and health of honey bee colonies (*Apis mellifera scutellata*) in Kenya

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Summary/Abstract:

Honey bees provide critical pollination services, income, and nutrition for rural Kenyans. There has been a long-standing interest in developing approaches to maximize honey production by Kenyan beekeepers. Since honey bees in Kenya are loosely managed (hives are colonized by migrating honey bee swarms, and colonies readily abandon their hives, or abscond), the primary management decision of beekeepers is the type of hive they use. Kenyan beekeepers primarily have access to three types of hives: traditional Log hives with fixed combs, Western Langstroth hives with moveable combs fixed in frames, and Kenyan top bar hives with moveable combs fixed to top bars. Here, we evaluated the effect of hive type on hive attractiveness to migrating swarms, colony health in terms of *Varroa* mite levels and prevalence of viruses, and the likelihood that the colony absconds. We found significant differences in hive attractiveness among the different hive types, possibly due to differences in the cavity size. Pathogen and parasite loads were correlated with colony age, but not hive type. Absconding rates were associated only with colony size: larger colonies were less likely to abscond. Given the differences in cost, replacement rates, equipment and training among the hive types, we recommend that a redesigned Kenyan Top Bar hive with a starting volume of 40L and a moveable separation board may be the best solution for Kenyan beekeepers, though a redesigned hive would need to be similarly evaluated. Furthermore, placing apiaries in

locations with abundant floral resources may reduce absconding rates, though absconding should not be completely eliminated since periodic breaks in brood production may serve as a natural mechanism for controlling parasite and pathogen loads.

Keywords (10):

Africa, health, honey production, management practices, parasite, pathogen, rural beekeeping, absconding

Short title (running head): Management of Kenyan honey bees

Introduction

Honey bees in Kenya provide critical pollination services, nutrition, and income for smallholder farmers and rural families. Honey bees and other pollinators contribute USD $3.2 million in ecosystems services to several major vegetable, fruit and nut crops in western Kenya alone (Kasina et al. 2009). Honey produced by both honey bees (*Apis mellifera*) and several stingless bee species provides nutrition (particularly during times of drought) and income for many East Africans and has important cultural and medicinal value (Macharia et al. 2010;; National Farmers Information Service of Kenya). However, Kenyan beekeepers report a much lower average honey production per hive than US beekeepers (9.3 kg/hive in Kenya vs 25.67 kg/hive in the US (National Agricultural Statistics Service 2014; National Beekeeping Station 2007). Many factors differ between beekeeping in the US and Africa, including the subspecies of bees (Hepburn & Radloff 1998; Ruttner 1987), the climate, the prevalence of honey bee parasites and pathogens (Muli et al. 2014) and the management practices (Crane 1999; Mbae 1999). In Kenya, as in most of East Africa, three hive styles are typically used by beekeepers: traditional log hives which have fixed combs as in a wild colony; Langstroth hives, which are the typical Western-style hive with movable-frames; and the Kenyan top bar hive (KTBH) which employs movable top bars rather than frames (Adjare 1990). The choice of hive type is the primary management decision made by the majority of African beekeepers: empty hives are occupied by migrating feral honey bee swarms, and beekeepers typically only disturb colonies at the time of honey collection, after which colonies often abscond, leaving the hive and migrating to a new location (Crane 1999; Mbae 1999). In this study,

we evaluated whether hive type affected occupation rates by feral swarms, colony health, and absconding rates across the wet and dry seasons.

The three hive types vary in structure, size, cost, and the amount of beekeeping expertise needed to construct and manage these hives (Figure 1, reviewed in Adjare, 1990). The log hive is the style traditionally and currently used by most Kenyan beekeepers; these are made of hollowed out logs and can vary greatly in size, volume and weight. Log hives are normally hung high in trees out of reach of predators. They are also the cheapest of the three hive types, typically ranging in price from USD$4-10 depending on their size and quality and can last 20 years or longer. Langstroth hives, either imported or locally-made, are approximately 40 liters in volume and are the most costly of the three styles at USD$50-70 and last typically 7-8 years. These hives require the most skill to construct and manage, and have been optimized for European honey bee stocks. The Kenyan Top Bar Hive (KTBH) was invented in1965 by C.J. Tredwell and Peter Paterson at Hampshire College of Agriculture and subsequently improved by G.F. Townsend in 1976 (Crane 1999). Their goal was to develop a hive that would serve as an intermediate approach to beekeeping for rural Kenyans with limited resources. The KTBH integrates elements of the traditional log hive, such as the shape and size, with the moveable comb feature of the Langstroth hive (Gentry 1982). However, it uses top bars rather than frames, which are cut to the width of the hive and provide the proper bee space between combs allowing them to be manipulated by the beekeeper for inspection and honey harvesting. These hives are usually50-55 liters in volume, cost approximately USD$40 and typically last about 7-8 years in Kenya (see methods).

There has long been discussion of whether the reduced honey yield in Kenyan beekeeping operations is due to a difference in management practices. However, attempts to improve the livelihoods of rural people in East Africa through beekeeping development projects have had limited success for more than 50 years. These projects primarily involve the introduction of Western-designed equipment (Langstroth and KTB hives) where otherwise beekeepers would use only traditional Log hives. Recommendations from government agencies and extension personnel, non-profit development groups from the West, and academic researchers all endorse the opinion that Kenyan beekeeping would benefit from adoption of modern moveable-comb hives (Adjare 1990; Ntenga & Mgongo 1991; UNDP-Kenya 2008; Wilson 2006). However, locally produced Langstroth hives are often of poor construction and beekeepers attempting to adopt them often receive little instruction. This has resulted in poor performance and abandonment (Muli et. al in review). Furthermore, the high cost of Langstroth and KTB hives puts them beyond the reach of most rural Kenyans. Indeed, of the reported hives, 96% are log hives (Hive Population and Production in Kenya, 2005, 2006, and 2007, Provincial Summaries).

Additionally, the type of hive might influence the health and behavior of the colony, and therefore its size and productivity. There is anecdotal evidence to suggest that moveable frame and/or moveable comb hives are less attractive to migrating swarms (E. Muli and M. Frazier, unpublished). Furthermore, bees in log hives with rough interiors are more likely to coat the walls with propolis, which has known antimicrobial properties and may reduce pathogen loads (Simone et al. 2009; Simone-Finstrom et al. 2010; Simone-

Finstrom & Spivak 2012). Colony size may also be influenced by the hive type, which could affect honey production and disease spread. Finally, absconding behavior (when the colonies cease brood rearing, consume all food stores, and abandon the hive) is common in Africa, and is typically triggered by disturbance and reduced nutritional resources (Grozinger et al. 2014). Hive type may also influence absconding behavior, thereby reducing the honey yields available to beekeepers. In this study, we test the hypotheses that hive type affects (1) colonization rates by migrating swarms, (2) hive health, including viral infection prevalence and *Varroa* loads and (3) absconding rates of established colonies.

Materials and Methods

Apiary Establishment

In October 2012, three apiaries (A, B and C) were established on the South Eastern Kenya University (SEKU) campus located 40 kilometers west of Kitui and 155 kilometers east of Nairobi, Kenya. The apiaries were within 2.5 kilometers of each other and each was surrounded by a sturdy wire fence approximately 2.5 meters in height for protection against predators such as honey badgers. In total, 25 Langstroth (Lan) hives, 25 Traditional Log (Log) hives, and 25 Kenyan Top Bar hives (KTBH) were distributed among the three apiaries and left to be occupied by migrating swarms, which typically move into the area during the short rains in October and November. All hives were

locally constructed in Kenya. Langstroth hives with a volume of 40 L were obtained from African Beekeepers Limited (Nairobi, Kenya) at a cost of Ksh. 4,500 (US $48.80, conversions done on March 15, 2015) per hive. They were outfitted with frames with starter strips of beeswax foundation, rather than full sheets of foundation, which is typical for hives of this type used in Kenya. KTBH with an approximate volume of 52.5 L were obtained from the International Centre of Insect Physiology and Ecology (*icipe)* at a cost of Ksh. 3,500 (US $37.96) per hive. A small amount of beeswax was applied to all top bars. Applying beeswax to frames and top bars serves to make the hives more attractive to passing swarms and to provide the bees with "guidance" on where to construct combs in order to minimize the building of combs across frames and top bars, which defeats the movable frame/comb advantage of these hives. Log hives were constructed by and purchased from Mulwa Mbithi, a beekeeper in Ukasi – Mwingi at a cost of Ksh. 1000 (US $10.84) per hive. They ranged in volume from 25 to 64 liters. The average volume of the log hives per apiary was 45±3.94L, 42±3.4L, 39±3.L, for apiaries A, B and C respectively. There was no significant difference in average log volume between apiaries (ANOVA, F-ratio=0.6135, p=0.5504). The interior of the log hives was rubbed with a ball of heated beeswax and propolis and smoked with the wood of 'Mutanga' (Kamba) as per traditional practices. See Figure 1 for more details about the hive types. Eight hives of each type were randomly placed within each apiary with the exception of one additional of each hive type placed in each apiary (apiary A had one additional Langstroth, B had one additional Kenyan Top Bar and C had one additional log hive). At the beginning of the study, an automatic weather station was established at the South Eastern Kenya University (SEKU). The HOBO U30-NRC Weather Station (Onset Computer, Bourne,

MA) recorded the atmospheric pressure, rainfall, temperature and relative humidity. Data was recorded per minute and later converted to daily maximum values.

Collection of Colony Parameters and Samples

All apiaries and hives were inspected weekly throughout the study to document hive occupation and absconding of previously established colonies. Routine data collection of established colonies began in early December 2012, and was only gathered on colonies that had been established for at least two weeks in order to minimize absconding due to disturbance. Data collected on occupied hives included weight, the number of occupied frames (Lan and KTBH only), nest area (taken for colonies following abscondance), and the presence and quantity of *Varroa* mites. *Varroa* mites were assessed using a standard sugar roll assay described in (Ellis & Macedo 2001), using the standard half-cup to collect approximately 350 bees. Additionally, for each established colony, foragers were collected as they returned to the hive entrance with visible pollen loads. In total, 30 foragers/colony were collected on ice, stored at -20°C during field collections, and sent to the Center for Pollinator Research (Pennsylvania State University, University Park, PA) for detection of viruses. Samples were stored in individual 2 mL cryogenic vials (VWR, Radnor, PA) in RNAlater (Life Technologies, Carlsbad, CA) or 95% ethanol.

Screening for Viral Infections

The protocol for screening for viruses was similar to that described in Muli et al 2014, with some modifications. While Muli et al pooled 5 bees/colony, we pooled abdomens from 20 bees per colony to increase the probability that viruses with low prevalence would be detected. This would allow a 90% probability of detecting any virus with 10% prevalence, a 65% probability of detecting viruses with 5% prevalence, and only a 20% probability of detecting viruses with 1% prevalence (Pirk et al. 2013) for each colony, but the probability of detecting virus infections across treatment groups/apiaries would be substantially higher due to the replication across colonies. For samples collected in April from Apiary A and C, abdomens were homogenized in 600 µL of TRI Reagent ® (Sigma-Aldrich, St. Louis, MI) using eight 2.0 mm zirconia beads and a Fastprep instrument (Qbiogene, Montreal, Quebec) for 3 cycles at max speed for 45 seconds each. Samples extracted with TRI Reagent ® were very difficult to clean, so the remaining samples (Apiary B, April collections, and all apiaries for June collections) were homogenized instead in 600 uL QIAzol (Qiagen, Valencia, CA) lysis buffer. Whole RNA was extracted from the homogenate for viral detection as per the manufacturer's instructions for TRI Reagent or QIAzol. Following extraction, purified RNA was suspended in 30 µL of sterile deionized H_2O. RNA concentration and purity was measured spectrophotometrically on a NanoDrop 1000 (Thermo Scientific, Waltham, MA).

cDNA was synthesized using random hexamers and Superscript III ® reverse transcriptase kit (Life Technologies, Carlsbad, CA) in 15 µL reactions using 200 ng of

RNA per reaction. The presence of eight viruses common in US and European honey bee populations (see Table 1 for viruses screened and the primers used) was tested with specific primers (Life Technologies) and REDtaq ® supermix (Sigma-Aldrich). A touchdown procedure was used for the RT-PCR to maximize primer specificity and account for slight differences in melting temperatures of primer pairs. The reactions were run on a Mastercycler Pro (Eppendorf, Hamburg) using the following program: 48°C for 45 min for reverse transcription; *Step 1:* 95°C for 2 min; *Step 2:* 10 cycles at 95°C for 30s, 65°C with a 1°C stepdown per cycle to 55°C for 1 minute, followed by 68°C for 2 minutes; *Step 3:* 30 cycles at 95°C for 30 s, 57°C for 1 min, and 68°C for 2 min; and *Step 4:* finally the reaction was held at 68°C for 7 min. This program is a slight modification of the program tested and confirmed by Chen and colleagues (Chen et al. 2006). Water controls and positive controls for ABPV, BQCV, DWV, IAPV, and VDV (positive samples for CBPV, KBV or SBV were not available) were included in each RT-PCR run. Reactions were imaged on 1% agarose gels using the Gel Doc™ XR+ System (Bio Rad, Hercules, CA).

<u>Statistical Analyses</u>

All statistical analyses were performed using JMP ® Pro 10 (SAS, Cary, North Carolina). Data points were determined to be outliers if they were more than 3.5 standard deviations from the mean.

Hive Occupation: Significant differences in the rate of occupation of hives by hive type was determined by survival analysis, where the rate of some event occurring (the ‘death’,

here it is hive occupation) is compared across groups. Log-rank tests were used make pairwise comparisons of the survival distributions of the occupation of the hive types. Survival analyses according to hive types were performed for all three apiaries pooled (Figure 2) and for each apiary separately (Figure S1). Our threshold for statistical significance for this set of data is p=0.017 as per the calculation of the Bonferroni threshold (k=3, threshold for whole model is p=0.05) for multiple comparisons of survival curves.

Infection Prevalence: To determine if season impacted the presence of viral infection, Pearson's Chi-square was used to test whether infection prevalence differed between June and April for all pooled data. A 2-sample test for proportions was used to determine whether infection rates were affected by season within apiaries and across hive types.

Absconding Rates: The number of occupied frames was first transformed by taking the Log_{10} of the count data. Then, the transformed data were subjected to a Wilcoxon Rank-sum with an approximation of a Chi-square distribution. To further gain insight into this relationship, a generalized linear model was constructed using a logit link function, with the log10 of the number of occupied frames as the predictor and the binomial variable (yes or no) "absconding" as the response variable

Since absconding peaked just after June, all hives screened in June were chosen for further analysis of the effect of viral infection and *Varroa* parasitization on absconding behavior. First, the absconding data were subjected to a two-sample test for proportions in order to test for a relationship between infection with more than one virus and absconding status, given that every hive had at least one virus. Secondly, mite count data

were log-transformed and tested for a relationship with absconding status with a Wilcoxon Rank-sums test. Further, the effect of location on absconding rates was assessed using a Pearson's Chi-square.

Results

Effect of hive type and apiary location on hive occupation

Based on temperate and rainfall patterns, it was possible to distinguish four seasons during the course of this study (see Figure 3). These seasons could be classified as high temperature and no rainfall (January and February), high temperature and very high rainfall (March and April), lower temperature and low rainfall (May, June and July), high temperature and no rainfall (August-September), lower temperature and low rainfall/short rains (October, November and December).

Though hives were placed in apiaries in October 2012, there was a marked increase in occupation of all hives in early April 2013 (Figure 2, around day 135 post hive establishment) corresponding directly with the middle of the reproductive swarming season (March-May) in Kitui region, Kenya. By 141 days after the hives were set out in the apiaries, corresponding to 9 April 2013, all Langstroth hives were occupied by swarms; therefore this date was chosen as the end-point for the analysis of occupation rates. At this end-point, 20 (80%) of all Log hives were occupied and only 4 (16%) of the KTB hives were occupied. Pair-wise comparisons confirmed that Langstroth Hives were

occupied most quickly, followed by Log hives, and lastly Kenyan Top Bar hives (Survival, Log-rank: Lan vs Log $p<0.0027$, Lan vs KTB $p<0.001$, and Log vs KTB $p<0.001$).

The same analysis performed on each apiary resulted in similar significant differences among hive types within each apiary, with the exception of Apiary C, where there was no observable difference in preference for Langstroth and Log hives (Figure S1). There were no significant differences in occupation rates between apiaries for individual hive types (Lan hives , Log-Rank, $p=0.5507$, $\chi^2=1.19$, DF=2; KTB hives, Log-Rank, $p=0.6065$, $\chi^2=1.0$ DF=2; Log hives, Log-Rank, $p=0.7166$, $\chi^2=0.666$, DF=2).

Effect of season, apiary and hive type on viral infection prevalence

April was chosen as a representative month for the first wet season the colonies experienced, while June corresponded to the first dry season that the colonies experienced. All colonies were tested for the presence of eight viruses in April and June to determine if there was a seasonal relationship for viral prevalence (see Table 1 for a listing of viruses, and Table S1 for a listing of virus infections in each colony at each time point).

In April, BQCV was the most prevalent virus, as it was found in 48.38% of infected colonies. However, we also detected one colony infected with DWV, one colony infected with SBV, and four colonies infected with VDV in April. In June, DWV was the most

prevalent with 97.5% of all colonies infected, followed by 52.5% of colonies having BQCV, while one colony was infected with ABPV and five colonies were infected with VDV (see Table S1). In no instances did we detect CBPV, KBV or IAPV.

In April, there was moderate viral infection prevalence with 50.4% of all colonies having at least one virus. In June there was a significant increase in viral infection prevalence, with 100% of colonies having at least one virus (Pearson's Chi-square, χ^2=22.501, DF=1, $p<0.0001$).

When comparing across the three apiaries, Apiary C had the lowest number of infected colonies in April, but 100% of the colonies in Apiary C were infected by June. Within each individual apiary, there was an increase in infection rates from April to June, but this was only significant for Apiary C (2-sample test proportions, Apiary A, p=0.2459; Apiary B, p=0.0823; Apiary C, $p<0.001$).

When comparing the three different hive types, there was also a significant increase in infection rates from April to June for both the Langstroth and Log hives, but the difference were not significant for the Kenyan Top Bar hives, likely due to the small sample size (Figure 4; 2-Sample Test for Proportions, DF=1; KTB p=0.2073, Lan p=0.0007, Log p=0.0007). However, there was no difference in infection rates by hive type for April in terms of number of infected colonies (Pearson's Chi-square, p=0.5885, χ^2=1.061) or numbers of viruses/colony (Pearson's Chi-square, p =0.584, χ^2=2.845). For June, all hives had at least one virus, and there was no difference in infection rates by hive type in terms of the number of viruses (Pearson's Chi-square, p=0.7074, χ^2=2.154).

***Varroa* loads**

Average mite loads increased linearly with time (Figure 5: Least Square Means, R^2=0.2258, p=0.0108). A proxy for size (occupied number of frames) does not scale linearly with mite loads alone (Log10 OccFrames: DF=1, F ratio = 0.0504, p=0.8231) but adding it as an additional predictor improves the first model by reducing the error in the model (RMSE without Log10 OccFrames = 0.352, RMSE with=0.347) without introducing redundant information or co-linearity into the model. The levels of *Varroa* in June were not dependent on hive type (Wilcoxon Rank Sums, p=0.349, χ^2=2.1, DF=2) or apiary (Wilcoxon Rank Sums, p=0.2305, χ^2=2.94, DF=2).

In contrast to recent studies in North America (Francis et al. 2013), New Zealand (Mondet et al. 2014), Hawaii (Martin et al. 2012), South Africa (Martin & Kryger 2001) and Kenya (Muli et al. 2014) there was no relationship observed between *Varroa* loads and the viral diversity (number of viruses) found in colonies during April (Figure S2, Regression, ANOVA, p=0.3949) or June (Regression, ANOVA, p=0.228.).

Factors impacting absconding rates

There was a marked increase in absconding in July and August 2013, which corresponds to the hot, dry season in Kenya. We labeled colonies that absconded in July-September as "absconding colonies" and those that did not abscond as "remaining colonies". We then examined colony size, virus infection prevalence and *Varroa* levels of these colonies

in June (the start of the dry season) to determine if there were any significant differences in these parameters between absconding and remaining colonies.

The relationship of hive size to absconding was assessed. Interestingly, remaining colonies had significantly more occupied frames in June (One-way Wilcoxon Rank Sums, Chi-square approximation, p=0.0175, χ^2=5.642, DF=1). Furthermore, constructing a Generalized Linear Model demonstrate that the number of occupied frames in June was a good predictor of whether a colony would abscond in July-September. The intercept term was significantly non-zero for both the GLM (p=0.0336, χ^2=4.51, parameter estimate=-3.52)

Additionally, the three apiaries exhibited significant differences in levels of absconding (Pearson's Chi-square, p=0.0122, χ^2=8.818). There were no differences in absconding between Apiaries A and B (Pearson's Chi-square, p=0.5839, χ^2=0.3) but, Apiary C had significantly higher absconding rate than either A or B (Pearson's Chi-square, p=0.044 and 0.0046, χ^2=4.055 and 8.04, respectively).

No other factors were correlated with absconding. Absconding and remaining colonies did not differ significantly in the number of viruses (Figure 6A; 2-Sample Test for Proportions, p=0.1716) or *Varroa* mite levels (Figure 6B; Wilcoxon Rank Sums, p=0.2363) in June. Including these variables in the GLM discussed above did not improve the model (data not shown).

Discussion

Determining which hive type is best for Kenyan beekeepers requires an assessment of the economic costs and benefits. Log hives are the least expensive option to purchase, and require the least amount of labor and training, while the Lan hives are the most expensive. Furthermore, Lan hives need to be replaced more frequently (7-8 years, compared to 20 for Log hives and 7-8 for KTB hives), again increasing the overall cost of these hives. In terms of honey production, when properly managed, KTB hives produce more honey than traditional hives in Ethiopia (Yirga & Teferi 2010) and Log hives in Kenya (Mulindo et al. 2008), while Lan hives are more productive than KTB hives (Beyene et al. 2015; Gebremedhn & Estifanos 2013), except, perhaps, in more arid regions (Mulindo et al. 2008). However, Lan hives require specialized equipment for honey extraction, which again increases the cost of these hives to the beekeeper. Therefore, if a beekeeper can afford the initial investment of the KTBH, the additional honey produced would likely far outweigh the cost differential. Given the importance of beekeeping to the nutrition, cultural life, and income of rural Kenyan households, it is vital that management practices are developed that optimize honey bee productivity and health while minimizing the labor and economic cost of beekeeping

Overall, our data indicate that Langstroth hives are most attractive to migrating swarms while KTB hives are least attractive, but otherwise there was no variation among the hive types in any of the other parameters we tested. Levels of *Varroa* mites and the prevalence of viral infections increased over time (from April to June) but did not differ

between hive types. Colonies were significantly less likely to abscond during the dry season if they had occupied more frames by June, and there was variation in absconding rates across location, but again, there was no effect of hive type.

Swarms preferred Langstroth hives, followed by Log hives and then the KTB hives (Figure 2). If we consider cavity volume (Lan hives were 40 L, Log hives averaged 45±3.94L, 42±3.4L, 39±3.L in Apiaries A, B, and C, respectively, and our KTB hives were approximately 52.5L), this is consistent with previous work indicating the swarming colonies of European honey bees prefer cavities of approximately 40L (Seeley & Morse 1976; Seeley & Morse 1978). We suggest that the KTBH may be the most economical route considering the intermediate cost and honey yield, after addressing two key issues: the volume, and thermoregulation. The utility and attractiveness of KTB hives would likely be greatly improved by reducing the cavity volume with a dividing board that may be moved to accommodate a growing colony. Alternatively, models with a queen excluder might be explored as Mulindo et al effectively improved the productivity of log hives this way(Mulindo et al. 2008). However, it has been reported that colonies housed in KTB hives may have difficultly thermoregulating and has been recommended that KTB hives be placed in shaded areas, and even in thatch huts (Gichora 2003). Alternatively, hive covers on KTB hives may be constructed out of materials and with varying configurations that improve colony thermoregulation, as explored in (Gichora 2003). In this study, it was found that modifying the cover by adding a soft timber insulation beneath the corrugated iron cover and painting the cover white significantly reduced the peak internal temperature of the hives. However, given that KTB hives in this

study frequently peaked at temperatures exceeding 35°C and even reaching 40°C, one should consider the climate before choosing to use KTB hives, as brood development occurs optimally at 35°C and wax melts at 40°C (Gichora 2003; Groh et al. 2004; Maier et al. 2003). It is also possible that reducing the cavity volume may also improve the thermoregulation by reducing the amount of air that needs to be moved, thereby reducing thermoregulatory stress; this is an area for future investigation.

Varroa loads and viral infection rates also correlated with season and/or colony age, but were not correlated with hive type or location (Figures 4 and 5). *Varroa* mites must develop in honey bee brood cells, and therefore mite production coincides with honey bee brood production. Previous studies have found that when more brood cells are available to a reproductive female, mite populations will steadily grow over time (Boot et al. 1994; Calis et al. 1999; Fuchs & Langenbach 1989). Thus, by causing a break in the brood cycle, absconding and relocating to a new hive may serve as a natural limit to *Varroa* infestation. Similarly, beekeepers in the US are encouraged to break a colony's brood cycle by either caging the queen or even re-queening if a colony is heavily infested (Hood 2000; Ruppert 2011). Thus, allowing Kenyan honey bee colonies to abscond and migrate may help reduce the loads of *Varroa* mites and other pathogens in honey bee populations. Interestingly, a recent study suggests that acaracide treatments, which can be quite expensive, are minimally beneficial in South African *A. mellifera scutellata* colonies (Strauss et al. 2015), and thus miticide treatments should not be encouraged.

The likelihood that a colony will abscond during a period of reduced forage appears to be influenced primarily by colony size rather than infection levels or the hive type. Larger

colonies were significantly less likely to abscond. These results are consistent with the findings of Winston and colleagues for Africanized honey bee stocks in French Guiana, South America. They found that colonies dealt with reduced forage by either hoarding honey (the typical European honey bee response) or absconding. In their study, Africanized colonies with fewer stored resources were more likely to respond to a reduced nectar flow by absconding (Winston et al. 1979). Interestingly, Winston et al also found that feeding colonies with sugar water was not a viable strategy for preventing absconding. They hypothesize that the more important factor in the hive's decision to abscond is likely nectar flow as opposed to honey stored (Winston 1993). In contrast, Schneider and McNally found that absconding/migrating colonies of *A. mellifera scutellata* in Botswana had larger food stores and population sizes than non-migrating colonies, 4-6 weeks prior to starting migration preparation (Schneider & McNally 1992). It is possible that the differences between the studies is due to the timing of the colony assessments: perhaps the larger colonies prepared for absconding/migration early by reducing brood production (in preparation for migration, queens will cease egg-laying, reviewed in Grozinger et al 2014), which would result in a smaller colony size at a later timepoint. While it is possible that we failed to observe migration preparation, this is unlikely since the data on colony size were collected at least 4 weeks prior to most of the abscondances. Thus, based on our results and Winston 1979, beekeepers should be able to reduce absconding due to limited resources by placing apiaries in areas with sufficient floral resources and nutrition. However, again, colonies should still be allowed to abscond in response to other stressors like parasites, pathogens, and predators to ensure the long-term survival and population health.

Beekeeping in East Africa is a complex and involves integrating information about the natural history and behavior of African honey bee sub-species, developing economically viable management strategies, and adjusting approaches to mitigate emerging challenges, such as the introduction of *Varroa* mites and climate change. This study is a first step in evaluating the role of hive type on honey bee colony performance and health, and our results suggest that modification of the KTB hives may provide the most optimal approach to improving beekeeper's economic gains. Future studies are needed to evaluate the utility of these modifications across regions that vary in flowering resources and climate. Finally, our study also suggests that current practices (not chemically treating for *Varroa* mites and allowing colonies to abscond and migrate) likely benefits honey bee populations, and thus should be continued.

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Table 2-1 **Primers used for molecular detection of bee pathogens.**

Primer	Primer Pair	Product Size (bp)	Reference
ABPV	F TTATGTGTCCAGAGACTGTATCCA R GCTCCTATTGCTCGGTTTTTCGGT	900	Benjeddou et. al. 2001
BQCV	F TGGTCAGCTCCCACTACCTTAAAC R GCAACAAGAAGAAACGTAAACCAC	700	Singh et. al. 2010
CBPV	FAGTTGTCATGGTTAACAGGATACGAG R TCTAATCTTAGCACGAAAGCCGAG	455	Ribiere et. al. 2002
DWV	FTCCATCAGGTTCTCCAATAACGGA R CCACCCAAATGCTAACTCTAAGCG	450	Yue and Genersch 2005
IAPV	F GCGGAGAATATAAGGCTCAG R CTTGCAAGATAAGAAAGGGGG	586	Di Prisco et, al, 2011
KBV	F GATGAACGTCGACCTATTGA R TGTGGGTTGGCTATGAGTCA	417	Stoltz et, al. 1995
SBV	F GCTGAGGTAGGATCTTTGCGT R TCATCATCTTCACCATCCGA	824	Chen et, al, 2006
VDV	F GATAGCGTCAGGGTATCGG R TTCATCTGCCGTTTGGTGGA	541	Moore et, al. 2011

Abbreviations: Acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Israeli acute bee paralysis virus (IAPV), Kashmir brood virus (KBV), sac brood virus (SBV), Varroa destructor virus (VDV). References: Benjeddou et al. (2001) Applied and Environmental Microbiology 67:2384–2387; Chen et al. (2005) Applied and Environmental Microbiology 71(1):436–441; Di Prisco et. al.(2011) Journal of General Virology 92: 151–15; Klee et al. (2007). Journal of Invertebrate Pathology 96: 1–10; Moore et al. (2011) Journal of General Virology 92: 156-161 Ribiere et al. (2002) Apidologie 33: 339–351; Singh et al. (2010) PLoS ONE 5(12): e14357. doi:10.1371/journal.pone.0014357; Stoltz et al. (1995)Journal of Apicultural Research 34: 153–160.doi:10.1371/journal.pone.0094459.t001; Yue and Genersch. (2005) Journal of General Virology 86: 3419-3424

Figure 2-1. Honey bee hive types commonly used in East Africa.

A. Langstroth hives. These are most commonly used in Western beekeeping. Contrary to typical Western practices, in Kenya these hives are often suspended from a tree or bar as shown. **B. Kenyan Top Bar hives.** These hives are also suspended from a tree limb to dissuade predators. The comb has no support structure and consists only of a bar at the top of the hive body from which the bees build comb **C. Traditional Log Hives**. This hive body is most commonly used in Kenyan beekeeping because they are inexpensive, long-lasting, and easy to maintain. These hives vary considerably in size as they are simply cut, hollowed-out logs, but the volumes of the hives are usually intermediate between the Langstroth and Kenyan Top Bar. (Photos from Maryann Frazier)

A

B

C

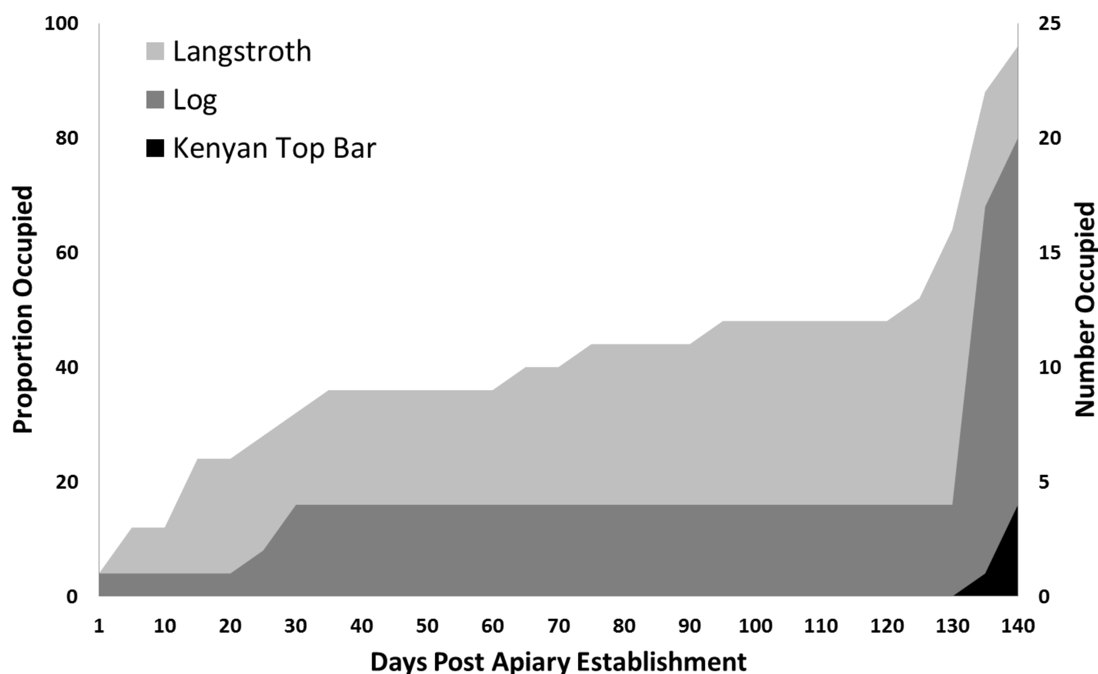

Figure 2-2. Effect of hive type on occupation rates.

We examined the time (days post-colony establishment) at which the different hives types were occupied by migrating colonies. 75 total hive bodies were placed in 3 apiaries at the South Eastern Kenya University (SEKU) in October 2012. Data collection was completed when 100% of the Langstroth hives were occupied, on April 9, 2013. Pair-wise comparisons reveal that Langstroth Hives were occupied most quickly, followed by Log hives, and lastly Kenyan Top Bar hives (Survival, Log-rank: Lan vs Log $p<0.0027$, Lan vs KTB $p<0.001$, and Log vs KTB $p<0.001$).

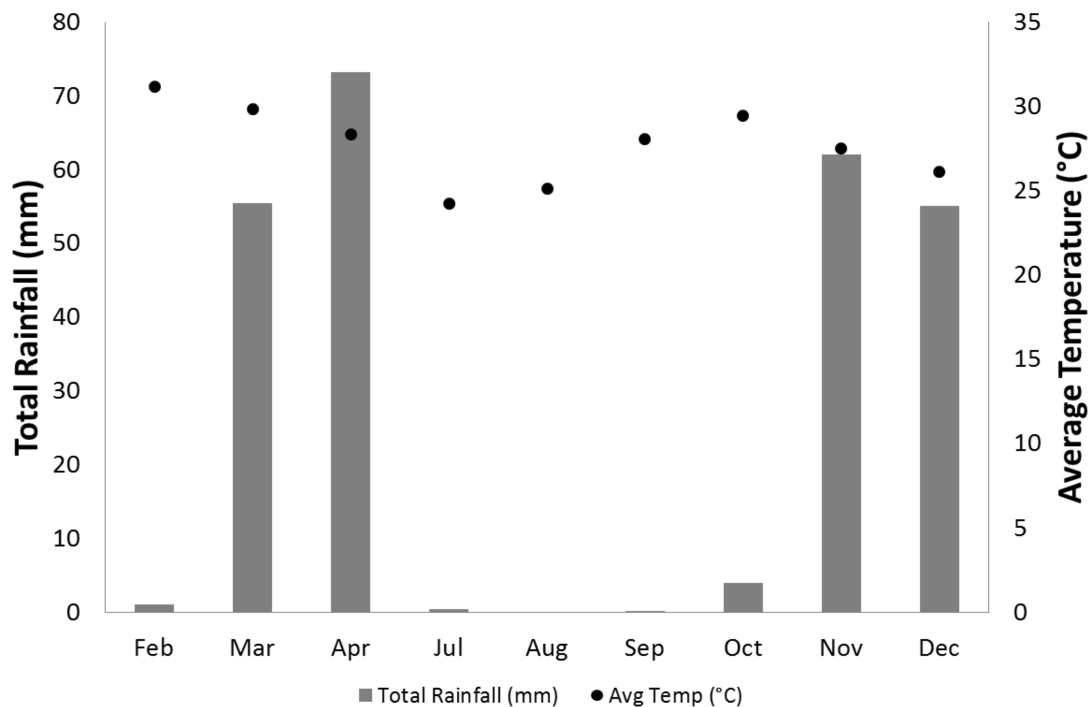

Figure 2-3. Rainfall and maximum daily temperature.

Temperature and rainfall were monitored daily using an automatic weather station established at the South Eastern Kenya University (SEKU). Over the course of the study, there were four distinct seasons: dry and hot in February, high rains and hot in March and April, very dry and cooler in July-September, and cooler with high rain in November-December. The reproductive swarming season falls in March-April, and the absconding season/migratory swarming season occurs in June-August. All three apiaries were established in October 2012 and bees were screened in April and June 2013 for the presence of viral infections. Standard errors were not included for temperature because they were so small they could not be represented graphically.

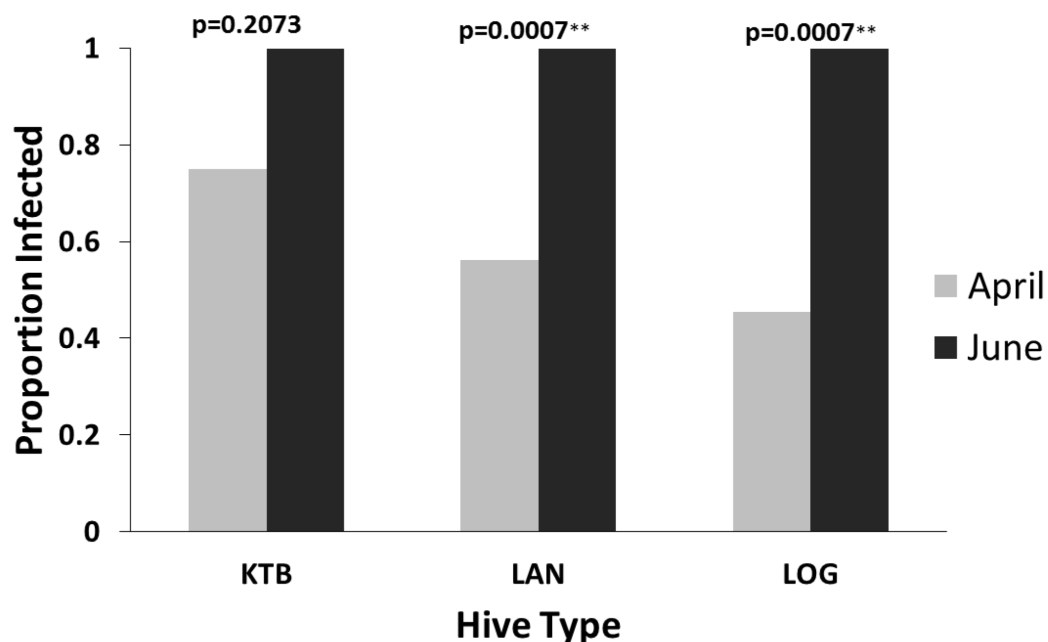

Figure 2-4. Effect of season and hive type on virus infection prevalence.

April was chosen as a representative month for the first wet season the colonies experienced while June corresponded to the first dry season month that the colonies experienced. While there was a general trend for higher prevalence of infection in June in all hive types, the difference was only significant in Langstroth and Log hives (2-Sample Test for Proportions, DF=1; KTB p=0.2073, Lan p=0.0007, Log p=0.0007).

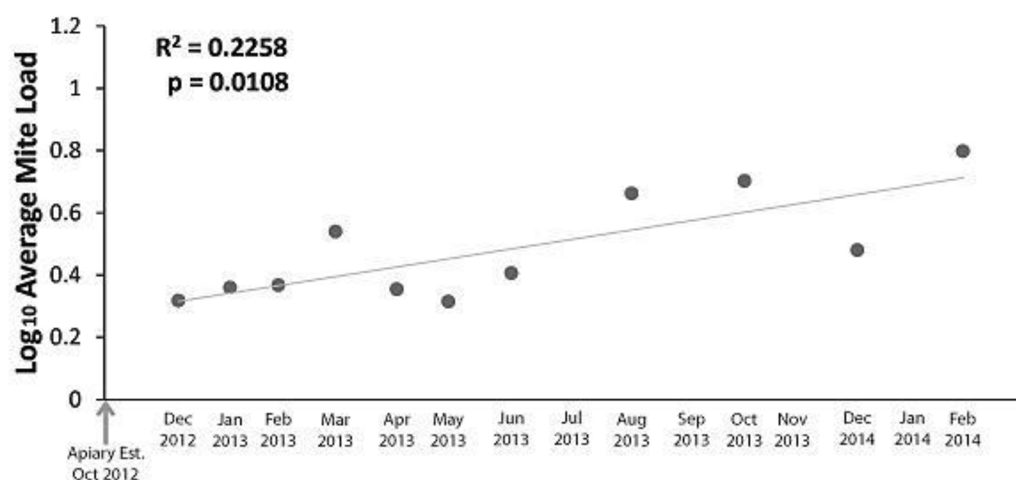

Figure 2-5. Log 10 Average *Varroa* load over time.

Apiaries were established in Octover 2012 and measurements started December 2012 to avoid disturbing young colonies. There was a clear linear relationship between mite counts and time, suggesting that mites are accumulating over the life span of the colony. The predictive power of the model was improved when the number of occupied frames was included (RMSE without Log10 OccFrames = 0.352, RMSE with=0.347) as an additional predictor variable (Least Square Means, R^2=0.2258, p=0.0108; Log10 occupied frames: DF=1, F ratio = 0.0504, p=0.8231).

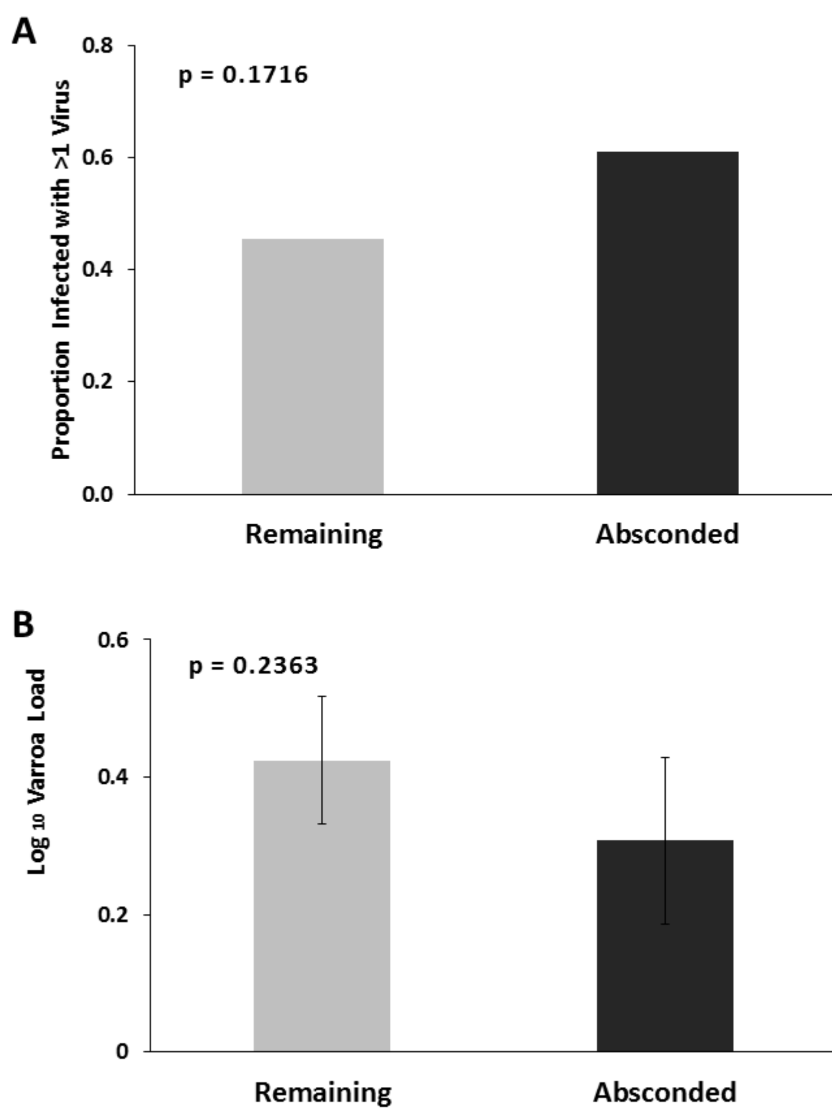

Figure 2-6. Effect of pathogen and parasite infection levels on absconding rates.

A. All colonies in June 2013 were infected with at least one virus and several were infected with multiple viruses (see Table S1). Colonies that absconded in July or August 2013 were not infected with more types of viruses in June than those that did not abscond (2-Sample Test for Proportions, p=0.1716). **B.** Whether a colony absconded in July or August had no relationship with its *Varroa* loads (log transformed) in June (Wilcoxon Rank Sums, p=0.2363).

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Sample	Hive Type	Month	ABPV	BQCV	CBPV	DWV	KBV	IAPV	SBV	VDV
AKB7	KTB	Apr	--	++	--	--	--	--	--	--
AKTB8	KTB	Apr	--	++	--	--	--	--	--	--
ALAN1	LAN	Apr	--	++	--	--	--	--	--	++
ALAN2	LAN	Apr	--	--	--	--	--	--	--	--
ALAN4	LAN	Apr	--	++	--	--	--	--	--	--
ALAN5	LAN	Apr	--	++	--	--	--	--	--	--
ALAN7	LAN	Apr	--	++	--	--	--	--	--	++
ALAN8	LAN	Apr	--	++	--	--	--	--	++	--
ALOG2	LOG	Apr	--	--	--	--	--	--	--	++
ALOG5	LOG	Apr	--	++	--	--	--	--	--	--
BKTB8	KTB	Apr	--	--	--	--	--	--	--	--
BLAN1	LAN	Apr	--	--	--	--	--	--	--	--
BLAN3	LAN	Apr	--	++	--	--	--	--	--	--
BLAN6	LAN	Apr	--	++	--	--	--	--	--	--
BLAN7	LAN	Apr	--	++	--	--	--	--	--	--
BLAN8	LAN	Apr	--	++	--	--	--	--	--	--
BLOG2	LOG	Apr	--	++	--	--	--	--	--	++
BLOG3	LOG	Apr	--	++	--	--	--	--	--	--
BLOG6	LOG	Apr	--	++	--	--	--	--	--	--
CKTB1	KTB	Apr	--	--	--	++	--	--	--	--
CLAN1	LAN	Apr	--	--	--	--	--	--	--	--
CLAN4	LAN	Apr	--	--	--	--	--	--	--	--
CLAN5	LAN	Apr	--	--	--	--	--	--	--	--
CLAN7	LAN	Apr	--	--	--	--	--	--	--	--
CLAN8	LAN	Apr	--	--	--	--	--	--	--	--
CLOG1	LOG	Apr	--	--	--	--	--	--	--	--
CLOG2	LOG	Apr	--	--	--	--	--	--	--	--
CLOG3	LOG	Apr	--	--	--	--	--	--	--	--
CLOG6	LOG	Apr	--	--	--	--	--	--	--	--
CLOG8	LOG	Apr	--	--	--	--	--	--	--	--
CLOG9	LOG	Apr	--	--	--	--	--	--	--	--
ALAN2	LAN	June	--	--	--	++	--	--	--	--
ALAN4	LAN	June	--	--	--	++	--	--	--	--
ALAN5	LAN	June	--	--	--	++	--	--	--	--
ALAN6	LAN	June	--	--	--	++	--	--	--	--
ALAN7	LAN	June	--	++	--	++	--	--	--	--
ALAN8	LAN	June	--	++	--	++	--	--	--	--
ALAN9	LAN	June	--	++	--	++	--	--	--	++
ALOG4	LOG	June	--	++	--	++	--	--	--	--
ALOG5	LOG	June	--	--	--	++	--	--	--	--

ALOG6	LOG	June	--	--	--	++	--	--	--	--
AKTB7	KTB	June	--	++	--	++	--	--	--	--
BLAN1	LAN	June	--	++	--	++	--	--	--	--
BLAN2	LAN	June	--	++	--	--	--	--	--	--
BLAN3	LAN	June	--	++	--	++	--	--	--	--
BLAN5	LAN	June	--	--	--	++	--	--	--	--
BLAN6	LAN	June	--	--	--	++	--	--	--	--
BLAN8	LAN	June	--	--	--	++	--	--	--	--
BLOG1	LOG	June	--	++	--	++	--	--	--	++
BLOG2	LOG	June	--	--	--	++	--	--	--	--
BLOG3	LOG	June	--	--	--	++	--	--	--	--
BLOG7	LOG	June	--	++	--	++	--	--	--	--
BLOG8	LOG	June	--	++	--	++	--	--	--	--
BKTB7	KTB	June	--	--	--	++	--	--	--	--
BKTB8	KTB	June	--	++	--	++	--	--	--	--
CLAN1	LAN	June	++	++	--	++	--	--	--	--
CLAN2	LAN	June	--	++	--	++	--	--	--	--
CLAN3	LAN	June	--	--	--	++	--	--	--	--
CLAN5	LAN	June	--	++	--	++	--	--	--	--
CLAN6	LAN	June	--	++	--	++	--	--	--	--
CLAN7	LAN	June	--	++	--	++	--	--	--	--
CLAN8	LAN	June	--	--	--	++	--	--	--	--
CLOG1	LOG	June	--	++	--	++	--	--	--	++
CLOG2	LOG	June	--	--	--	++	--	--	--	++
CLOG3	LOG	June	--	++	--	++	--	--	--	--
CLOG5	LOG	June	--	--	--	++	--	--	--	--
CLOG6	LOG	June	--	--	--	++	--	--	--	--
CLOG8	LOG	June	--	--	--	++	--	--	--	--
CLOG9	LOG	June	--	++	--	++	--	--	--	++
CKTB1	KTB	June	--	--	--	++	--	--	--	--
CKTB7	KTB	June	--	++	--	++	--	--	--	--

Table 2-2 Raw infection data by hive

This table shows raw infection data for each hive screened for ABVP, BQCV, CBPV, DWV,KBV, IAPV, SBV, AND VDV. If a hive was found to be infected with a particular virus, it was denoted with a '++', and if it was uninfected, it was denoted with a '--'. April and June were chosen as representative moths of the wet and dry seasons, respectively.

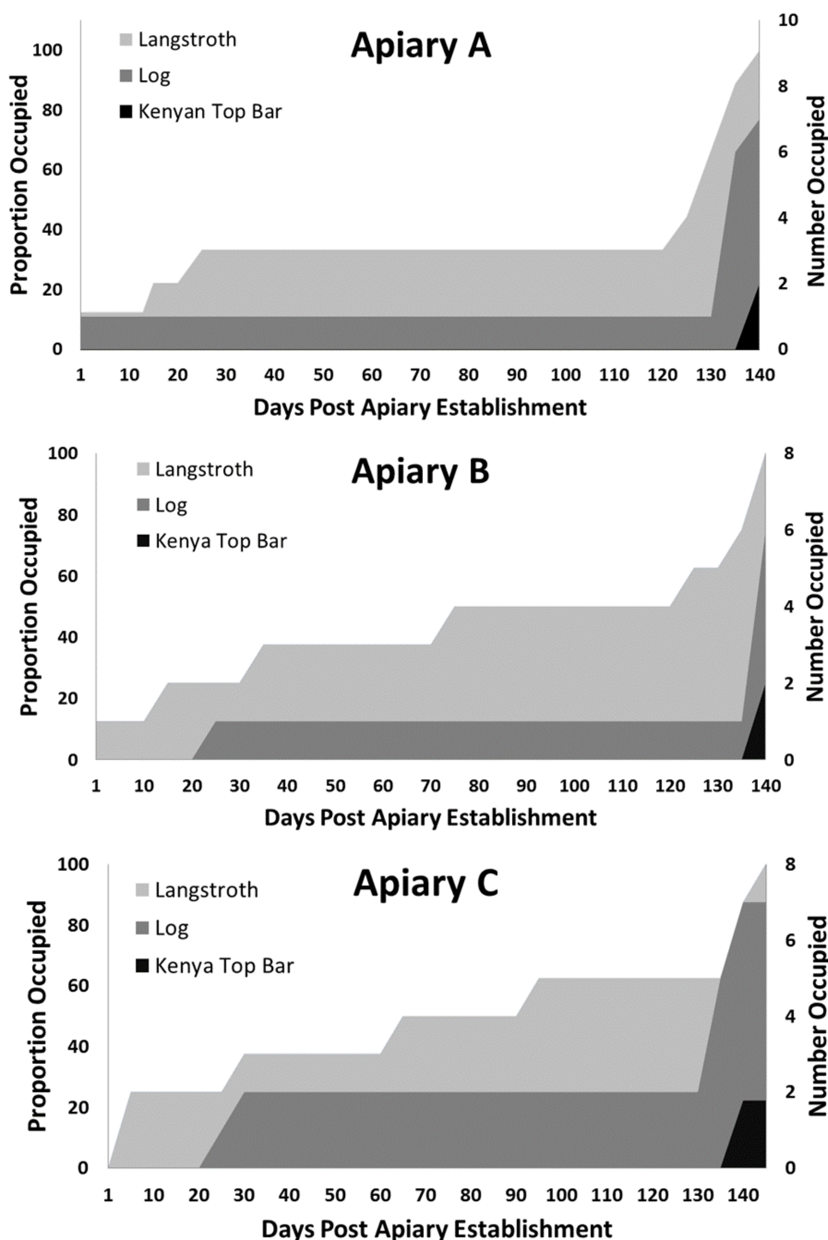

Figure 2-S1. Occupation of hive types within apiaries.

We examined occupation rates for the three different hive types within each of the three apiaries. Our new threshold for statistical significance is p=0.017 as per the calculation of the Bonferroni threshold for multiple comparisons of survival curves. The over-all trend was maintained in Apiary A (Survival, Log-rank, Lan vs. Log P=0.0200, Lan vs. KTB p<0.0001, Log vs KTB p=0.0037), and Apiary B (Lan vs. Log p=0.0063, Lan vs. KTB p<0.0001, Log vs. KTB p=0.0091) but in Apiary C the Langstroth and Log hives had no discernible difference in occupation rates (Lan vs. Log p=0.9430, Lan vs. KTB p<0.0001, Log vs. KTB p<0.0001).

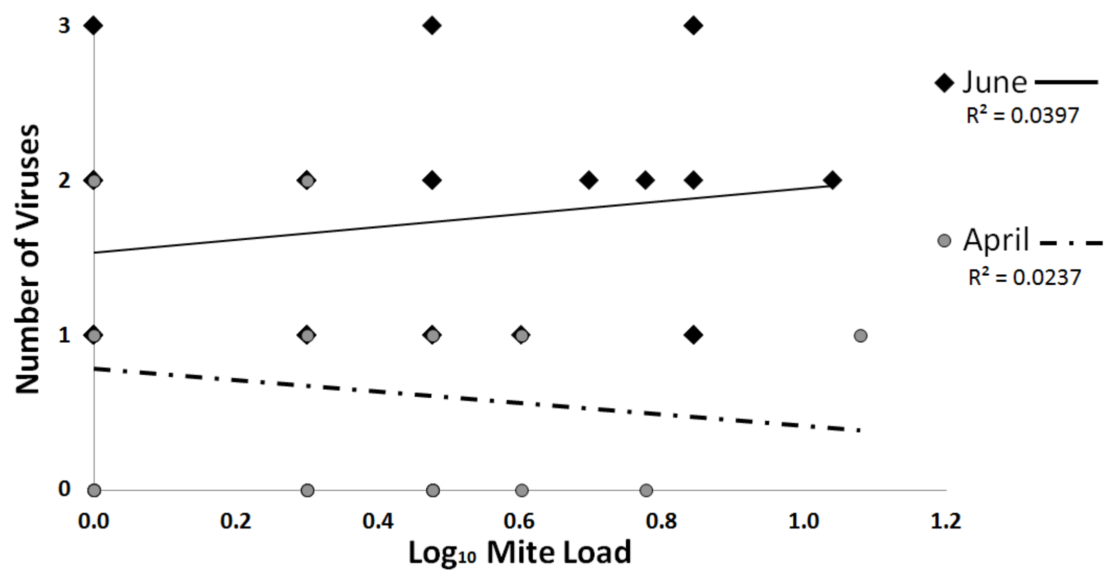

Figure 2-S2. Log 10 *Varroa* loads regressed against number of viruses a colony was infected with in June and April.
No relationship was found between number of viruses and *Varroa* loads in April (Regression, ANOVA, p=0.3949) or June (Regression, ANOVA, p=0.2280).

Chapter 3
Ecology of interspecies transmission of deformed wing virus between bumble bees and honey bees

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Abstract

Many pollinator populations are in decline or otherwise threatened by a multitude of factors including disease. The existing evidence shows that viral pathogens are a large part of these threats and many of them infect both honey bees and native pollinators. However, the population dynamics of viruses in honey bee and native pollinator hosts remain to be fully evaluated. For example, current evidence suggests that viruses are easily transmitted across host species and do not form host species-specific populations. While it is clear that viruses can replicate in some of their native pollinator hosts, it remains to be determined if the resulting virions are infective. Here we ask for the first time whether virions that replicate within a bumble bee species are infectious to conspecifics, using a native bumble bee species (*Bombus impatiens*) and a common pathogen that is found across pollinator populations (Deformed Wing Virus). Our data suggest that virions isolated from honey bees are more infectious than those isolated from bumble bees, and passage through bumble bee hosts may select for specific viral strains. However, these results are fairly preliminary, and require further investigations. Additionally, we highlight outstanding questions in this field and future research directions

Introduction

It has been estimated that pollination services account for up to 9.5% of the global value for agricultural production, amounting to approximately 35% of global crop production (Gallai et al. 2009; Klein et al. 2007). Indeed, in the US alone, insect pollination, including all non-*Apis* pollinators, was estimated to be responsible for USD$15.2 billion of directly dependent crops in 2009, with USD$3.44 billion of this being attributed to insects other than honey bees and leaf-cutter bees. An increasing population in the U.S. means an increased demand for food and a continued demand for insect pollination (Calderone 2012). Thereby, a market has developed around rental and transport of honey bees and *Bombus* species in order to supplement crop pollination in the US and Europe (Carreck et al. 1997; Sumner & Boriss 2006). However, other wild insects are responsible for the pollination of many crops, and are often more efficient than managed bees (Breeze et al. 2011; Garibaldi et al. 2014; Greenleaf & Kremen 2006; Kremen et al. 2002; Morandin & Winston 2005; Winfree et al. 2007). Unfortunately, managed honey bees and *Bombus* species are infecting native bees and wild conspecifics with a range of pathogens (Colla et al. 2006; Graystock, Yates, Darvill, et al. 2013; Graystock, Yates, Evison, et al. 2013; Graystock et al. 2014; Singh et al. 2010).

Many pathogens historically thought of as honey bee pathogens are spilling over into native pollinator populations. Several studies have demonstrated that these pathogens can cause sub-lethal effects and reduced longevity in native pollinator species, such as *Bombus terrestris* and *Bombus pascuorum* in Europe and (Genersch et al. 2006; Graystock, Yates, Darvill, et al. 2013). While it remains to be determined the extent to

which these "spillover pathogens" are impacting wild pollinator populations, there is great concern that these pathogens contribute to the observed declines in wild pollinator populations (Cameron et al. 2011; Goulson et al. 2006; Goulson et al. 2008); indeed, one-half of the bumble bee species which have been surveyed in the US and Europe show evidence of decline (Cameron et al. 2011; Potts et al. 2015). Thus, it is critical to evaluate the impacts of these pathogens on a broad array of pollinator host species, and understand how transfer through these non-*Apis* hosts effect the virulence and population dynamics of these pathogens. .

One of the most well-described and commonly identified pathogens that spills over from managed honey bee to wild pollinator populations is Deformed Wing Virus (DWV) an iflavirus in the order *Picornavirales* (Fürst et al. 2014; Ravoet et al. 2014; Singh et al. 2010). As with most honey bee viruses, DWV causes a number of sub-lethal and lethal effects on honey bees (reviewed in McMenamin & Genersch 2015), including learning deficits, deformed wings, shortened and bloated abdomens, premature death, and even colony collapse, in conjunction with *Varroa* mite infestations (Shen, Yang, et al. 2005; Dainat & Neumann 2013; de Miranda & Genersch 2010; Francis et al. 2013; Nazzi et al. 2012; Yang & Cox-Foster 2005). . The effects of DWV on other pollinators species are not well described but several studies have identified DWV in populations of bumble bees (Fürst et al. 2014; Levitt et al. 2013; McMahon et al. 2015; Singh et al. 2010), and DWV infections have been found in all tissues of infected *Bombus huntii* workers (Li et al. 2011).

The transmission routes of DWV in non-Apis pollinator species such as bumble bees remains to be determined. Several studies have demonstrated that DWV can be transferred between species via pollen, and thus infected honey bees may transmit DWV to wild bumble bees by contaminating flowers that both species are visiting (Chen et al. 2006; Shen, Cui, et al. 2005; Singh et al. 2010). Interestingly, phylogenetic analyses of DWV in honey bee and non-Apis pollinator species indicate that there is no host-species segregation of viral quasi-species, and rather viral strains are grouped by geographic region (Fürst et al. 2014; Levitt et al. 2013; Ravoet et al. 2014; Singh et al. 2010). These studies suggest that the viruses may be circulating within honey bee populations, and then these repeatedly spill-over to infect bumble bee populations, but do not circulate within the bumble bee populations. Interestingly, DWV can replicate within bumble bee hosts (Fürst et al. 2014; Levitt et al. 2013; Li et al. 2011), which suggests that the virus that replicates within bumble bees is either not infectious or cannot outcompete viruses introduced from the honey bees.

Here, we begin to investigate whether DWV from a heavily infected honey bee colony can (a) infect bumble bees (*Bombus impatiens*) and (b) whether infected bumble bees produce virions that are infectious to other bumble bees. In this experiment we isolated virus from honey bee workers with deformed wings and then orally infected bumble bee workers. After confirming that the bumble bee workers became infected, inocula were prepared from these individuals and a second cohort was orally inoculated with this isolate.

Methods

<u>Experimental infections.</u>

Two bumble bee colonies (colonies A and B) were purchased from Koppert Biological Systems (Howell, MI) and kept in a climate controlled dark room with pollen and 50% sucrose *ad libitum*. After the colonies acclimated, 21 individuals from each colony of approximately the same size were chosen randomly from atop the brood nest. For each colony, 3 workers were placed into small plastic cylindrical cages of approximately 17.7 cm in diameter by 15 cm high, which were provided with a ball of pollen and 50% sucrose *ad libitum*. Thus, each colony produced 6 cages, for a total of 12 cages.

After 24 hours, 3 cages from each source colony were randomly assigned to either the sucrose control group, or the virus-fed group, generating a total of 6 control and 6 treatment cages. The virus fed group were provided with 500 µL 50% sucrose and 50% sucrose in virus extract (for preparation, see below), respectively. One day after treatment, all cages were again fed 50% sucrose *ad libitum* and pollen was replenished as needed. After 8 more days, bees were collected onto dry ice. Note that honey-bee-collected pollen was used for these studies; this pollen could have contained viruses (Singh et al. 2010). However, since only bees in the treatment groups showed infection while control bees were uninfected (see results), it suggests the pollen was not the route by which the bumble bees were infected with the virus.

Two cohorts (Cohort 1 and Cohort 2) were generated in this study chronologically, both were treated as above. For Cohort 1, treatments were sucrose and virus extracted from honey bees with deformed wings. For Cohort 2, treatments were sucrose and virus extracted from infected bumble bees from cohort 1. Individuals collected from colony A in Cohort 2 were fed extract taken only from Cohort 1 individuals originally taken from colony A, while individuals collected from colony B in Cohort 2 were fed extract taken only from Cohort 1 individuals originally taken from colony B. This created two distinct biological replicates to assess (though note that both colonies in Cohort 1 were fed the same original honey bee extract). See Figure 1 for a schematic of the experimental design.

Preparing Innocula

Cohort 1 Inoculum. Bees in Cohort 1 was treated with sucrose and virus extracted from honey bees as in (Galbraith et al. 2015) with deformed wings. To extract the virus, 2 thoraces from deformed individuals were homogenized per 1 mL of deionized water using eight 2.0 mm zirconia beads and a Fastprep instrument (Qbiogene, Montreal, Quebec) for 3 cycles at max speed for 45 seconds each. The homogenate was centrifuged at 15,000 rpm for 15 seconds to clarify the homogenate, and the supernatant was placed on a 0.2 µm filter and a syringe was used to move the extract through the filter into a fresh tube. The filtrate was then mixed with equal parts sucrose. PCR analysis (see below and Figure 2) confirmed that these extracts contained deformed wing virus.

Cohort 2 Inoculum. Bumble bees from the infected, treatment groups of Cohort 1 were chosen. The guts and ovaries were dissected out and the eviscerated abdomen was cut in half. Half of the abdomen was placed in 400 µL Quiazol (Qiagen, Valencia, CA) lysis buffer for RNA extraction and stored at -80°C for later analysis, while the other half was stored at -80°C in RNAlater for later use. These bees were screened for DWV infections via PCR (see below for screening methodology). Once infected individuals were identified, the remaining halves of eviscerated abdomens of these individuals were selected for inoculum preparation. The dissect abdominal tissues taken from two individuals from the same source colony were removed from RNAlater, washed in Ringer's solution (128 mM NaCl, 18 mM CaCl2, 1.3 mM KCl, 2.3 mM NaHCO3) then homogenized per 1 mL of deionized water as previously described. Preparation then continued as above.

Screening for Viral Infections.

RNA isolation from thoraces and eviscerated abdomens. The thoraces of honey bees and halves of eviscerated bumble bee abdomens were screened in the same manner. Tissues were homogenized in 400 µL of Quiazol lysis buffer as described above for inoculum preparation in water and whole RNA was extracted as per the manufacturer's instructions. RNA purity and quantity was assessed spectrophotometrically using Nanodrop 1000 (Thermo Scientific, Waltham, MA).

RNA isolation from inocula. 200 µL of inoculum were mixed thoroughly with 600 uL of TRIzol® LS (Sigma-Aldrich, St. Louis, MI). 200 µL of chloroform were added to the

homogenate, vortexted for 15 seconds and then incubated on the benchtop for 5 minutes. Samples were then centrifuged at 15,000 rpm at 4°C for 20 minutes. The aqueous phase was then removed and whole RNA extraction was completed with the RNeasy Mini Kit (Quiagen) as per the manufacturer's instructions.

cDNA Synthesis and reverse transcription PCR (RT-PCR). cDNA was synthesized using random hexamers and Superscript III ® reverse transcriptase kit (Life Technologies, Carlsbad, CA) in 15 µL reactions using 500 ng of RNA per reaction. Samples were then screened for DWV (see Table 1)for primers used, Life Technologies, Carlsbad , CA). Note that multiple primers were used for DWV (see Table 2). 1 µL of each sample and primers were combined with REDtaq® Supermix (Sigma Aldrich) and run on a Mastercycler Pro (Eppendorf, Hamburg) using the following program: *Step 1:* 95°C for 2 min; *Step 2:* 40 cycles at 95°C for 30 s, 60°C for 1 min, and 68°C for 1 min; and *Step 3:* finally the reaction was held at 68°C for 7 min as in (Y. P. Chen et al. 2006). Positive controls from infected honey bees and non-template controls were included for all reactions.

Real time RT-PCR. New cDNA was prepared as above and 2 µL were used per reaction. Viral titers and an endogenous control gene (β-actin) were assessed using real time using SYBR® Green (Life Technologies) master mix as per the manufacturer's instructions using the 7900HT Fast Real-Time PCR System and SDS software 2.3 (Applied Biosystems, Waltham, US). Positive controls and non-template controls were included for all reactions. For each sample, Ct values were assessed. If the SDS software scored a

reaction as having Ct values that were "undetermined" or above 30, the virus was considered to not be present.

RNA Quality Check. In addition to assessing the RNA quality using the Nanodrop, we also examined quality by running a random subset of the cages on an agarose gel. A random cagewas chosen from each treatment group for both cohorts, RNA from these individuals was pooled, and a DWV positive honey bee control was used. Of the samples run on the gel, one included infected bumble bee whole RNA and one infected honey bee whole RNA. A DNA ladder and 200 ng of whole RNA from each sample was loaded onto a 1% non-denaturing agarose gel, run at 60 V for 45 minutes, stained with ethidium bromide and imaged using the Gel Doc™ XR+ System (Bio Rad, Hercules, CA).

Results

Primers Used in RT-PCR

Refer to Table 2 for detailed information on the primers for DWV detection. Primers were selected that spanned the DWV genome, and recent studies demonstrated that intra-species variation in DWV sequence varies across the 10Kb genome, with some sites more conserved than others (Cornman et al. 2013). Briefly, RT-PCR was performed with a primer pair targeting the RNA-dependent RNA polymerase at the 5' end of the genome at positions 9247-9697, a highly conserved region (RdRp,). Real time RT-PCR was

performed using two primer pairs, the first of which targeted the Internal Ribosomal Entry Site (IRES, at basepair 154-249), a region at the variable 5' end. The second pair used in real time RT-PCR analysis targeted the helicase region of the genome at nucleotide position 6255-6965, a site with a relatively high proportion of segregation sites (see Figure 1, from Cornman et al. 2013).

Screening with RT-PCR

Extracts. The aqueous extract prepared from the two symptomatic honey bees with deformed wings honey bees was positive for DWV (Figure 3). The aqueous extracts which were fed to the Cohort 2 bees were prepared with individuals positive for DWV from Cohort 1 and were positive for DWV. The sucrose extract was negative for DWV (Figure 3).

Cohort 1. RT-PCR screening identified one individual from each virus-infected cage that was infected with DWV, with the exception of a single cage where all three individuals were infected.(Figure 4). None of the bees from the sucrose-fed control cages were infected. Since bees from each source colony were equally likely to be infected, there was no apparent source-colony bias

Cohort 2. RT-PCR screening identified only one individual that was infected with DWV. None of the bees from the control cages were infected. (Figure 5)

Screening with real-time RT-PCR

Extracts. Aqueous extract from symptomatic honey bees screened with DWV primers targeting the helicase gene showed an average Ct value of 26.32 compared to the positive control's Ct value of 22.22. This extract also showed presence of actin, indicating host material was present in the prepared inoculum (Ct=18.44 compared to a positive control Ct of 15.00). Extracts from infected bumble bees from Cohort 1 (which was fed to the Cohort 2 bees) showed no signal for virus and some signal for actin (Colony A extract Ct=22.7, B extract = 22.7).

When the same samples were screened using primers for DWV targeting the IRES, no samples had Ct values below 34. (See Table 2 for raw infection data and Ct values).

Cohort 1 and 2. All samples had Ct values above 30 or undetermined when screened via real time RT-PCR with either primer set. However, all samples showed normal actin levels with Ct values ranging from 14.15-16.35 compared to a positive control of 16.4

RNA Quality Check

For all four randomly selected samples, 1 from each treatment in each Cohort, and the positive control, there were no signs of significant degradation of endogenous RNAs as both the small subunit and large rRNA subunit bands were intact with minimal smear

following (Figure 6). Size estimates are left out as the rRNA was not denatured and so migration is likely to be unpredictable, and RNA strands of a given size are known to run at faster speeds on agarose gels than DNA of a similar size (Wicks 1986).

However, the positive control had 2 larger bands matching at about 2kb and 1.65 kb and the samples had very dim bands at about 1.65 kb, potentially indicating DNA contamination. It is worth noting that the 28S bands of the DWV-fed groups are dimmer than the 28S bands for the sucrose control groups and that all samples deviated from the 2:1 ratio for 28S:18S intensity. This deviation is likely because the 28S ribosomal subunit in insects is known to be very heat labile, and will break into two pieces that co-migrate with the 18S region (Winnebeck et al. 2009). Furthermore, viral infection in honey bees has been shown to disrupt rRNA transcript abundance, though whether this causes an increase or decrease in abundance varies between studies (Boncristiani et al. 2013; Johnson et al. 2009)

Discussion

Overall, our results provide intriguing preliminary data suggesting that infection of bumble bees with DWV extracted from honey bees results in infection with a only subset of DWV strains in bumble bees, and these strains are only weakly able to infect additional bumble bee hosts. Oral inoculation with DWV isolated from deformed honey bee workers resulted in the infection of one individual from 5 of the 6 cages, and

infection of all 3 individuals from the 6th cage (8 bees out of 18). However, inoculating a second cohort of bumble bees with virus isolated from these infected bumble bees resulted in only one infection (1 bee out of 18). Furthermore, these viruses were detected only by primers targeting the conserved RdRp region of DWV, while primers targeting the more variable helicase and IRES regions did not demonstrate infections in either bumble bee Cohort 1 or 2. Obviously, given the small number of replicates, the substantial variation between primers, and the low infection rate of the Cohort 2 bees, these studies would need to be repeated to support this interpretation of the data.

It is curious that only one individual became infected per cage in most of Cohort 1, as one might expect more variation. In cages of three queenless bumble bee workers, workers will aggress each other until one worker establishes reproductive dominance, while the other bees remain subordinate with inactivated ovaries (Amsalem et al. 2015). Thus, in our studies, it is possible that the reproductive dominant individual was more likely to become infected, perhaps because she consumed more food to support ovariole development, and/or this individual reduced investment in immune function to produce eggs (Adamo et al. 2008; Brunner et al. 2014; Seehuus et al. 2006). Alternatively, the virus infection may have reduced the likelihood that the bee would be able to establish dominance. A comparison of ovarian activation rates and oocyte size would have allowed us to determine whether there was an association between dominance status and infection rates

Though viral RNA was identified using the RdRp primers in both Cohort 1 and 2, it is unknown if these represented actively replicating viruses. In order to confirm active

replication, it would have been necessary to screen for the negative strand of the virus. To do this, I would have synthesized cDNA from the whole RNA of infected individuals with primers specific for DWV that were tagged with random sequences as in (Yue & Genersch 2005). Then, I would have performed positive and negative strand specific PCR to determine whether the infected bumble bees had active infections. However, given that virus was detected in the fat bodies after being washed, it is likely that the infection was 'real', as the virus had to bypass the gut and somehow establish itself in the fat bodies. Furthermore, it has been previously demonstrated that DWV can actively infect *Bombus impatiens* workers (Levitt et al. 2013).

The fact that PCR results changed depending on area of the genome being targeted is possibly due to generation of a new quasi-species of the virus during the bumble bee infections, whether due to selection or drift. Deformed Wing Virus has a monopartite positive sense ssRNA genome at about 10kb with a single open reading frame. Typical of picorna-like viruses, DWV replicates by producing a large poly-protein that is post-translationally processed by viral-transcribed proteases to produce functional proteins. The 3' end of the genome encodes highly conserved genes for transcription proteins, including the RNA-dependent RNA polymerase, while the 5' end is highly variable and contains the IRES and relatively variable genes that encode structural proteins (Cornman et al. 2013; Lanzi et al. 2006).

DWV, like all ssRNA viruses, exists as quasi-species due to high lability of the genome and susceptibility to mutation. In fact, it has been shown that transmission route has the effect of strain selection. For example, transmission of the virus in honey bees by *Varroa*

mites seems to reduce strain diversity and select for a certain, more virulent strain of DWV (Martin et al. 2012; Moore et al. 2011; Ryabov et al. 2014). It is possible that in this study, selection was in place for quasi-species that could pass the peritrophic membrane lining the guts of bumble bees and establish infections on bumble bee fat body tissue. Considering the importance of secondary structure of the IRES in allowing host-specific utilization of ribosomes, it is also possible that this selection event acted on the IRES(Moore et al. 2011; Ongus et al. 2006). This could explain why we saw no signal when targeting the IRES. To test this hypothesis, viral isolates from the deformed honey bees and infected bumble bees would have to be sub-cloned and subjected to deep sequencing in order to get a detailed picture of the quasi-species present in the bumble bees. The sequences would then have to be bionformatically analyzed to look for sequence segregation, or places in the genome where the consensus sequence from a given group differ from the original isolate. It may be possible to subject the sequence data to F and D statistics in order to look for markers of selection as well (Holsinger & Weir 2009; Holsinger 2012). As mentioned above, lack of signatures for selection may indicate that neutral forces (i.e. genetic drift) may have resulted in quasi-species differentiation.

Our studies also suggest that the strains of virus the replicate within the bumble bee is not as capable of infecting additional bumble bee hosts as the strains of viruses replicated within honey bees. While inoculation with honey bee virus extract resulted in infections in 8/18 individual, inoculation with bumble bee virus extract resulted in infections in only 1/18 species. Considering the lower amount of actin mRNA in the inocula extracted from

Cohort 1 bumble bees as compared to the inoculum prepared from symptomatic honey bees, it is possible that this difference is due to fewer virions being present in the extract. If infected bumble bees do indeed produce DWV virions that are less infectious than DWV virions produced by honey bees, it could explain the associations found between managed honey bee colonies and the presence of DWV in adjacent native pollinator populations. This is because we would expect host populations that are exposed to a more infectious viral quasi-species (i.e. that which is being introduced by managed honey bee colonies) to be more highly infected than a host population that is exposed to a less infectious viral quasi-species (i.e. that which is circulating in bumble bees). Indeed, this could also explain the lack of host-species differentiation of viral quasi-species. If the virus cannot become established in the new host species, then that which is present is likely to be a result of frequent spill-overs. Therefore, strains present in honey bees and native bees would appear to be undifferentiated.

Note, more research has to be done to look at how effective DWV in bumble bees is at infecting nest mates and other conspecifics. However, understanding mechanisms mediating virus-host interactions and resultant effects on ecology and evolution will allow us to develop management practices to mitigate negative impacts these viruses have on honey bee and wild bee populations.

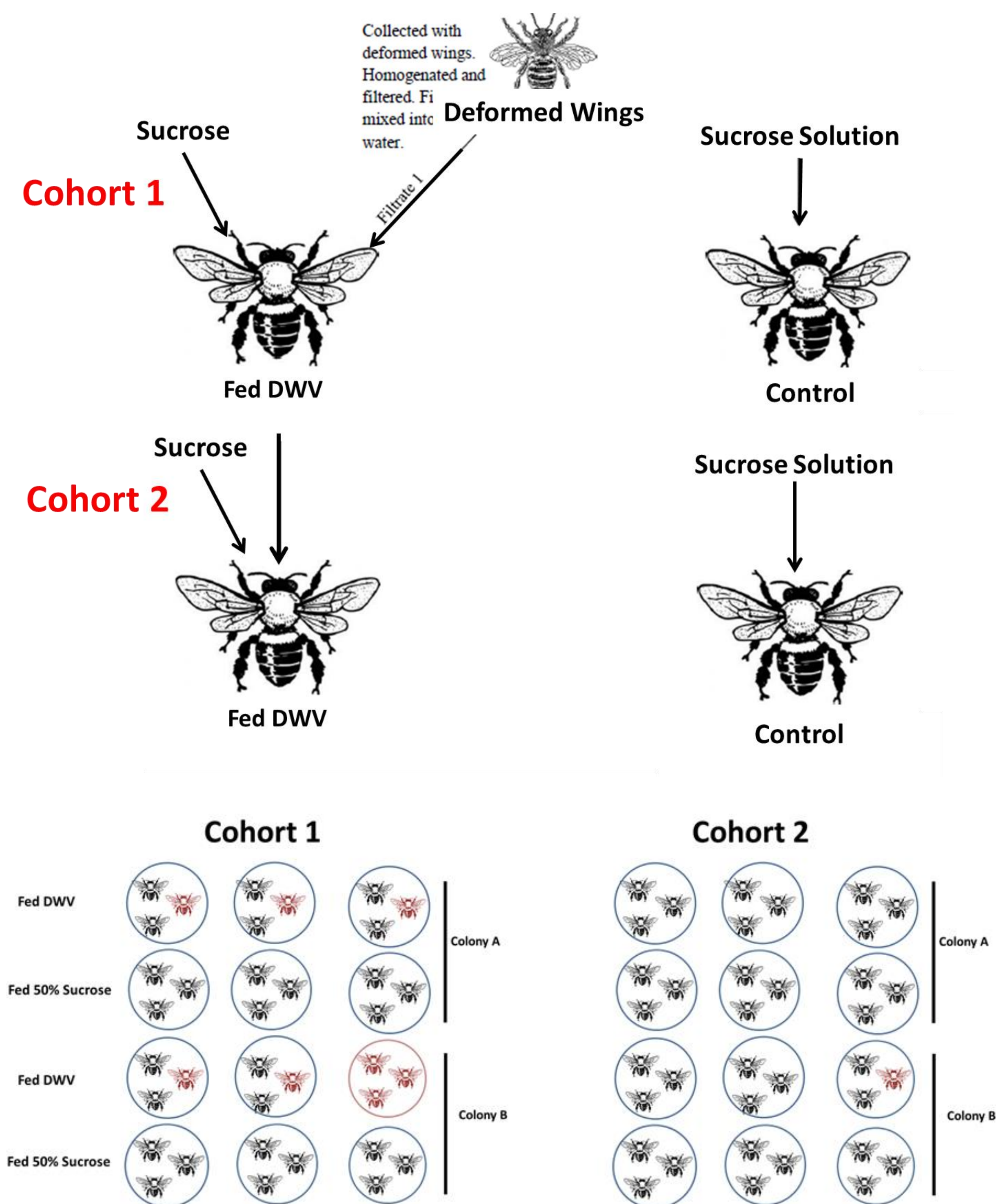

Figure 3-1. Experimental design and summary of results

A. For a full description, see materials and methods. Briefly, we mimicked a spill-over by orally inoculating *Bombus impatiens* workers with virus extracted from Deformed Wing Virus (DWV)s symptomatic honey bees., and ran a sucrose-fed control in parallel. This group, referred to as Cohort 1, was allowed to live with sucrose and pollen *ad libitum* for 8 days to allow the infection to progress. At the end of 8 days, all individuals were collected onto dry ice and screened for DWV. Infected individuals were then

used to prepare a new inoculum which was used to orally inoculate a second cohort composed of their sisters. B. Bees in black are uninfected with DWV and bees in red are infected with DWV.

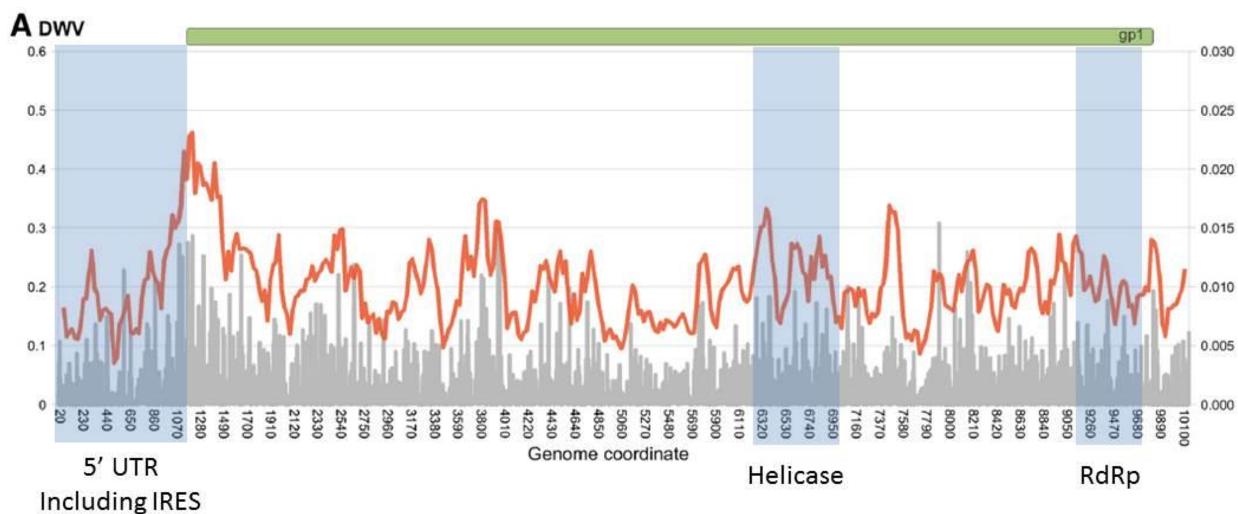

Figure 3-2 Genomic variation at targets of primer pairs

This is a slightly modified figure taken from (Cornman et al. 2013) showing haplotype diversity at SNPs (orange line, right axis). Here we see that Cornman found the RdRp to have very low haplotype diversity, as expected for an RNA virus, while the helicase and 5’UTR containing the IRES, have spikes of high diversity.

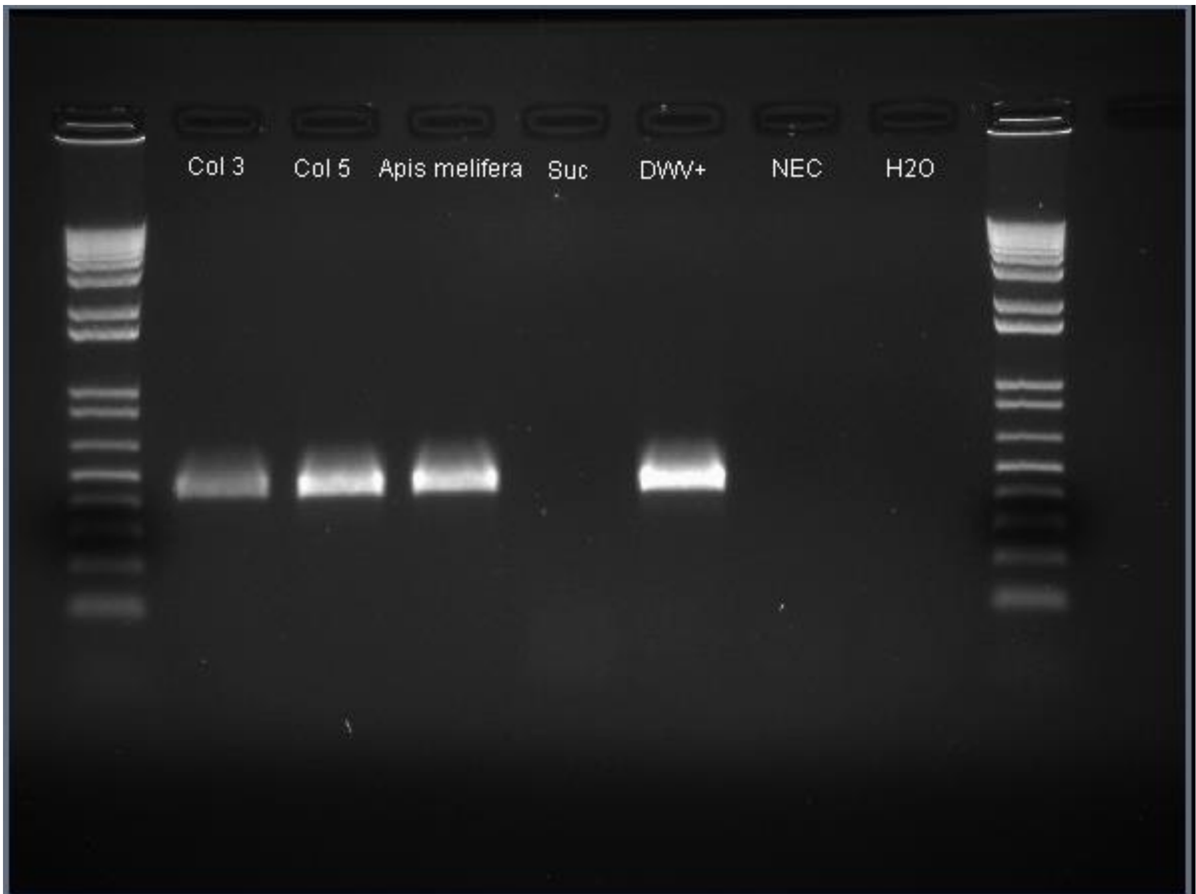

Figure 3-3. Screening inocula for DWV.

Lanes 1 and 2 are the inocula prepared from cohort 1 individuals and fed to Cohort 2 individuals from colonies A and B, respectively (colony 3 is A, and colony 5 is B).Lane 3 is the inoculum extracted from honey bee thoraces and is clearly positive for DWV. Later analysis via real time RT-PCR showed it was positive for BQCV. The non-enzyme control and the H2O were run with DWV primers.

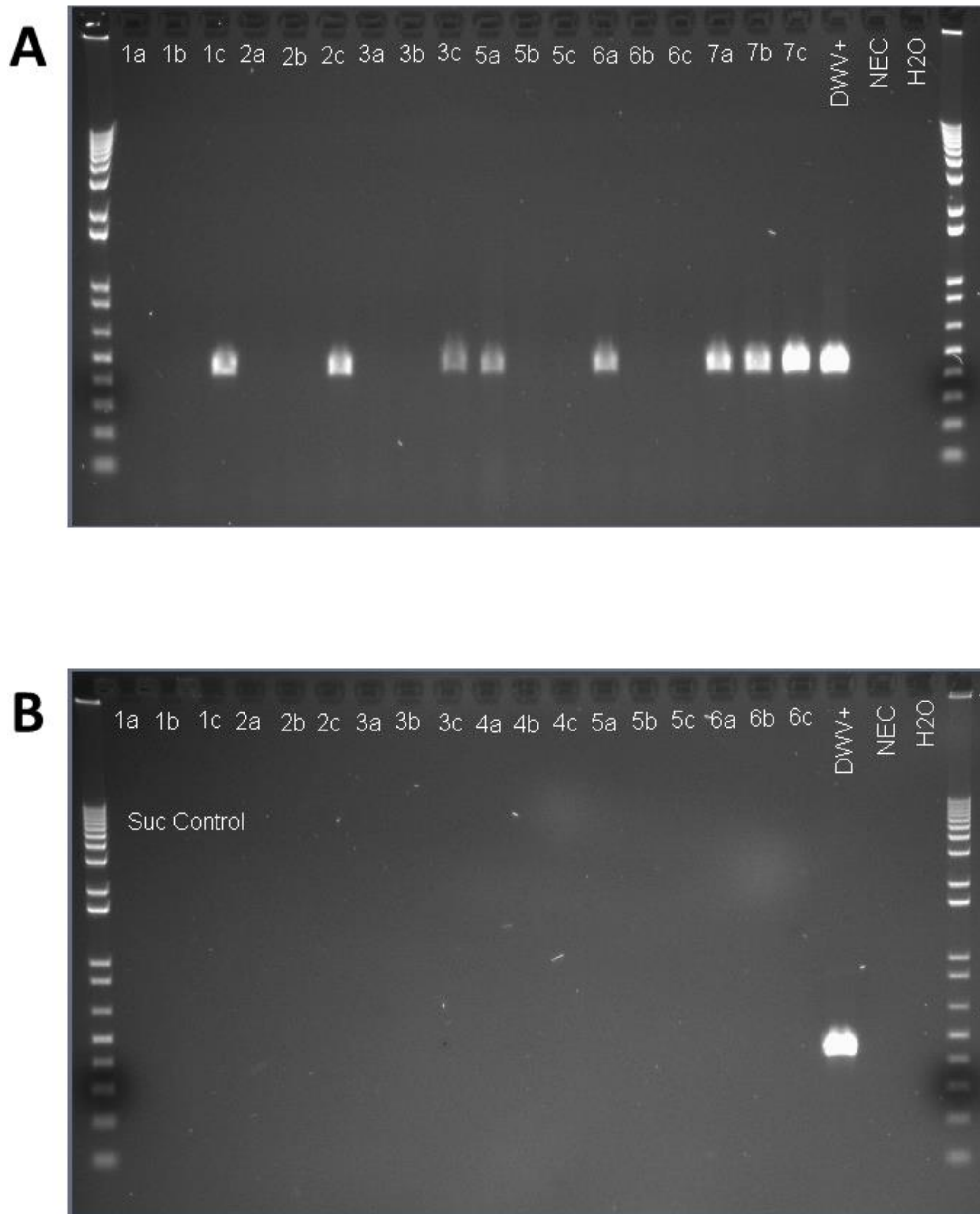

Figure 3-4. Individuals from Cohort 1 treatment and control.

A. Individuals from Cohort 1 orally inoculated with DWV were screened for infection. Numbers 1, 6 and 7 were from Colony B; 1 and 6 were combined for preparation of the inoculation for Cohort 2 individuals from Colony B. Lanes 2, 3 and5,were from Colony A; 2 and 5 were used preparation of the

inoculation for Cohort 2 individuals from Colony B. **B.** Individual's in Cohort 1 fed sucrose only were also screened for DWV. No infections were found. Groups 1, 3, and 4 were from Colon A. Groups 2, 5 and 6 were from Colony B. H2O and NEC were run with primers for DWV. The NEC was pooled RNA from all samples.

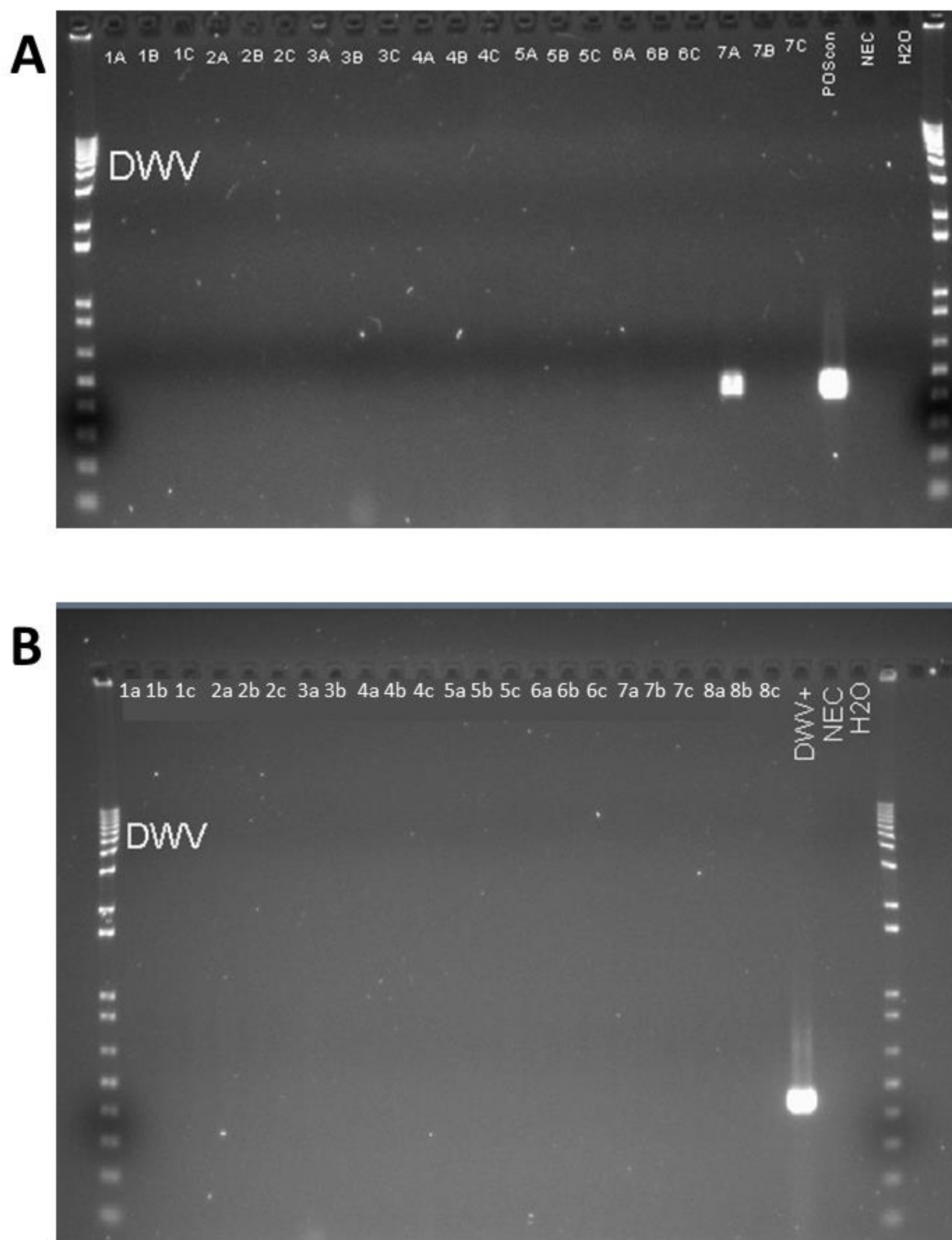

Figure 3-5. Cohort 2 treatment and control

A. Individuals were fed DWV extracted from sisters from Cohort 1. Groups 1,3, 5 and 7 were from colony B. Groups 2, 4 and 6 were from Colony B. 1 infection was found in cage 7, from Colony B. **B.** Cages being fed only sucrose were run in parallel with the treatment. The DWV label denotes only that primers specific for DWV were used,

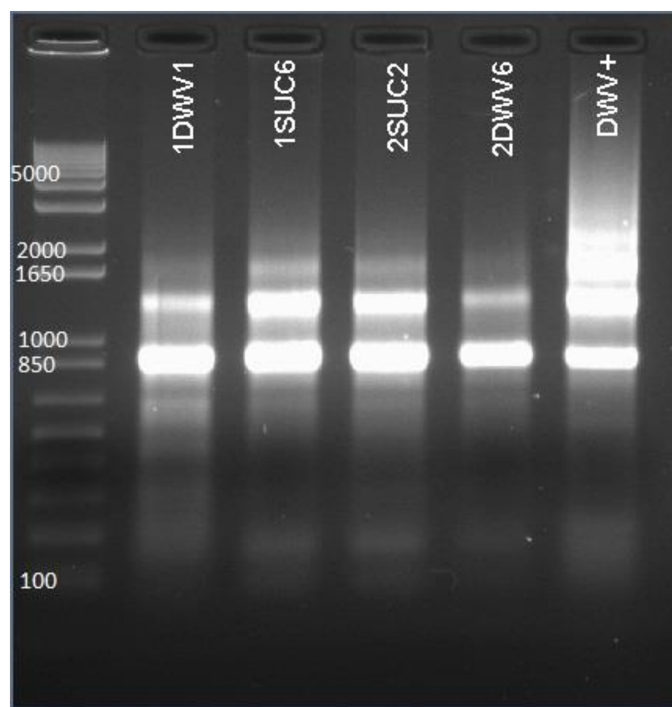

Figure 3-6. RNA quality check of random samples.

Whole RNA from individuals from 4 randomly chosen cages and the positive control was run on a non-denaturing gel. 1DWV1 is an infected individual from cage 1 from Cohort 1. 1Suc6 is an uninfected individual from cage 6 in cohort 1, fed only sucrose. 2SUC2 is an uninfected individual from cage 2, Cohort 2 and fed only sucrose. 2DWV6 is an uninfected individual from cage 6, Cohort 2 and was orally inoculated with DWV.. The two bands aligning with DNA standards of about 850 bp and 1500 bp are the 18S and 28S ribosomal subunits. The 28S:18S intensity ratio is below the expected 2:1, likely due to the known fragility of the insect 28S subunit which tends to break into two pieces that co-migrate with the 18S subunit.

Table 3-1 Primer pairs used in this study.

Virus/Gene	Primer Pair	Product Size (bp)	Target	Reference
Honey Bee Actin	F TTGTATGCCAACACTGTCCTTT R TGGCGCGATGATCTTAATTT	98	Beta-actin	(Simone et al. 2009)
Bombus Actin	F GGAGAAACTTTGTTACGTCGCC R CGCACTTCATGATCGAGTTG		Beta-actin	Amsalem, personal communication
DWV	F TCCATCAGGTTCTCCAATAACGGA R CCACCCAAATGCTAACTCTAAGCG	450	RdRp	Yue and Genersch 2005

	F ACAAGTTGGAGTTCACTATC R CTAAAGGTACATTCATACATAAG	95	IRES	Moore et al 2011
	F ATCAGCGCTTAGTGGAGGAA R TCGACAATTTTCGGACATCA	702	Helicase	Chen et al 2005
Abbreviations: Deformed wing virus (DWV), RNA dependent RNA polymerase (RdRp), Internal Ribosomal Entry Site (IRES) References: Simone et al. (2009) The Society for the Study of Evolution 63(11):3016-3022; Chen et al. (2005) Applied and Environmental Microbiology 71(1):436–441; Moore et al. (2011) Journal of General Virology 92: 156-161; Singh et al. (2010) PLoS ONE 5(12): e14357. doi:10.1371/journal.pone.0014357; Yue and Genersch. (2005) Journal of General Virology 86: 3419-3424				

Table 3-2 PCR data by sample

Sample	Colony	PCR with RdRp primers	qRT PCR with IRES primers	qRT PCR with Helicase primers	qRT-PCR with Actin primers
Cohort 1					
Honey Bee Extract		P	undet	26.316	19.18
Negative Control		-	undet	undet	36.4
Positive Control		P	undet	22.22	16.36
C1.1	**3**	-	undet	undet	15.933
C1.2		-	undet	undet	16.062
C1.3		-	undet	undet	15.915
C2.1	**5**	-	undet	undet	15.432
C2.2		-	undet	undet	15.495
C2.3		-	undet	undet	14.691
C3.1	**3**	-	undet	undet	15.620
C3.2		-	undet	undet	15.183
C3.3		-	undet	undet	15.328
C4.1	**3**	-	undet	undet	14.567
C4.2		-	undet	undet	15.176
C4.3		-	undet	undet	15.245
C5.1	**5**	-	undet	undet	14.710
C5.2		-	undet	undet	14.779
C5.3		-	undet	undet	15.164
C6.1	**5**	-	undet	undet	14.670
C6.2		-	undet	undet	14.690
C6.3		-	undet	undet	14.843
T1.1	**5**	-	undet	undet	15.318
T1.2		-	undet	undet	14.518
T1.3		P	undet	undet	14.221
T2.1	**3**	-	undet	undet	14.390
T2.2		-	undet	undet	14.417
T2.3		P	undet	undet	15.872

T3.1	3	-	undet	undet	16.035
T3.2		-	undet	undet	16.280
T3.3		P	undet	undet	14.825
T4.1	5	-	undet	undet	14.793
T4.2		-	undet	undet	14.910
T4.3		P	undet	undet	15.165
T5.1	3	P	undet	undet	15.263
T5.2		-	undet	undet	15.526
T5.3		-	undet	undet	14.346
T6.1	5	P	undet	undet	14.627
T6.2		-	undet	undet	14.581
T6.3		-	undet	undet	14.873
T7.1	5	P	undet	undet	14.871
T7.2		P	undet	undet	14.898
T7.3		P	undet	undet	14.152
Sample	**Colony**	**PCR with RdRp primers**	**qRT PCR with IRES primers**	**qRT PCR with Helicase primers**	**qRT-PCR with Actin primers**
Cohort 2					
Colony A Extract		P	undet	undet	22.830
Colony B Extract		P	undet	undet	22.780
Negative Control		A	undet	undet	36.400
Positive Control		P	undet	undet	16.360
C1.1	**3**	-	undet	undet	16.354
C1.2		-	undet	undet	15.286
C1.3		-	undet	undet	15.879
C2.1	**5**	-	undet	undet	16.043
C2.2		-	undet	undet	15.878
C2.3		-	undet	undet	14.478
C3.1	**5**	-	undet	undet	14.587
C3.2		-	undet	undet	14.635

C3.3		-	undet	undet	15.446
C4.1	**3**	-	undet	undet	14.973
C4.2		-	undet	undet	15.109
C4.3		-	undet	undet	15.133
C5.1	**5**	-	undet	undet	15.300
C5.2		-	undet	undet	15.303
C5.3		-	undet	undet	14.717
C6.1	**3**	-	undet	undet	14.583
C6.2		-	undet	undet	14.831
C6.3		-	undet	undet	14.616
T1.1	**5**	-	undet	undet	14.983
T1.2		-	undet	undet	14.738
T1.3		-	undet	undet	14.703
T2.1	**3**	-	undet	undet	14.873
T2.2		-	undet	undet	14.816
T2.3		-	undet	undet	14.879
T3.1	**5**	-	undet	undet	15.242
T3.2		-	undet	undet	15.371
T3.3		-	undet	undet	14.637
T4.1	**3**	-	undet	undet	14.687
T4.2		-	undet	undet	14.686
T4.3		-	undet	undet	14.541
T5.1	**5**	-	undet	undet	14.790
T5.2		-	undet	undet	14.739
T5.3		-	undet	undet	15.920
T6.1	**3**	-	undet	undet	15.937
T6.2		-	undet	undet	14.733
T6.3		-	undet	undet	14.685
T7.1	**5**	P	undet	undet	14.656
T7.2		-	undet	undet	15.441
T7.3		-	undet	undet	1[illegible].792

Here PCR data is presented by sample, by cohort, and by source colony. RT-PCR samples were imaged on 1% agarose gels and observed for presence or absence of a band corresponding to DWV. If it was present, here the sample is denoted with a P, otherwise a negative is denoted with '-'. For all real time data, Ct values are reported where given, otherwise the sample is denoted as having no signal with 'undet' meaning undetermined.

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Chapter 4
Conclusions and Future Directions

Beekeeping is an ancient vocation that has provided us with nutrition and some of the earliest form of industry (Bloch et al. 2010)). Even in modern agriculture, the services and goods provided to us by managed colonies are invaluable. Despite our long-standing history with apiculture, we are still parsing out the most effective management methods. This is particularly true in the face of a wide array of emerging and re-emerging threats facing bees including pesticides, pathogens, parasites, poor nutrition (often attributed to our monoculture cropping system), and our management practices. In this thesis I started investigating two of these factors by asking how management practices and disease impact hive health in Kenya, and how pathogen transmission between honey bees and non-Apis bee species influences the virulence and population dynamics of that pathogen.

The industry of East Africa, including Kenya, relies primarily on agriculture. Due to this, many families live on smallholdings where they also keep bees as an additional income and nutrition source. However, it is generally agreed upon that these smallholders are not utilizing honey bees to their maximum potential. Therefore, there has been a longstanding push to incorporate Western apicultural practices into East Africa. Our results here indicate that this may not be ideal, specifically in the Mwingi district of Kenya. In this district, the primary factor controlled by the beekeeper is the hive type used, where beekeepers select from the Western Langstroth (Lan), Kenya Top Bar (KTB) or the

Traditional Log (Log) hive. Thus, we investigated the effect of hive type on colony behavior and health.

We found a significant impact of hive type of hive occupation rates, and this seemed to be more related to the volume of the hive cavity. We found no effect of hive type on pathogen and parasite loads. Interestingly we did not find an effect of pathogens or parasites on colony health or longevity.. We did find levels of Varroa mites increase over time, which suggest that absconding behavior may serve as an important behavioral defense mechanism against brood-parasites by breaking the brood cycle and leaving pathogens and parasites behind in the old nest. Given considerations of cost, hive body durability and lifespan, ease of use and honey extraction, and the amount of honey produced, our recommendation is that beekeepers use redesigned KTB hives. To address issues of attractiveness to migrating swarms and previously recorded complications with thermoregulation, we suggest a KTB be fitted with A) a moveable division board and B) a white painted corrugated iron top cover with soft timber insulation. Future studies need to be done to evaluate such a modified hive and to precisely evaluate the cost/benefit of each hive type, accounting for initial costs and honey production.

The parasites and pathogens that we investigated were likely newly introduced to Kenya over the last decade. Thus, large-scale epidemiological studies should be done to fully characterize the ecology and the impact of the pathogens and parasites on East African honey bee populations, as the impacts may change with time. We did find a significant increase in DWV infected colonies in the dry season following the first wet season the colonies experienced in this study. This may imply that there are some ecological trends

that diseases follow in Western honey bee populations as well as East African populations, despite very different environments and management styles. Namely, that periods of nutritional stress (Winter in the West and the period of dearth in East Africa) may correlate with higher incidence of pathogens.

In Chapter 3 of this thesis we went on to investigate the effect of cross-species transmission on the virulence and population dynamics of a common honey bee virus, DWV. Several recent studies have demonstrated that populations of wild *Bombus* species are becoming infected with this virus in the US and Europe. However, how these cross-species infections affect the ecology and evolution of DWV has not been explored. Interestingly, studies have shown the DWV strains segregate according to geographic region rather than host, suggesting that the viruses, though competent to replicate in bumble bee hosts, are not established as independent populations within these host. Instead, DWV population in bumble bees may be generated by continuous re-infection from honey bees.

Our results from this study are preliminary but suggest that virions produced by an infected bumble bee may be less infectious than those being shed by an infected honey bee, and therefore the former may be outcompeted by the latter. However, our sample size was small and the analysis was complicated by differing results from different primers. Thus, this study should be repeated using a greater number of biological replicates (in terms of both honey bee viral isolates and independent passages through a larger number of bee species), Furthermore, the viral quasi-species should be characterized before and after infecting bumble bees. Increasing the number of replicates

will also confirm or refute that indeed virus isolated from bumble bees is less infectious than that which is isolated from deformed honey bees. Furthermore, a third treatment group should be included in Cohort 2, which is given the original honey bee-derived isolate to control for trial biases arising from things like source-colony age differences (i.e. source colonies were older at the time of collection of Cohort 2).

On a broader level, the results of these studies suggest that we reevaluate how we currently manage honey bees (i.e. high population densities, frame swapping, monoculture crop fields, pesticide exposure, miticide usage, etc). This includes a need for more investigations on how apiculture is impacting disease ecology and, conversely, the role that native bees play in the ecology of these diseases. One concern is that modern beekeeping may be selecting for more virulent pathogens which then may have a greater health impact on native bees while also threatening our managed honey bee populations. If DWV populations produced in honey bees are indeed more virulent than populations produced in bumble bees, it may be due to current apicultural practices (e.g. high population densities, swapping of frames, combining of colonies etc). These might select for more virulent pathogens, as these practices lower the barrier to transmission, which thus increases within-host virulence (Ebert 1998). To test the effects of management practices on pathogen virulence and incidence, a controlled field study would have to be run. Specifically, an experiment could be conceived where one group of honey bee colonies is managed normally, one group in high-density with high frame movement between colonies, and a third group with very low density of hives and little management.

The spillover of these pathogens represents not only a major threat to food security and agriculture, but also to the ecosystem at large, as previous research shows that removing even a single pollinator from a system reduces the fitness of constituent flowering plants. Therefore, we must push forward in describing the extent to which native pollinators are being infected by these pathogens, and the extent to which these pathogens may be associated with changes in species ranges or population health.