Detection of pathogen spillover between managed honey bees (*Apis mellifera* L.) and native pollinators (*Bombus spp.*) through quantification of RNA viruses

by

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Dedicated to my grandfather,

Michael Hyska, who has always been looking

Out for me to complete this thesis

And my grandmother, Catherine Hyska,

Who is always looking out for him

And to my parents,

Brian Hyska, Lori Robson and Jude Davies

Who are responsible for getting me here.

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Abstract

RNA viruses are a contributor to recent honey bee disappearances and may have spilled over to bumble bees from honey bees (HB). This hypothesis is addressed by comparing the prevalence and intensity of seven viruses in bumble bees captured in proximity to and isolation from managed honey bee colonies. Additionally, sampling method for bees and different storage variables are compared for accuracy in estimating viruses in field-caught specimens. Prevalence was lower in isolated bumble bees for DWV, BQCV and SBV and higher for IAPV. KBV, ABPV and CBPV infections were rare. Virus intensity was higher in HB-exposed sites than unexposed sites for one virus and never higher in bumble bees than in honey bees. This suggests that spillover is likely, but viral dynamics are complicated and movement may occur in both directions. Additionally, specimens should be stored at -80^{o}C with no medium recommended for relative preservation of host and viral RNA.

General Introduction

Honey bee viruses are thought to be in the process of spilling over from managed honey bee colonies into native North American pollinators such as bumble bees, and these viral infections may relate to recent range reductions and disappearances of bumble bee species. Several picorna-like RNA viruses first characterised in honey bees such as Deformed Wing Virus (DWV), Black Queen Cell Virus (BQCV), Kashmir Bee Virus (KBV), Sacbrood Virus (SBV), Israeli Acute Paralysis Virus (IAPV), Acute Bee Paralysis Virus (ABPV) and Chronic Bee Paralysis Virus (CBPV) have been detected in bumble bees worldwide, and though research has been done to look at possible transmission routes such as shared floral resources and other insect vectors, little is actually known about the epidemiology of these viruses. The research reported in this thesis attempts to address the theory of pathogen spillover from honey bees to bumble bees and investigates different methods for handling field-caught specimens in order to maximize their accuracy in analyzing and comparing quantities of RNA. The thesis is written in paper style with a general literature review (chapter 1), two manuscripts (chapter 2 and 3), and a general discussion (chapter 4). The literature review provides background and context for the research contained within the thesis, as well as the rationale for the importance of this study. The two manuscripts are written for publication in peer reviewed journals. The general discussion provides a synthesis and deliberation of resultant conclusions and suggests future directions for research in this field.

Chapter 1 serves as an introduction to my thesis in which I review the body of knowledge on the effects picorna-like RNA viruses have on their honey bee hosts and the current theories as to their contribution to recently reported bee disappearances. I cover published theories on the transmission of these viruses and theories of pathogen spillover. I also review the importance of bee pollination services and the current information on how best to study RNA viruses in field-caught specimens.

Chapter 2 was a study developed to determine the likelihood of pathogen spillover from managed honey bee colonies to native bumble bee species by quantifying viral copies in field-caught specimens and comparing populations in terms of prevalence and intensity of infection with seven different RNA viruses. Bumble bees captured in proximity to sampled honey bees were theorized to have lower viral prevalence and higher viral intensity than associated honey bees and higher viral prevalence and lower viral intensity than bumble bees captured in isolation from infected honey bees, if the tenets of basic pathogen spillover were to be met. DWV and SBV fit this model in terms of prevalence, but BQCV was detected in 100% of all honey bee and honey bee-exposed bumble bee specimens and IAPV was more prevalent in isolated bumble bees than in those exposed to honey bees. Contrary to expectations, average intensities in bumble bees were never higher than in honey bees.

Chapter 3 was a two-part study investigating capture and storage techniques for their accuracy and efficacy in preserving RNA for viral detection and quantification studies. The first part dealt with the effects different storage temperatures (-20^{o}C and -80^{o}C), storage media (95% ethanol, RNAlater™, and no medium) and wait times until storage (up to 48hrs) had on the relative preservation of host and viral RNA. It was determined that although DWV and BQCV preserved well under most treatment combinations, specimens should be stored at -80^{o}C without the use of storage medium in order to ensure the best relative preservation of both viral and host RNA needed for proper PCR analysis. Additionally, although viruses were still detectable up to 48 hours until storage in order to get the most accurate estimates of viral titres they should be stored as soon as possible after collection. The second part dealt with the possibility that passive bee bowl traps, commonly used for collecting field specimens, may act as a contamination source between uninfected and infected bees sharing the same bowl. Experimental trials determined that uninfected alfalfa leafcutting bees did not test positive for DWV and BQCV as a result of sharing a bee bowl with infected honey bees. BQCV primers did however detect what is possibly a unique RNA target in leafcutting bees used in these experiments.

Chapter 4 is a general discussion of the research contained within this thesis and suggestions for future research directions.

CHAPTER 1. Literature Review

Honey Bee Losses or Colony Collapse Disorder

Beekeepers have been dealing with overwintering losses of their honey bees for as long as they have been keeping them (Oldroyd 2007). These losses have been attributed to pesticides, habitat destruction, climate change and various pests and pathogens (Neumann and Carreck 2010) including mites, fungal spores, bacteria and viruses. Over the winter of 2006-2007, a decidedly new phenomenon occurred in the US, that resulted in mass disappearances of worker bees that left behind relatively empty hives filled with abandoned brood and food stores and, most surprisingly, a suspicious absence of dead adult bees (Hayes Jr et al. 2008, Evans et al. 2009, Ellis et al. 2010). Reports of these losses by beekeepers were widespread across the states, with some losses reported at near 100% (Oldroyd 2007). Historically, occasional periods of high percentage losses are not unheard of, but having such a high proportion of beekeepers affected was uncommon (Ellis et al. 2010). During the following winter more than 750,000 colonies were estimated to have collapsed with similar symptoms (Hayes Jr et al. 2008). These enigmatic symptoms associated with the losses were named colony collapse disorder, or CCD (Ellis et al. 2010, Ratnieks and Carreck 2010).

Following the initial reports, controversy immediately arose as to whether or not this represented a legitimate new problem or was simply a resurgence of a previously well-described disease outbreak, such as the “Isle of Wight” disease from 1906 (Neumann and Carreck 2010). Reports in general media, sparked great alarm as to what this might mean for

large agricultural industries that are maintained through pollination services provided by bees. Globally, insect pollination services are valued at more than 160 billion USD annually, or in other words 9.5% of the agricultural market for human food (Gallai et al. 2009). Upwards of 130 billion USD can be attributed to the contributions of bees to the pollination of various fruit, vegetable, nut, stimulant and edible oil crops (Gallai et al. 2009, Ellis et al. 2010). These fruit, vegetable and seed crops account for 87 important global food crops contributing 35% of global food production by weight, and are often almost totally reliant (~90%) on bee pollination services (Klein et al. 2007). Almonds, for example, are 100% reliant on insect-pollination and valued at 2 billion dollars annually alone (Ratnieks and Carreck 2010). Additionally, one cannot discount the indirect influence insect pollination has on the pollination of fodder crops, such as clover and alfalfa, for livestock (Ellis et al. 2010). It can be argued that massive amounts of overwintering loss currently attributed to colony collapse disorder may drastically decrease the economic viability of beekeeping, and that there may be drastic effects on these crops currently reliant on bees for pollination. Therefore, studies as to the causes of this "mysterious phenomena" are duly warranted.

It is also important to note that, though the term CCD was created to describe the particular set of symptoms observed in the United States to characterize a specific type of colony loss, high losses are also occurring in Canada, Europe, the Middle East and Japan (Neumann and Carreck 2010) where those specific symptoms are not observed. In Germany, for instance, some beekeepers started experiencing high levels of winter mortality (near 100%) in 2002/2003 (Genersch et al. 2010). Much more so now than in the past, honey bees are traded worldwide, and this global movement has been hypothesized to have contributed to the

problem. Pathogens, which certain species or populations of honey bee may have evolved resistance to, are now being spread to naïve populations as a result. In some cases, as with viral infections, parasites and pathogens may be working synergistically to activate latent infections in new hosts, bringing about mass bee deaths (Martin et al. 2012). Due to the fact that large beekeeping operations are often the most affected (Hayes Jr et al. 2008) and colonies in proximity to those experiencing symptoms of CCD are more likely to eventually exhibit these symptoms themselves (Evans et al. 2009), it was thought likely the cause was contagious.

Israeli Acute Paralysis Virus (IAPV) in particular was tied to CCD, being found more often in colonies exhibiting the symptoms of CCD than those without it, whereas most other viruses existed across all colony types (Cox-Foster et al. 2007). However, this did not represent a causal link and was problematic in that the diagnostic symptoms for CCD were not unique enough to the disorder (Anderson and East 2008). Another study found increased levels of Black Queen Cell Virus (BQCV), Deformed Wing Virus (DWV), Kashmir Bee Virus (KBV), and Acute Bee Paralysis Virus (ABPV), as well as the non-viral pathogens *Nosema apis* and *Crithidia mellificae* in CCD colonies, and argued that synergistic relationships between these pathogens are truly detrimental for the honey bee (Cornman et al. 2012), but the same study did not find increased levels of IAPV in dying colonies. Pesticide residues and mites also were not more concentrated in CCD colonies suggesting they are not a primary cause of the disorder in and of themselves (Evans et al. 2009). Work by Desai et al. (2016) showed that different wintering environments could also interact with pathogenic infections to increase bee mortality over the winter, suggesting that environmental factors may also complicate the picture.

On a more global scale, winter losses reported in Switzerland and Germany are attributed to infections with Acute Bee Paralysis Virus (ABPV) (Berthoud et al. 2010) and Deformed Wing Virus (DWV) linked with varroa mite infection (Berthoud et al. 2010, Genersch et al. 2010, Dainat et al. 2012a). In Denmark infection with ABPV, KBV and IAPV appeared to be a more predictive marker of colony death than DWV (Francis et al. 2013). This study showed that varroa mite infestation often led to high viral titres in bees, but was not always required for the virus to reach high levels. When considering the northern hemisphere as a whole, simultaneous infection with DWV and *Varroa destructor* (Anderson and Trueman) is most commonly linked with overwintering losses (Dainat et al. 2012b, Desai et al. 2016).

As most studies cannot agree on any single cause of colony loss, it is generally believed that a combination of stressors are involved (Potts et al. 2010). However, most studies do agree that viruses are in some way involved (Genersch and Aubert 2010). Indeed, even bees from "CCD colonies" not only have higher viral loads, but often exhibit a larger diversity of pathogenic infections (Evans et al. 2009, Cornman et al. 2012).

Worldwide Bumble Bee Disappearances

Although honey bees receive the majority of the media coverage, dwindling populations of several bumble bee species have been reported worldwide as well, with declines beginning far sooner than the advent of colony collapse disorder (Goulson et al. 2008). Unfortunately, government statistics on the status of wild non-*Apis* pollinators are lacking (Meffe 1998) with several species in need of protection without the legislation and documentation to grant it to them (Winfree 2010). Bumble bees are abundant, efficient generalist pollinators responsible for

pollinating a large range of generalist and specialist plants, so their threatened status would likely have a large effect on floral communities relative to less common pollinator guilds (Memmott et al. 2004, Goulson et al. 2008). Memmott et al. (2004) show through their models that this expected reduction in plant diversity should not be more than a linear relationship with disappearing bee species, which makes sense given pollinator redundancy in the system (Potts et al. 2010). Since bees do not represent all pollinating insect species, alarm due to their reported disappearances is somewhat sensationalized and does not necessarily spell the doom for agricultural industries and wild ecosystems that some are presupposing (Ghazoul 2005). For example, although bee species are in decline in the Netherlands, British hoverflies, another wild insect pollinator, appear to be increasing in diversity (Biesmeijer et al. 2006, Stokstad 2006). However, despite the fact that bees are not the only pollinators to receive threatened status, and even though it is hard to quantify the exact economic contribution of pollination services provided by bees, their significant contribution to pollination should not be of minor concern (Steffan-Dewenter et al. 2005).

Worldwide there are 250 species of bumble bee, many of which are now in decline, and several of which are listed as endangered (Williams and Osborne 2009). Most is known about bumble bee declines for European species where good historical records of species distributions exist. The UK has seen significant range contractions of selected southern *Bombus* species since the 1960s (Williams 1982). In Hungary, almost half of their native species are in decline, with ten species labelled as endangered (40%), seven of which are critically so (Sárospataki et al. 2005). In Britain and Ireland, late emerging species like *B. distinguendus, B. ruderarius* and *B. sylvarum* have been undergoing drastic declines since the 1980s (Fitzpatrick

et al. 2007). While several species are in decline this does not mean that bumble bees in general are at risk but simply that the species assemblage is shifting (Sárospataki et al. 2005, Fitzpatrick et al. 2007). What this might mean for natural landscapes and native plant species reliant on insect pollination is speculative at best (Meffe 1998), but is no less important than the economic consequences of the loss of commercial pollination services. In the UK, wild insect pollinated plants are already exhibiting decline, with wind and water pollinated species filling the void (Stokstad 2006). Additionally, though it would prove difficult to quantify, reduction in "free" pollination services provided by wild bees would likely lead to lower yields of some agricultural crops (Meffe 1998). For example, wild pollinators increase the efficiency of managed honey bees pollinating hybrid sunflowers (Greenleaf and Kremen 2006), so their removal should result in yield loss.

In North America, some species such as *B. affinis, B. terricola, B. frankilini, B. occidentalis* and *B. pensylvanicus* have demonstrated drastic declines in range (Goulson et al. 2008, Brown 2011, Cameron et al. 2011). In Southern Ontario specifically, these species and five others have declined during the last 35 years (Colla and Packer 2008). Like in Britain, this represents roughly half of Southern Ontario's *Bombus* species. In Illinois, *B. terricola* as well as *B. variabilis, B. borealis* and *B. ternarius* have been extirpated from the area (Grixti et al. 2009), whereas *B. ternarius* is increasing its range in other parts of North America (Colla and Packer 2008). These declines are thought to coincide with shifts in habitat use for agriculture, though it is thought pesticides and pathogens could still be involved (Grixti et al. 2009). Shifts in latitudinal thermal limits on species' ranges brought about by climactic changes are also likely to have had an effect on these range changes (Kerr et al. 2015).

Bees are vulnerable and have a high risk of extinction, stressors aside, due to their haplodiploid mode of reproduction resulting in decreased genetic diversity, their reliance on the floral resources of pollen and nectar for survival and their utilization of building materials from diverse localities (Potts et al. 2010, Winfree 2010). Declining bee species are correlated with late emergence, narrow specialized ranges, and being at the edges of their ranges (Goulson et al. 2005, Williams and Osborne 2009). In Europe and Asia, bee disappearances have been attributed most frequently to habitat loss and reduction in suitable forage (Edwards and Williams 2004, Goulson et al. 2005, Fitzpatrick et al. 2007), but general effects of climate change and certain pesticides (neonicotinoids) have been investigated as well (Potts et al. 2010). Likewise, in Brazil extirpation of the native species, *B. bellicosus* is associated with habitat destruction and climate change (Martins and Melo 2010). In North America, it is thought the likely culprits are the same pathogens attributed to honey bee losses (Williams and Osborne 2009, Winfree 2010, Fürst et al. 2014). Cameron et al. (2011) link bumble bee declines to infection with the gut parasite *Nosema bombi.* Brown (2011) ascertains that pathogens are likely to be driving declines all over the world. In a study by Szabo et al. (2012) testing the theory of pathogen spillover from commercialized greenhouse bumble bees, at risk bee species declined when sharing environments near greenhouses. This data is consistent with pathogen spillover as a driver of decline, and though it did not completely explain these declines they found no support for pesticide use or habitat loss as causes. Though pesticides could affect bees as individuals, it did not appear to lead to declines in their range, and many of the bees tested were from urban areas where they would have already suffered from drastic habitat fragmentation (Szabo et al. 2012).

It is not unreasonable to think that with heavily infected honey bees being transported within North America on a regular basis (Graystock et al. 2013b), they may be spreading their pathogens into native North American populations. Varroa mites, which are heavily implicated in the collapse of honey bees, are not known to affect bumble bees. But, a suite of picorna-like RNA viruses that had previously been characterized in honey bees, have been consistently found to additionally infect bumble bees, making these viruses a likely culprit in bumble bee losses (Bailey and Gibbs 1964, Potts et al. 2010, Singh et al. 2010, Fürst et al. 2014, McMahon et al. 2015, Dolezal et al. 2016, Tehel et al. 2016). This notion is further supported by evidence that the same genetic strain of Deformed Wing Virus is found in honey bees and bumble bees caught in proximity to each other (Fürst et al. 2014).

RNA Viruses

There are currently 24 viruses that have been described in honey bees, 16-18 of which are likely distinct species (de Miranda et al. 2013), many of which have been identified in bumble bees and other pollinating insects as well (Singh et al. 2010, Manley et al. 2015, McMahon et al. 2015, Dolezal et al. 2016). The majority of them are RNA-like in structure and fit within a couple of common families of insect-infecting viruses within the superfamily Picornaviridae (Grabensteiner et al. 2001, Palacios et al. 2008). Several belong to the newly described family of invertebrate viruses, the Dicistroviridae (Mayo 2002). All are somewhat broadly related to picorna-like viruses found in a wide range of plants and animals (Evans and Hung 2000, Grabensteiner et al. 2001).

Honey bee viruses often exist as latent, low-level infections maintained within "healthy" bees (Bailey 1967, Bailey and Woods 1974, Dall 1985, Chen et al. 2006b, Genersch and Aubert 2010, de Miranda et al. 2013) and were not detected in great prevalence until the refinement of qPCR methodology. This may also have been due to the fact, that under the electron microscope, they all have the same spherical to oval appearance consisting of a single-strand of RNA adorned with capsid proteins (Chen et al. 2006a). As a group, these viruses are widespread in the host tissues, including the hemolymph, gut, body tissues, ovaries and spermatheca. (Chen et al. 2006b). Though their visible symptoms sometimes differ greatly, all are suspected to result in decreased survivorship of the colony (Martin 2001), especially when seen in combination with other pathogens or stressors (Bakonyi et al. 2002). Additionally, infection with more than one virus commonly occurs (Shen et al. 2005a, Shen et al. 2005b, Baker and Schroeder 2008, Welch et al. 2009, Singh et al. 2010, Runckel et al. 2011, Desai and Currie 2016, Desai et al. 2016), and low temperatures are thought to increase their lethality (Prisco et al. 2011). The following are seven of the most commonly studied RNA viruses in honey bees.

Deformed Wing Virus (DWV)

Deformed Wing Virus (DWV), a member of the genus *Iflavirus*, is one of the most common viruses discovered in both honey bees and bumble bees worldwide (Allen and Ball 1996, Tentcheva et al. 2004, Genersch et al. 2006, Baker and Schroeder 2008, Genersch and Aubert 2010), although it was first isolated in the early 1980s in Japan (Allen and Ball 1996). Visible symptoms include the development of malformed and ostensibly useless wings, bloated abdomens, and similar paralysis symptoms associated with infection with several other honey

bee viruses (Lanzi et al. 2006, De Miranda and Genersch 2010, Fürst et al. 2014). Asymptomatic colonies of honey bees may still harbour viral titres greater than $1x10^8$ copies per bee making them prone to increased winter mortality (Highfield et al. 2009).

Amongst honey bees, which are thought to be its original host (Wilfert et al. 2016), DWV is found in all life stages, including symptomless adults (Chen et al. 2005). Chen et al. (2005) found that concentrations of the virus were highest in the pupal stages and lowest in adult drones, where infection was also the least prevalent. In fact, it is hypothesized that pupae must contain a threshold level of viral infection in order for visible symptoms to appear at the colony level (Bowen-Walker et al. 1999). Suppression of virus titres using RNAi reduces the proportion of bees with deformed wings and increases honey bee survival (Desai et al. 2012). Infections within adult bees are often sequestered to the head and gut regions, although the virus can also be found in the thorax and wings of bees presenting wing deformities (Lanzi et al. 2006). However, in asymptomatic infections, virus is not detectable in the head (Yue and Genersch 2005) in honey bees or bumble bees (Genersch et al. 2006). Also, within queen and drone bees it can be found in the reproductive structures (Fievet et al. 2006). Infection with low levels of virus in the adult stage does not typically affect survivorship but results in them becoming carriers until their death (Martin 2001). Viral titres of DWV tend to increase in the fall in association with varroa, shortly before the overwintering period, and so are thought to be a good predictor for overwintering losses (Gauthier et al. 2007, Prisco et al. 2011, Runckel et al. 2011, Dainat et al. 2012b, Nazzi et al. 2012, Desai and Currie 2016).

The full genome of DWV was first sequenced by Lanzi et al. (2006) and is 10140 nucleotides in length with a single large open reading frame (ORF) at the 3' end encoding three

important structural proteins, followed by a poly(A) tail. Sequence comparisons put the virus squarely within the genus *Iflavirus* (Iflaviridae) (Lanzi et al. 2006).

In honey bees, DWV is closely associated with the mite, *V. destructor* (Allen and Ball 1996, Martin et al. 2012), with a particular strain of this virus, named VDV-1, replicating within the mites themselves (Ongus et al. 2004). In the absence of varroa, DWV often exists as an asymptomatic and covert infection at the colony level (Sumpter and Martin 2004, De Miranda and Genersch 2010, Prisco et al. 2011). It can also be transmitted in contaminated larval food (Yue and Genersch 2005).

Black Queen Cell Virus (BQCV)

Black Queen Cell Virus (BQCV) is characterized by deaths in immature honey bee queens and typically presents as blackened, rotting queens within their rearing cells (Bailey and Woods 1977). Viral titres tend to peak in mid-summer and decrease in the later fall months (Gauthier et al. 2007, Runckel et al. 2011) and mainly infect adult bees (Tentcheva et al. 2004). Within bumble bee hosts (*Bombus huntii)*, infection is widespread throughout the tissues of wild caught adults, including drones (Peng et al. 2011) but infected bees are asymptomatic.

The full genome of BQCV was first sequenced by Leat et al. (2000) and is 8550 nucleotides (nt) long with a poly-A tail and two Open Reading Frames (ORFs). Sequence comparisons grouped it with other picorna-like viruses with insect hosts such as the *Drosophila* C virus (DCV) (Leat et al. 2000), in the viral family Dicistroviridae (Bonning 2009). The major structural, or capsid, proteins are formed from the 3' end ORF (Evans and Hung 2000, Leat et al.

2000), whereas it is thought that the replicase protein is encoded by the 5' ORF (Leat et al. 2000).

KBV-IAPV-ABPV Complex

The three viruses (KBV, ABPV and IAPV) exist as a distinct complex within the Dicistroviridae and the Genus *Aparavirus* (Bonning 2009). The full genome of KBV was first sequenced by de Miranda et al. (2004) and is 9506 nt long with two ORFs and a poly(A) tail. The major structural proteins are formed from the 3' end (Evans and Hung 2000, De Miranda et al. 2004). The full genome of ABPV was first sequenced by Govan et al. (2000) and is 9470 nt long consisting of two ORFs separated by a short 184 nucleotides with an additional poly-A tail. The major structural, or capsid, proteins are encoded by the 5' ORF, whereas the replicase protein is encoded by the 3' ORF. Sequence comparisons grouped it with cricket paralysis-like viruses (CPV) (Govan et al. 2000). The full genome of IAPV was first sequenced by Maori et al. (2007) and is 9487nt in length with 2 ORFs, similar to the other members of the Dicistroviridae.

One of the first bee viruses to be described, Kashmir Bee Virus (KBV) was first discovered in India (Bailey and Woods 1977) and has multiple strains (Hung et al. 2000, De Miranda et al. 2004). Visible symptoms of infection with this virus are minor twitching and occasional paralysis. However, the virus most often persists as a latent infection that may increase in the later fall months (Tentcheva et al. 2004, Ward et al. 2007, Runckel et al. 2011). It can also persist in dried fecal material for at least four days (Hung 2000). When it does become virulent, however, it kills its host within 3 days, acting much quicker than other honey bee

viruses (Bailey et al. 1979). Lethality of this virus, when it does occur, appears to be triggered by the varroa mite (Ward et al. 2007).

Acute Bee Paralysis Virus (ABPV) has long since been categorized in honey bees and studied extensively in the laboratory setting (Bailey and Gibbs 1964). Despite this, it is not typically found in high prevalence in the field, often persisting at low viral titres (~$1x10^5$ copies per bee) until the onset of symptoms (Highfield et al. 2009, Genersch and Aubert 2010). This is perhaps owing to the fact that lethal symptoms act within 5-6 days of the virus reaching high levels of infection within the individual (Bailey et al. 1963, Bailey and Gibbs 1964, Martin 2001). Symptoms include trembling and an acute form of the paralysis associated with the other paralysis viruses, the onset of which is simply more rapid. Viral titres of ABPV appear to be lowest in the early summer months (Ball and Allen 1988, Gauthier et al. 2007), peaking in August and dropping off in the late fall (Tentcheva et al. 2004, Runckel et al. 2011).

Given the rapid onset of ABPV symptoms it may be hard to capture a bee in the field exhibiting high intensity of infection. Though there is no existing proof to support it as a causative agent of CCD, its rapid onset fits the symptoms. ABPV can be spread through fecal material, where the virus particles can remain viable for months (Bailey and Gibbs 1964). Additionally, varroa mites and contaminated food fed to larvae by infected adult carriers are probable transmission pathways (Ball and Allen 1988).

Israeli Acute Paralysis Virus (IAPV) is the most recently characterized virus of those discussed in this review, and was only recently deemed a separate virus (Maori et al. 2007). Discovered in Israel in 2004 (Maori et al. 2007), it has since been sequenced from bee samples around the world, and was even deemed a causative agent of Colony Collapse Disorder in the

United States of America (Bonning 2009) although IAPV infections have been detected in US colonies prior to the onset of CCD (Chen and Evans 2007). Visible symptoms include the trembling and paralysis associated with the other paralysis viruses, as well as hair loss on the thorax and a darkening at the tip of the abdomen (Maori et al. 2007). Viral titres tend to increase in the later fall months (Runckel et al. 2011).

Sacbrood Virus (SBV)

Sacbrood Virus (SBV) exists as symptomless infections among adults in the hive, which can pass it on to new generations of offspring through hypopharyngeal gland secretions (Bailey 1969). Lethal symptoms occur only in the larval stages and involve halted growth and ultimately, petrification of the brood. Infection results in larvae that look like a fluid-filled "sac" and end up in the cell as a hard brown scale (Bailey 1969, Grabensteiner et al. 2001). Unapparent infections of SBV occur regularly in pupae (Dall 1985). The virus is found to be more prevalent in adults (Tentcheva et al. 2004), perhaps as a direct result of its only being lethal to the larval stage, though titres are roughly equal on average between infected pupae and adults (Gauthier et al. 2007). Viral titres tend to peak in mid-summer and decrease in the later fall months (Gauthier et al. 2007, Baker and Schroeder 2008, Runckel et al. 2011).

SBV was the first honey bee virus to be sequenced and is 8832 nt long with a single ORF (Grabensteiner et al. 2001). The major structural protein is formed from the 5' end of its ORF (Evans and Hung 2000, Grabensteiner et al. 2001). Sequence comparisons group it within the genus *Iflavirus*, along with DWV (Bonning 2009).

Chronic Bee Paralysis Virus (CBPV)

Chronic Bee Paralysis Virus (CBPV) has been categorized for as long as ABPV, the major difference being that the onset of the trembling and paralysis symptoms take longer (7-8 days) to manifest (Bailey et al. 1963, Bailey 1967, Ribière et al. 2010). Symptoms also include a bloating of the abdomen, dislocation of the wings, dysentery and eventual death. CBPV symptoms are thought to best describe the "Isle of Wight disease" that occurred in Britain in the early 1900s (Ribière et al. 2010). There is also a second set of visible symptoms associated with the virus, termed "Mal noir", "Schwarzsucht" or "little blacks" (Ribière et al. 2010), that involves losing the hair from the abdomen (Bailey 1967). These black-appearing bees are attacked through biting by other members of the hive until they are forced out and eventually die from the paralysis symptoms (Ribiere et al. 2007). Hundreds of dead bees lining the ground about the hive can be evidence that CBPV infection has reached lethal doses, which sometimes are as high as 10^{13} copies (Ribiere et al. 2007), within the colony (Bailey 1967).

CBPV is widespread, being found in honey bees from every beekeeping continent besides South America (Bailey 1967, Ribière et al. 2010). Viral tires are highest in midsummer, although the virus probably persists as low-level infections in the majority of colonies throughout the year (Tentcheva et al. 2004, Ribière et al. 2010). It can be found in many of the bee's tissues, such as the gut, hypopharyngeal glands, honey sacs, head and body tissue (Ribière et al. 2010). CBPV only manifests in adults, and is of little detriment to the brood stages. CBPV is unlike the other RNA viruses found in bees and though it shares some characteristics with the viral families *Nodaviridae* and *Tombusviridae* it has not yet been classified (Ribière et al. 2010).

Pathogen Spillover

Pathogen spillover occurs when an organism is introduced into a new environment and its associated parasites and pathogens spread to novel host species in that environment. Often these pathogens are spread by anthropogenic means and have a potential to be severely detrimental to new naïve host populations (Daszak et al. 2000). Woolhouse et al. (2005) established a "basic reproduction number, R_0", and stated simply that if $R_0 < 1$ (meaning the virus does not fully replace itself with each replication) in the new host then it is likely to only contract the infection from the original source population, limiting the spread throughout this host, but if $R_0 > 1$ in the new host it in turn is capable of further spreading the infection itself. However, when infecting a new host, they also point out that the pathogen should be less infectious to it at first. Sometimes, given time and especially with RNA viruses, the pathogen can adapt to its host and mutate to form a distinguishable strain (Ebert 1998). RNA viruses, for this reason, are the most likely pathogens to spread to new hosts (Woolhouse et al. 2005). Since spillover of *Nosema ceranae,* a microsporidian parasite of bees, has been demonstrated from honey bees to bumble bees in Europe and North America (Graystock et al. 2013a), it seems extremely likely viruses could be spilling over as well. In the case of *N. ceranae,* it became more infective to its new bumble bee host than it had been to its original honey bee host (Graystock et al. 2013a). Similarly, the intestinal trypanosome *Crithidia bombi* is found to be more infective to naïve bumble bee hosts (Imhoof and Schmid-Hempel 1998). Fortunately, a pathogen that shows up in high prevalence among living members of a new host is likely to be less serious, as highly pathogenic infections are more likely to kill their host before they can spread the disease (McCallum and Dobson 1995).

Spread of viruses

Disease can easily take hold in a honey bee colony due to the amount of direct contact between large numbers of workers in a limited space and their particular social structure that facilitates food exchange and distribution of pheromones (Chen et al. 2006a). Within a colony, viruses can either be spread horizontally amongst members of coexisting generations, or vertically (from mother to egg or from male to sperm). Given that vertical transmission often favours latent expression of the disease rather than overt symptoms (Chen et al. 2006a, De Miranda and Genersch 2010), it would make sense for the majority of these viruses to be transmitted vertically. Infections with particular viruses in offspring often reflect the viruses found in their mothers (Chen et al. 2006b, De Miranda and Fries 2008). Transovarial transmission has been demonstrated for KBV and SBV (Shen et al. 2005a). Furthermore, virus particles have been detected in the semen of adult drones and the spermathecae of queens (Chen et al. 2006a, De Miranda and Fries 2008). Infected semen has been demonstrated to lead to the infection of ovaries in mated queens, such that horizontal transmission can occur during reproduction as well (De Miranda and Fries 2008). Detection of virus particles, not only on the surface of the eggs, but within surface-sterilized eggs, argues that offspring can even become infected prior to oviposition (Chen et al. 2006a).

Many studies have demonstrated that these viruses are transmitted horizontally as well (Shen et al. 2005a, Chen et al. 2006a, De Miranda and Genersch 2010), which leads to their potential to infect novel hosts. Contaminated food (ie. pollen, honey stores, brood food and royal jelly) are potential sources of transmission for DWV, BQCV, KBV, SBV, IAPV, ABPV, and CBPV (Shen et al. 2005a, Chen et al. 2006a, Singh et al. 2010), and bees robbing food from dead

or weak infected colonies could risk spreading virus particles to their colonies (Shen et al. 2005a). Virus particles remain viable in food kept at room temperature for months (Singh et al. 2010). Additionally, fecal material has also often tested positive for DWV, BQCV, KBV and CBPV (Hung 2000, Chen et al. 2006b, Ribiere et al. 2007).

It has long been hypothesized that honey bees were the original host, and that viruses that infect them are now showing up in bumble bees (Genersch et al. 2006, Ward et al. 2007, Singh et al. 2010, Peng et al. 2011, Evison et al. 2012, Gamboa et al. 2015, Manley et al. 2015, McMahon et al. 2015, Dolezal et al. 2016, Tehel et al. 2016). Recently it has been demonstrated that among the genus *Apis,* DWV is a re-emerging and widespread virus with the European honey bee (*Apis mellifera*) acting as its original host (Wilfert et al. 2016). Fürst et al. (2014) argue that since sympatric honey bees and bumble bees share the same strains of DWV and the prevalence of this virus is higher in the honey bee, DWV is likely spilling over into native bumble bees from imported honey bees. In fact, DWV and several of the other known honey bee viruses have been detected in non-*Apis* Hymenoptera and other arthropods as well (Levitt et al. 2013) and it is argued that multiple spillover events may have already occurred (Manley et al. 2015). The issue of potential pathogen spillover from honey bees to native species is the main question addressed by this thesis.

Varroa mite

Most of the studies of transmission of these viruses has been within honey bee colonies, where it is often transmitted by another parasite, the varroa mite (*Varroa destructor* which is

named *Varroa jacobsoni* in the earlier literature (Anderson and Trueman 2000)). *V. destructor* has been introduced to the Americas at least twice, where it became a huge problem for colonies of *Apis mellifera* (Oldroyd 1999). Mites can both transmit some of the viruses, as well as activate latent infections in pre-existing hosts, through the suppression of the immune system (Ball and Allen 1988, Bowen-Walker et al. 1999, Nordström 2003, Chen et al. 2004, Shen et al. 2005b, Genersch and Aubert 2010, Neumann et al. 2012). Varroa mites make excellent vectors of DWV, and concentrations of the virus in mites can be higher than that in honey bees (Bowen-Walker et al. 1999). Additionally, the relatively low virulence of DWV towards mites means fewer mites are required to transmit the infection to a new host colony (Sumpter and Martin 2004). KBV and SBV have also been detected in mite saliva (Shen et al. 2005a). Varroa mites can transmit viral infections from mite-to-mite (Chen et al. 2006a), horizontally but not vertically (Nordström 2003). Certain viral strains can replicate within the mites, such that they have become a reservoir host, rather than simply a vector (Ongus et al. 2004, Yue and Genersch 2005, Di Prisco et al. 2011, Neumann et al. 2012).

Varroa mites acting in conjunction with these viruses are one of the most promising avenues of study looking at the potential causes of colony loss in honey bees (Martin et al. 2012). Varroa mites have been positively tested for ABPV (Bakonyi et al. 2002, Genersch and Aubert 2010), CBPV (Celle et al. 2008), KBV (Hung and Shimanuki 1999, Chen et al. 2004, Shen et al. 2005a), IAPV (Di Prisco et al. 2011) and SBV (Tentcheva et al. 2004, Shen et al. 2005a), but are the most associated with, and harbour the highest titres of, DWV (Tentcheva et al. 2004, Gauthier et al. 2007). The characteristic symptoms associated with economic loss from DWV do not tend to occur in the absence of infestation with *V. destructor,* and likewise *Varroa* was

relatively harmless to *Apis cerana* in the absence of viral infections (Sumpter and Martin 2004, De Miranda and Genersch 2010, Martin et al. 2012). Despite this clear association, recent studies suggest DWV can bring about winter colony losses without need for varroa co-infection (Highfield et al. 2009, Locke et al. 2012). Moreover, varroa mite infestation appears to have no effect on the viral load of KBV, IAPV and ABPV in Hawaiian honey bees (Martin et al. 2012). Additionally, mites have not been shown to have any association with BQCV and CBPV infections (Tentcheva et al. 2004).

Varroa mites are not known to establish populations in bumble bee colonies (Highfield et al. 2009), so whereas the mites may act as a reservoir host to keep active infections going within and among honey bee colonies, spillover into bumble bees and other insect species must be occurring through other means.

Pathogen Spillover in Bumble Bees

Promising research as to how bumble bees may acquire viral infections from honey bee hosts involve shared flower use (Singh et al. 2010), as well as transmission through other intermediary hosts such as ants (Celle et al. 2008) and small hive beetles (*Aethina tumida* Murray (Coleoptera : Nitidulidae)) (Eyer et al. 2009). Wax moth (*Galleria mellonela* Fabricius (Lepidoptera : Pyralidae)) larvae sampled from honey bee hives tested positive for IAPV and BQCV (Triyasut et al. 2015). Pollen of common bumble bee forage such as clover (*Trifolium* L. and *Melilotus* spp. L.), goldenrod (*Solidago* spp. L.), thistles (*Cirsium* spp. Sommier & Levier) and burdock (*Arctium minus* Bernh*)* test positive for DWV, BQCV and SBV. Additionally, IAPV is capable of spreading through simple contact made during simultaneous flower visits (Singh et

al. 2010). Several studies show that once pathogens or parasites establish in bumble bees they can readily be spread to other bumble bee colonies and species. For example, the tracheal mite, *Locustacarus buchneri* Stammer, probably spilled over from commercial bumble bees from Japan into native bumble bees in Europe (Goka et al. 2006). Bumble bees from commercial greenhouses in North America appear to spread *Crithidia bombi* and *Nosema bombi* into wild populations through shared flower use (Colla et al. 2006, Otterstatter and Thomson 2008, Murray et al. 2013) and thus pose a high risk for native bumble bee communities (Meeus et al. 2011). Finally, in Patagonia, the protozoan *Apicystis bombi* has been transmitted from imported *Bombus terrestris* Harris into native bumble bee populations (Arbetman et al. 2013).

Study of RNA Viruses in the Field

Though there have been many studies conducted on RNA viruses, few investigate the ideal means of handling and storing them for accuracy and reliability in quantitative experiments. Many studies involving these RNA viruses are done from lab-based sampling and experiments using lab-reared colonies and bees, often sourced from managed hives. In these cases, RNA integrity of honey bees is best preserved when stored at -80^{o}C or -20^{o}C. If the sample is to be stored at 4^{o}C, crushed or sliced samples submersed in RNAlater^{TM} (Ambion, Austin, Texas, USA) can similarly preserve the integrity of the RNA. Some methods, such as addition of 70% ethanol which is commonly done in field collection for ecological work, leads to degradation of RNA integrity. In general RNA virus quality is best preserved at -80^{o}C (Chen et al. 2007).

Though it is still possible to detect DWV and BQCV in samples held at room temperature for up to 5 days, this approach can severely compromise use in quantitative experiments. Bees captured in the field are best kept at low temperatures, such as in coolers of dry ice, or in high-salt buffers (such as RNAlater) that can aid in preservation of RNA integrity. Also of note, is that viral RNA appears to be less prone to degradation than host mRNAs (Dainat et al. 2011). This could have implications for use of the relative CT method of quantification.

CHAPTER 2. Comparisons of RNA virus prevalence and intensity among bumble bees (*Bombus spp.*) caught in proximity to and isolation from managed honey bee (*Apis mellifera* L.) colonies

Abstract

Recent declines of bee species worldwide have raised concerns as to the fate of major commercial and wild flowering plants dependent on these bees for pollination. In North America, declines in native species have been attributed to pathogen spillover from imported pollinators used for greenhouse and crop pollination. Among these pathogens are a small group of RNA viruses that result in reduced survivorship of colonies. However, little is known about the distribution of these viruses in different habitats. This study focuses on seven of these viruses that are known to occur in commercial honey bees (*Apis mellifera* L.), and examines their prevalence and concentration in native bumble bees (*Bombus spp.* Latreille) (Hymenoptera: Apidae, Bombini) from different landscapes. Sampling of bumble bees and honey bees occurred at sites in proximity to and isolation from contact with managed honey bee colonies throughout the foraging season. RNA was extracted from each specimen and analysed for viral quantities using RT-qPCR. The viruses KBV, ABPV and CBPV appeared in very low prevalence and intensity among sampled bee populations. DWV, BQCV, SBV and IAPV however, were found in high prevalence and intensity in honey and bumble bees alike. When comparing populations of honey bees, bumble bees exposed to honey bees and bumble bees secluded from honey bees, prevalence was typically lower in bumble bees than honey bees and lowest in those bumble bees isolated from honey bees with the following exceptions: BQCV was similar in prevalence among honey bees and bumble bees sharing an environment with

honey bees and IAPV was more prevalent in isolated bumble bees than those in close proximity to managed honey bees. Comparisons of intensity using concentrations of viral RNA found in 1µg RNA samples showed that virus copies were equal among different bumble bee species for three of the four main viruses, with the exception being BQCV, which appeared in lower concentrations in bumble bees secluded from honey bees than those in close proximity to them. Prevalence data from DWV, BQCV and SBV supports the hypothesis that viruses are spilling over from managed honey bees to native bumble bees when found in proximity to managed honey bee colonies, and this may also be true of IAPV which does not fit the expected pattern, though a bumble bee specific variant may exist. Comparisons of viral intensity showed only one virus (BQCV) had higher intensity in sites with managed bees than in isolated sites. However, some honey bees did differ in viral intensity from some bumble bee species and some bumble bee species differed from each other even within isolated sites in terms of DWV, SBV and IAPV intensity, suggesting species may differ in regards to the amount of exposure in the environment or level of immunity. The wide geographical spread of viruses makes it clear that native bumble bee species may be at risk from infection. Further research should focus on the impact infection with these viruses has on fitness in native pollinator populations.

Introduction

Bees represent the best adapted pollinators in the animal kingdom, and so are heavily utilized for the pollination of many agricultural crops (Klein et al. 2007), as well as pollinating a significant proportion of wild flowering plants (Meffe 1998, Memmott et al. 2004). Insect

pollination services are responsible for 9.5% of the agricultural food market, totalling approximately 160 billion US dollars annually (Gallai et al. 2009), the majority of which is provided by bees (Gallai et al. 2009, Ellis et al. 2010). Simulations of decline in generalist pollinators, like bumble bees, within pollination networks demonstrate that native plant diversity may also expect declines (Memmott et al. 2004, Goulson et al. 2008).

Of the 250 species of bumble bee existing in the world, several are reported to be in decline, endangered or at risk of extinction (Williams and Osborne 2009). Parts of the UK report range contractions or extirpations for up to 50% of their native species (Fitzpatrick et al. 2007), and have listed 40% of their species endangered (Sárospataki et al. 2005). In North America, similar levels of species loss have been observed at least in Ontario and Illinois (Colla and Packer 2008, Grixti et al. 2009). Disappearances of bumble bees are explained through habitat destruction (Edwards and Williams 2004, Goulson et al. 2005, Fitzpatrick et al. 2007, Martins and Melo 2010), climate change and possible pesticide use (ie. neonicotinoids) (Potts et al. 2010). Additionally, it is thought that pathogens, such as RNA viruses, threatening managed honey bee colonies, could affect native bumble bee communities as well (Williams and Osborne 2009, Winfree 2010, Fürst et al. 2014).

To date more than 24 distinct species of virus have been characterized in honey bees (de Miranda et al. 2013), of which seven are the most common and therefore the subject of this study: Deformed Wing Virus (DWV)(Lanzi et al. 2006), Black Queen Cell Virus (BQCV)(Leat et al. 2000), Kashmir Bee Virus (KBV)(De Miranda et al. 2004), Sacbrood Virus (SBV)(Grabensteiner et al. 2001), Israeli Acute Paralysis Virus (IAPV)(Maori et al. 2007), Acute Bee Paralysis Virus (ABPV)(Govan et al. 2000) and Chronic Bee Paralysis Virus (CBPV)(Ribière et al. 2010). All seven

viruses belong to the viral superfamily Picornaviridae (Palacios et al. 2008) and consist of a single strand of RNA shaped into a sphere and coated with capsid proteins (Chen et al. 2006a). Most are part of the newly classified family Dicistroviridae (Mayo 2002), with the exception of DWV and SBV which are of the genus *Iflavirus* (Iflaviridae) (Bonning 2009) and CBPV, which shares little similarity with the other honey bee viruses and is as of yet unclassified (Ribière et al. 2010). All the viruses have the capacity to exist as latent infections within colonies (Bailey 1967, Bailey and Woods 1974, Dall 1985, Chen et al. 2006b, Genersch and Aubert 2010, de Miranda et al. 2013) permeating most tissues of all castes (Chen et al. 2006b). Though their overt symptoms differ in terms of life stage targeted and physical symptoms, all lead to decreased survivorship of the colony, with models predicting virus-weakened honey bees that overwinter as a colony being particularly vulnerable in the overwintering stages (Martin 2001). All seven of these viruses have been shown to infect bumble bees (Bailey and Gibbs 1964, Genersch et al. 2006, Ward et al. 2007, Potts et al. 2010, Singh et al. 2010, Peng et al. 2011, Evison et al. 2012, Fürst et al. 2014, Gamboa et al. 2015, McMahon et al. 2015, Dolezal et al. 2016, Tehel et al. 2016). It has been theorized that with increased global trade of honey bee hives, honey bee viral pathogens are spilling into native bumble bee communities. Fürst et al. (2014) argue that this seems likely given that DWV strains are the same in honey bees and bumble bees caught in proximity.

Though the exact means of spread is not defined, shared flower use (Singh et al. 2010) or other potential insect vectors such as small hive beetles (Eyer et al. 2009), wax moth larvae (Triyasut et al. 2015) and ants (Celle et al. 2008) represent promising avenues of study. IAPV has been demonstrated to spread simply through contact during shared flower use, and DWV,

BQCV and SBV are found in the pollen of common bumble bee forage (Singh et al. 2010). Some virus particles are hardy and can persist for months in contaminated food or fecal matter, such that a bumble bee robbing the honey stores of a honey bee hive could possibly pick up the infection in this manner (Bailey and Gibbs 1964, Hung 2000, Yue and Genersch 2005, Singh et al. 2010).

Although these viruses were first characterized in honey bees, it is not known for sure that is where they truly originated. It is possible *Bombus* hosts act as reservoirs for the infections (Sachman-Ruiz et al. 2015) and viruses could be in the process of re-entering the original host population through 'pathogen spillback' (Daszak et al. 2000). Theory predicts that due to the high levels of infectivity required to overcome the species barrier, a pathogen should be less infective, as well as less prevalent, in a new host species. At the same time, higher doses of the pathogen may be required for it to be infective to the new host, suggesting intensities should be higher in a novel infected host (Woolhouse et al. 2005). This study attempts to elucidate whether or not viruses are spilling over from managed honey bee colonies into native bumble bee communities by comparing the prevalence and intensity of seven different viruses in bumble bees caught in presence or absence of honey bee colonies maintained in proximity to their home ranges. Virus quantities in wild bumble bees are also compared to levels found in the honey bees within the same sites.

Several factors such as timing of capture, bee species and capture method could potentially have confounding effects on assumptions made about differences in prevalence and intensity of viruses. DWV and IAPV viral titres in honey bees are thought to increase in later fall months, whereas BQCV and SBV typically peak around mid-summer (Runckel et al. 2011).

Though bumble bee species such as *Bombus terricola* and *B. borealis* appear to be in decline, others like *B. ternarius* are increasing their range (Colla and Packer 2008, Grixti et al. 2009). If viruses are thought to be responsible for the disappearance of declining bee species, susceptibility must differ between species. Whereas viruses often exist as latent low-level infections (Bailey 1967, Bailey and Woods 1974, Dall 1985, Chen et al. 2006b, Genersch and Aubert 2010, de Miranda et al. 2013), at high viral titres hosts experience overt symptoms that result in a weakened state (Lanzi et al. 2006, Maori et al. 2007, De Miranda and Genersch 2010, Fürst et al. 2014), which may affect their ability to be captured by certain methods.

The specific objectives of this study were: (1) to compare virus prevalence and intensity between populations of the hypothetical original host, the European honey bee ,*Apis mellifera,* and populations of bumble bees captured in proximity to and isolation from honey bees; (2) to compare virus prevalence and intensity in bumble bee species sampled from sites with a presence or absence of honey bee colonies; (3) to compare two common methods of sampling bumble bees in relation to their likelihood of capturing infected bees; (4) to compare temporal patterns of virus prevalence and intensity in bumble bees from sites in the presence and absence of honey bees.

Materials and Methods

Sample collection/identification

Bees were collected in the summer of 2012 (July – August) from three sites that contain honey bee colonies (HB-exposed) in or near Winnipeg, Manitoba: “U of M Point” (N49º50.12’

W97º7.10'), "Glenlea" (N49º38.47' W97º7.20) and "Charleswood" (N49º50.12' W97º17.53'), as well as three non-honey bee sites (HB-absent), two within Sandilands Forest, Manitoba: "Platford" (N49º28.163' W096º23.481') and "404 Sandilands" (N49º23.310' W096º17.726'), and a third "Red Deer" (N50º45.89' W94º11.293') located about 30km north of Kenora, Ontario. This project examined viruses in all species of bumble bees (*Bombus* spp.) and honey bees (*Apis mellifera* L.) that were captured. Other species of bees were kept but not analysed. Sites without honey bee hives were chosen based on observations of bumble bee presence, adequate distance from known honey bee hive practices (~30km), as well as the presence of a number of excellent forage plants including, clover (*Trifolium repens* L.), bee balm (*Monarda didyma* L.), thistle (*Cirsium arvense* [L.] Scop.) and goldenrod (*Solidago sp*. L.). All sites are well out of foraging range (>10km) from each other to avoid any possible overlap in flight range of study colonies.

Bees were collected at each site with 7-10 day intervals using both a passive bee bowl sampling method as well as opportunistic sampling with a sweep net. Thirty plastic bowls were set in straight transects at each site according to Canpolin protocols for bee bowl sampling (NSERC-CANPOLIN 2009; coloured plastic bowls 15-17.5 cm in diameter and filled 2/3 with water mixed with DAWN™ original scent dishsoap [conc. 3 drops/litre], and placed 3 metres apart alternating blue, yellow and white). Areas of open ground were chosen for transects to ensure they were visible to bees and were in limited competition with flowers. Bowls were set up each morning (9h30m – 11h30m) and removed at the end of the day (between 16h30m and 18h30m; at least seven daylight hours after set-up). Following bee bowl set-up, sweep net sampling was carried out opportunistically in the same general area. Bees captured in nets

were frozen in a cooler of dry ice, to both euthanize the bees and preserve RNA viruses in their tissues, and then transferred to a sample cup for further storage on dry ice. Ten bees, of each genus (ie. *Bombus*, and *Apis*) were captured where present each sampling day. Contents of the bee bowls were collected by filtering them through a strainer and were also kept on dry ice for further processing. Each site was sampled in this fashion once every 7-10 days for the duration of the summer beginning on 07/05/2012 and ending on 09/05/2012.

Upon return to the lab later in the same sampling day, collections from the bee bowls were thawed in 95% ethanol and all bees were separated from the sample. Bumble bees were identified to species using taxonomic traits described by Colla et al. (2010). All bumble bee samples were transferred to individual 1.5 ml Eppendorf tubes with a 0.5 ml volume of RNAlater-ICE™ (Life Technologies Inc., Burlington, ON) to better preserve any viruses present. Honey bee samples were also stored in tubes, though not individually and without RNAlater-ICE™. All bees were then stored and kept at -80ºC until processing for RNA extraction.

RNA extraction

Individual bees were submersed in liquid nitrogen to prevent RNA degradation and crushed into a fine powder using a mortar and pestle. Depending on the size of the bee, a 15-35 mg sample of the powder was weighed out, transferred to a 1.5 ml Eppendorf tube and stored at -80°C to await RNA extraction. Additionally, some samples were crushed directly in 1ml of lysis buffer (100 µl buffer RLT [Qiagen]: 1 µl β-Mercaptoethanol [Sigma-Aldritch]) using a Retsch MM400 bead homogenizer. Five metal beads were used per bee, and the machine was set to 30 cycles/sec for 3-4 10 min. runs, rotating samples between runs to ensure equal

homogenization. Heads were removed from bees before crushing as it has been suggested they contain inhibitors for the RNase integral to qPCR study (Boncristiani et al. 2011). Likewise, legs were removed from bumble bee specimens to be sent away for DNA-barcoding (Canadian Centre for DNA Barcoding, University of Guelph) for species that could not be correctly identified.

RNA extraction from the bee powder was performed using an RNeasy extraction kit (Qiagen, Valencia, Ont., Can.) according to the manufacturer's protocols and mixing 1:100 β-mercaptoethanol with Buffer RLT for tissue lysis. Twenty to 25 µl of molecular grade water was used to elute the sample from the column in order to maximize RNA concentration. Concentration of extracted RNA samples was determined by measuring the optical density using Nanodrop 2000 software and a ThermoScientific NanoDrop 2000 spectrophotometer. Samples with a concentration of less than 200 ng/µl were discarded from analysis, as they contained less than the 2 µg/10 ul elution required for cDNA synthesis.

cDNA synthesis

Extracted RNA was converted to cDNA by adding oligo dT (0.025 µg/µl; Invitrogen) and 1µl dNTP mix (0.5mM; Promega) as non-specific primers to a 1 µg/ml concentration of the RNA sample. This mixture was then annealed through incubation at 65ºC for 5 min. followed by 5 min. on ice to halt the reaction. Four µl of 5x first strand buffer (0.2M; Invitrogen), 2 µl 0.01M DTT (Invitrogen) and 1µl RNaseOUT (2U/µl; Invitrogen) were then added to the mixture and incubated at 37ºC for 2 min., after which point 1 µl M-MLV (10U/µl; Invitrogen) was added to

make a total volume of 20 µl. This final mixture was elongated through incubation at 37ºC for 1 hour followed by a 15 min. denaturation step at 70ºC.

RT-qPCR

Each cDNA sample was analyzed for copies of four different RNA viral targets: Deformed Wing Virus (DWV-174; accession #: KF378605.1), Black Queen Cell Virus (BQCV-310; AF183905.1), Sacbrood Virus (SBV-335; KC007374.1) and Israeli Acute Paralysis Virus (IAPV-203; EU224280.1) using a CFX96 (BioRad) real-time qPCR machine to perform absolute qPCR reactions. A subsample of available cDNA was analyzed for three other RNA viral targets: Kashmir Bee Virus (KBV-200; AY275710.1), Chronic Bee Paralysis Virus (CBPV-296; EU122229.1) and Acute Bee Paralysis Virus (ABPV-197; AF150629.1) as viral presence was found to be very rare in those samples tested for these viruses (*see* Table 2.1 *for a list of forward and reverse primers used to detect targets*).

Ninety-six-well optical plates were prepared with 20 µl volumes made up of 0.5M 2xpower SYBR Green PCR Master Mix (Bio Rad), a mixture of the appropriate forward and reverse virus primers (0.05M), the appropriate cDNA sample (0.01M) and 0.4M ddH_20. A plate held a maximum of twenty-two samples, each run in triplicate with an additional positive control involving actin amplification (Actin-181; table 2.1). The cDNA was deemed degraded if the actin cycle threshold (CT) was above 23.5 and the sample was discarded from further analysis. Wells were also set aside on each plate for negative primer and no template controls as well as five concentrations for a standard curve (selected based on expected gene copies to be found in samples, and rerun if gene copy numbers amplified outside of the standard curve).

Standard curves were produced from ten-fold serial dilutions of synthesized target sequences (GBLOCKS^{TM}) beginning with a sample known to contain 10^9 gene copies. Plates were run on the following program: 95ºC for 10 min. followed by 40 cycles of 95ºC for 15s, 55ºC for 30s and 72ºC for 30s followed by a disassociation step that produced a melt curve. If the melt curve was not represented by a single sharp peak the sample was discarded from analysis, as this shows something other than the target sequence was amplified. Copies of the viral sequence were replicated from the sample over the course of the amplification program and translated into a real-time curve of the quantity of virus found over time. From this curve a CT, or cycle threshold, was taken for each sample. CT values were compared to the standard curve in order to estimate a number of viral copies in each sample for each virus tested. CT values >35 were outside the ability of the machine to accurately detect target presence and so were treated as a result of 0 viral copies. Each virus tested for a given set of samples was run on a separate plate.

Statistical Analysis

Samples were first characterized as either being positive or negative for infection with each of the seven different viruses, in order to be analyzed for prevalence. Prevalence data was initially compared between three bee classes: honey bees, bumble bees from a site in proximity to honey bees (HB-exposed) and bumble bees from a site isolated from honey bees (HB-absent). Additionally, differences in prevalence were compared between all six sites (U of M Point, Glenlea, Charleswood, 404 Sandilands, Platford and Red Deer), by capture method (bee bowl or sweep net), by species and by timing of capture represented by week of capture within the field season (07/05/2012-09/05/2012). All statistical comparisons were done using logistic

analysis with the CATMOD procedure (SAS 9.3), including examination of interactive effects of bee class (HB-exposed vs HB-absent), bee species (comparison between bumble bees and honey bees or among bumble bee species), capture method and week of capture. Reduced models were run where interactions were not significant. Pairwise comparisons were Bonferroni-corrected.

Intensity (mean number of gene copies per µg of RNA in infected bees) was measured using the number of copies amplified from the 2µg of RNA used in the PCR reaction, and then standardised to copies/µg of RNA. Comparisons were made between species captured at HB-exposed sites and between species at HB-absent sites for the four most prevalent viruses found within samples (DWV, BQCV, SBV, IAPV). Values were log-transformed for analysis and were compared using a repeated measures ANOVA using the restricted maximum likelihood method (REML) with compound symmetry as the covariance structure (PROC MIXED SAS 9.3). Week was treated as a repeated measure and site as the subject in the model. Within bumble bees, intensity was compared between pooled samples of all bees from each type of site, as well as between capture methods (bowl and net) and timing of capture. Interactive effects between factors were tested and significant groups within the dataset were determined using Bohn-Holm least squares differences.

Results

Prevalence

Effect of Bee Class/Site

When considering first the prevalence of each of the viruses in honey bees and bumble bee communities at sites with honey bees (HB-exposed), prevalence of DWV in sampled honey bees was greater than in bumble bees ($\chi^2 = 49.89$, df = 1, $p<0.0001$) (Fig 2.1 A). However, prevalence among all three HB-exposed sites was similar when pooling honey and bumble bees together ($\chi^2 = 4.58$, df = 2, $p = 0.1014$) or within honey bees and bumble bees when considered separately by site ($\chi^2 = 2.52$, df = 2, $p = 0.2832$) (Fig. 2.1A). When comparing bumble bees sampled from HB-exposed sites to bumble bees in HB-absent sites, however, there was a significantly higher DWV prevalence in bumble bees sharing an environment with honey bees ($\chi^2 = 5.6$, df = 1, $p = 0.018$) (Fig. 2.1B) than in those where honey bees were absent, and no difference in DWV prevalence in bumble bees among the three HB-absent sites ($\chi^2 = 0.179$, df = 2, $p>0.05$).

Prevalence of BQCV did not differ between honey bees and bumble bees at HB-exposed sites ($\chi^2 = 0.58$, df = 1, $p = 0.4465$), with all but a few of the sampled bumble bees from the UofM Point site testing positive for the virus (Fig 2.2A). Prevalence did not differ among HB-exposed sites when all bees were pooled for each site ($\chi^2 = 0.06$, df = 2, $p = 0.9717$) or differ among individually considered sites between honey and bumble bees ($\chi^2 = 0.1$, df = 2, $p = 0.9504$) (Fig. 2.2A). Again when testing differences in prevalence between HB-exposed and HB-

absent bumble bees it was shown that there was a much higher prevalence of BQCV in bumble bees at HB-exposed sites ($\chi^2 = 65.14$, df = 1, $p<0.0001$) (Fig. 2.2B) than in HB-absent sites, with this lower prevalence not differing significantly between the three HB-absent sites ($\chi^2 = 0.807$, df = 2, $p>0.05$).

SBV was more prevalent in honey bees than in bumble bees sampled from HB-exposed sites ($\chi^2 = 4.85$, df = 1, $p = 0.0277$) (Fig 2.3A), regardless of which of the three sites they were sampled from ($\chi^2 = 0.79$, df = 2, $p = 0.674$). Prevalence among sites for honey bees and bumble bees also remained the same across all three HB-exposed sites ($\chi^2 = 2.19$, df = 2, $p = 0.3346$) (Fig. 2.3A). SBV infection was more prevalent in bumble bees sampled from HB-exposed sites than those from HB-absent sites ($\chi^2 = 6.7$, df = 1, $p = 0.0096$) (Fig. 2.3B), though once again there was no significant difference in the prevalence of SBV in bumble bees among the three HB-absent sites ($\chi^2 = 1.06$, df = 2, $p>0.05$).

IAPV was more prevalent in honey bees than bumble bees at HB-exposed sites ($\chi^2 = 55.63$, df = 1, $p<0.0001$), but in contrast to the other viruses there was significant variation among HB-exposed sites ($\chi^2 = 15.04$, df = 2, $p = 0.0005$), where IAPV prevalence was higher in both honey bees and bumble bees at the U of M Point site. However, relative differences between honey bees and bumble bees were similar across all sites as indicated by a lack of interaction between site and bee class ($\chi^2 = 1.82$, df = 2, $p = 0.4016$) (Fig. 2.4A). Contrary to what was seen with the other viruses, IAPV was also significantly less prevalent in bumble bees sampled from HB-exposed sites than from HB-absent sites ($\chi^2 = 5.97$, df = 1, $p = 0.0146$) (Fig.

2.4B). There was no effect of site on the prevalence of IAPV in bumble bees among the HB-absent sites ($\chi^2 = 1.15$, df = 2, $p>0.05$).

The prevalence of KBV was so low across all treatment groups that it was not compared between HB-exposed and HB-absent sites. Of 377 bees tested for this virus, two bumble bees from the honey bee site at Glenlea, and one bumble bee each from the non-honey bee sites (Platford and 404 Sandilands) were the only ones to test positive for the virus, with all 79 honey bee specimens examined testing negative for the virus. CBPV and ABPV prevalence and intensities were also not compared due to low proportions of a reduced sample size of bees testing positive for CBPV and ABPV. For CBPV, 3 out of 28 honey bees and 5 out of 91 bumble bees mostly from the U of M Point site tested positive and for ABPV (0 out of 47 honey bees and 2 out of 97 bumble bees both of the species *B. rufocinctus* from HB-exposed sites tested positive).

Effect of Species

Prevalence of DWV differed among bumble bee species ($\chi^2 = 17.52$, df = 6, $p = 0.0076$), and there was also a significant interaction between species captured in HB-exposed and HB-absent sites ($\chi^2 = 26.86$, df = 6, $p = 0.0002$). Upon closer inspection with a series of pair-wise chi-squared tests (Bonferroni-corrected for significance) the low prevalence of DWV infection among any specimens of the species *B. griseocollis* represented the only significant effect of species ($\chi^2 = 11.35$, df = 1, $p=0.0008$), and prevalence of DWV among the other *Bombus* species

was not significantly different, ranging from 22 to 49%. Furthermore, there was significantly higher prevalence in HB-exposed then HB-absent bumble bees of the species *B. rufocinctus* (χ^2 = 9.61, df = 1, p = 0.0019) and *B. ternarius* (χ^2 = 18.63, df = 1, p<0.0001), indicating that differences among species cannot always be separated from the effects of bee class (Fig. 2.5A).

The prevalence of BQCV (χ^2 = 4.39, df = 5, p = 0.4943) did not vary among species of bumble bees and there were no interactive effects with bee class (χ^2 = 1.97, df = 5, p = 0.8537). BQCV, as previously stated, was consistently more prevalent in all species of bumble bee captured from HB-exposed sites (Fig. 2.5B).

Similar to DWV prevalence, the number of bumble bees infected with SBV varied among species (χ^2 = 14.94, df = 6, p = 0.0208), but there was no interaction with bee class (χ^2 = 8.77, df = 6, p = 0.1868). SBV prevalence was significantly lower in *B. griseocollis* than any other species (χ^2 = 6.23, df = 1, p = 0.0126), and highest in *B. sandersoni*, though this species was only significantly higher than *B. rufocinctus* (χ^2 = 4.02, df = 1, p = 0.045) and *B. griseocollis* (Fig. 2.5C).

Lastly, the overall prevalence of IAPV (χ^2 = 9.09, df = 6, p = 0.1684) did not vary among species, but there was a significant interaction between species and bee class (χ^2 = 13.39, df = 6, p = 0.0373). Pair-wise comparisons revealed that in *B. sandersoni* (χ^2 = 6.42, df = 1, p = 0.0113) and *B. vagans* (χ^2 = 11.84, df = 1, p = 0.0006) IAPV was more prevalent in HB-absent bumble bees, whereas all other species exhibited no significant differences between HB-exposed and HB-absent bumble bees (Fig. 2.5D).

Seasonal trends

To look at the effect that time during the season had on virus prevalence, samples were compared across weeks in which they were captured, with an 8 week period representing the entirety of the field season. The week of capture alone appeared to have no effect on the prevalence of DWV ($\chi^2 = 5.13$, df = 7, p = 0.6447), though there was a significant interaction between week and bee class ($\chi^2 = 29.4$, df = 14, p = 0.0092). For honey bees, prevalence began high in early July and increased to be maintained around 100% in August, but for bumble bees DWV prevalence declined from July to August, with prevalence in HB-absent bumble bees dropping the most precipitously (Fig. 2.6A).

Timing of capture had no overall effect on the prevalence of BQCV in bee samples ($\chi^2 = 0.54$, df = 6, p = 0.9973), nor was there a significant interaction with bee class ($\chi^2 = 10.08$, df = 12, p = 0.6088). After the first two weeks of July, prevalence in both honey bees and HB-exposed bumble bees is consistently at a high level. However, although prevalence in HB-absent bumble bees drops to 0 and begins to increase again in August these differences do not affect the significance of the model, with differences being mainly attributable to bee class (Fig. 2.6B).

Likewise, timing of capture had no effect on SBV prevalence ($\chi^2 = 8.08$, df = 7, p = 0.326), nor its interaction with bee class ($\chi^2 = 20.87$, df = 14, p = 0.1051). Although, prevalence in honey bees tends to increase and prevalence in bumble bees decrease over the summer (Fig. 2.6C), these differences are not significant as they were for DWV. Also, within most weeks differences in prevalence were not significant between bee types.

Lastly, prevalence of IAPV does appear to be reliant on timing of capture ($\chi^2 = 25.32$, df = 7, p = 0.0007) and there was no interaction between timing and bee class on these weekly differences ($\chi^2 = 19.42$, df = 14, p = 0.1494). The prevalence of IAPV in bumble bees decreases from week to week throughout the season, and though the prevalence in honey bees increases slightly in July, it also begins to decrease in August (Fig. 2.6D).

Effect of Capture Method

When considering all species pooled together, capture method (bee bowl vs. sweep net) had no effect on virus prevalence within samples for any of the main viruses tested; prevalence of DWV ($\chi^2 = 1.01$, df = 1, p = 0.3512), BQCV ($\chi^2 = 0.00$, df = 1, p = 0.9871), SBV ($\chi^2 = 2.82$, df = 1, p = 0.0932) and IAPV ($\chi^2 = 2.26$, df = 1, p = 0.1325). There was, however, a significant interactive effect between capture method and bee class on the prevalence of DWV ($\chi^2 = 6.20$, df = 2, p = 0.0450) with nets capturing more infected bumble bees in HB-exposed sites ($\chi^2 = 6.22$, df = 1, p = 0.0126) (Fig. 2.7A). With BQCV, comparison of capture methods was not affected by bee class ($\chi^2 = 0.09$, df = 2, p = 0.9584) (Fig. 2.7B). Likewise no interaction occurred between bee-class and capture method for SBV ($\chi^2 = 0.73$, df = 2, p = 0.6953) (Fig. 2.7C) or for IAPV ($\chi^2 = 3.09$, df = 2, p = 0.2315) (Fig. 2.7D).

Intensity

Species comparisons

Virus intensity was examined to see if exposure treatments affected virus concentration in infected bees (DWV, BQCV, SBV, IAPV). When all bumble bees were pooled from HB-exposed and HB-absent sites and compared there was no significant difference in intensity of DWV infection ($F = 0.14$, $df = 1, 4$, $p = 0.7249$)(Fig. 2.8). However, when examining all bee species captured within HB-exposed sites (including honey bees), there were significant differences in intensity among species ($F = 2.71$, $df = 8, 148$, $p = 0.008$). Honey bees, *Apis mellifera*, had the highest DWV intensity (Fig. 2.9A) but it was only significantly higher than *B. ternarius* (Bonholm LSD) and no differences in intensity were found among bumble bee species at the HB-absent sites ($F = 1.429$, $df = 6, 46$, $p = 0.224$) (Fig. 2.9B).

When comparing pooled values for all bumble bee species, those bumble bees found in HB-exposed sites had higher average intensities of BQCV than those in HB-absent sites ($F = 24.81$, $df = 1, 4$, $p = 0.0076$) (Fig. 2.8). There were also differences in the intensity of infection among different species within HB-exposed sites for BQCV ($F = 14.65$, $df = 7, 232$, $p<0.0001$) with *A. mellifera* having a significantly higher intensity of infection on average than every bumble bee species except for *B. bimaculatus* and *B. griseocollis* (Fig. 2.10A). BQCV also differed in intensity among bumble bee species at HB-absent sites ($F = 4.768$, $df = 7, 67$, $p = 0.0002$) with *B. fervidus* (n=2) showing a higher average intensity than all other bumble bee species besides *B. vagans* and *B. perplexus* (Fig. 2.10B).

For pooled values of all bumble bee species, SBV intensities did not differ between HB-exposed and HB-absent bumble bees ($F = 5.38$, $df = 1, 4$, $p = 0.0812$) (Fig. 2.8). SBV intensities also did not differ among all species captured at HB-exposed sites including *A. mellifera* ($F = 0.901$, $df = 9, 133$, $p = 0.526$) (Fig. 2.11A) or among all bumble bee species at HB-absent sites ($F = 1.644$, $df = 7, 64$, $p = 0.137$) (Fig. 2.11B).

For pooled bumble bee species, IAPV intensities in bumble bees at HB-exposed sites did not differ from HB-absent sites ($F = 0.18$, $df = 1, 4$, $p = 0.6944$) (Fig. 2.8), When comparing all species at HB-exposed sites IAPV infection intensity was greater in honey bees than in *B. ternarius* but did not differ from other species ($F = 5.13$, $df = 5, 81$, $p = 0.0004$) (Fig. 2.12A) (Bonholm LSD) and intensity among bumble bee species did not vary within HB-absent sites ($F = 1.398$, $df = 6, 41$, $p = 0.239$) (Fig. 2.12B).

Effect of Method and Timing of Capture

Considering only pooled groups of bumble bees, species caught in sweep nets had slightly higher intensities of DWV infection than those caught in bowls ($F = 8.53$, $df = 1, 5$, $p = 0.033$) (Fig 2.13). Additionally, there were overall differences in DWV concentration throughout the season ($F = 4.88$, $df = 6, 7$, $p = 0.0282$) (Fig. 2.14). In general, DWV infections tended to decrease from the beginning of July towards the end of July and then began to increase again throughout August. In mid-August no infected bumble bees were captured from HB-absent sites. There was also an interaction between bee class and week ($F = 7.98$, $df = 4, 7$, $p = 0.0096$)

and intensity fluctuated between the two groups of bees with HB-exposed bumble bees being more infected one week and less infected the next (Fig. 2.14A).

SBV intensity also varied with seasonal timing of capture ($F = 7.79$, $df = 7, 10$, $p = 0.0022$) and intensity of infection in bumble bees tended to increase as the summer progressed (Fig. 2.14C). However, there was no interaction between timing of capture and bee class ($F = 1.29$, $df = 5,10$, $p = 0.3405$) and no effect of capture method ($F = 0.74$, $df = 1,5$, $p = 0.4285$) (Fig. 2.13).

Unlike DWV and SBV, the intensity of BQCV ($F = 3.1$, $df = 7,9$, $p = 0.1817$) and IAPV ($F = 2.37$, $df = 6,4$, $p = 0.2113$) did not vary with seasonal timing of capture (2.14B and 2.14D respectively). Measured intensities of BQCV ($F = 0.91$, $df = 1,5$, $p = 0.3849$) and IAPV ($F = 0.2$, $df = 1,4$, $p = 0.6761$) were likewise not affected by capture method.

Discussion

In this study, I compared the prevalence and relative intensity of seven different RNA viruses in bumble bees captured at sites that were in isolation from managed or feral honey bee colonies and in proximity to managed honey bee colonies, as well as foraging honey bees captured at sites with managed colonies. Evidence was provided that the four viruses (DWV, BQCV, SBV and IAPV) that were commonly found in sampled honey bees were present in most bumble bee species as well, even in those isolated from direct contact with honey bees. Patterns of virus prevalence were not always as would be expected with classical pathogen spillover in the direction of honey bees to bumble bees. Virus prevalence was higher in honey

bee populations than in bumble bees for DWV, SBV and IAPV, but for BQCV prevalence was roughly equal for honey bees and HB-exposed bumble bees. Likewise, although prevalence in bumble bees from HB-exposed sites was greater than in bumble bees from HB-absent sites for DWV, BQCV and SBV, the opposite was found for IAPV. In general, virus intensity among bumble bees was similar between HB-exposed and HB-absent bumble bees, with BQCV being the only virus to show significantly higher intensities in bumble bees found in HB-exposed than in HB-absent bumble bees. Additionally, although there were some interactions with site, bumble bee species, capture method and timing of sampling on viral prevalence and intensity, it is unlikely that they alter conclusions made from direct comparisons made between honey bees, HB-exposed and HB-absent bumble bees.

Deformed Wing Virus (DWV), first sequenced in honey bees by Lanzi et al. (2006) and soon after detected in bumble bees as well (Genersch et al. 2006), was first suggested to be "spilling over" from managed honey bees into native *Bombus* species in Great Britain on the basis of similar strains showing up but in lower prevalence in bumble bees (Fürst et al. 2014). In this study DWV prevalence in honey bees was also greater than in bumble bees from HB-exposed sites. Additionally, prevalence of DWV in bumble bees in HB-exposed sites was greater than in HB-absent bumble bees, which is what we would expect to happen if DWV was only recently invading isolated bumble bee species. This was also true of SBV. BQCV, on the other hand, was less prevalent in HB-absent bumble bee populations, but reached 100% prevalence in bumble bee populations at two of the HB-exposed sites. The high prevalence of BQCV in bumble bees found in HB-exposed sites could suggest that if BQCV were spilling over from honey bees to native bumble bees, the process would have had to have begun a while back to

have reached this degree of permeation. Peng et al. (2011) also surmised that directionality of the spillover of BQCV detected in *Bombus huntii* was hard to determine, and it is still possible that in some instances "spillback" of the pathogens into honey bees may explain high prevalence (Daszak et al. 2000). Sachman-Ruiz et al. (2015) demonstrated that commercial *Bombus impatiens* could act as reservoirs for several pathogens including honey bee viruses, and it is likely wild populations of *Bombus* could as well. However, since lower prevalences of DWV, BQCV and SBV were found in bumble bees from HB-absent sites relative to sites with honey bees it suggests that on the whole, the pathogen spillover hypothesis is still possibility for those three viruses.

None of the viruses detected in any significant frequency in honey bees were absent from HB-absent bumble bee populations. This observation argues for an alternate transmission method besides simple direct contact. It may still be possible that in some instances, honey bee ranges have spread farther than documented and expected, which could result in some incidental contact between *Apis* and *Bombus*. Indeed, early and late in the field season rare observations of honey bees were made at both the Platford and 404 Sandilands sites that may be a result of sparse forage at those times of years necessitating longer ranges of searching behaviour in honey bees. However, for the Red Deer site, which is ensconced in the middle of the boreal forest and surrounded by physical barriers such as small lakes and coniferous vegetation bumble bees here are quite unlikely to have received direct contact with honey bees. Virus transmission through shared flower use (Singh et al. 2010) could explain how these viruses "jumped" to this habitat if it could be determined that they did not already exist in populations here. It is also possible that the ability of some of these bee viruses to infect other

species may increase their range of transmission as well (Celle et al. 2008, Eyer et al. 2009, Triyasut et al. 2015). Although it has been suggested that DWV is a virus that has its origins in *A. mellifera* (Wilfert et al. 2016), given the wide spread geographical distribution of this virus this may not be true for it or other viruses that are widely dispersed in wild bumble bee populations and other insects.

Israeli Acute Paralysis Virus (IAPV) has also recently been characterized in honey bees (Maori et al. 2007). Although IAPV prevalence and intensity was higher in honey bees than bumble bees at HB-exposed sites, it appears simple pathogen spillover from honey to bumble bee may not explain the presence of this virus in bumble bee populations. It was found in higher prevalence in bumble bees from HB-absent sites than in HB-exposed sites which does not support what would be expected from pathogen spillover going from honey bees to bumble bees. IAPV exists as a complex of viruses along with KBV and ABPV (Bonning 2009), and for this study the same reverse primer was used to detect all three viruses. In fact, KBV and ABPV share 70% of their genome (De Miranda et al. 2004). Though it is clear these viruses are related, and likely that they share a common progenitor, little is understood about their evolutionary origins. Given the ease with which an RNA virus can mutate (Ebert 1998), it is quite possible that IAPV detected in bumble bees is of a different strain, or is a yet uncharacterized virus particular to bumble bee species. KBV detected in honey bees and associated varroa mites has been shown to have variations in its nucleotide sequences (Hung et al. 2000). Likewise, several distinct lineages of SBV exist, which could be a result of host switching (Grabensteiner et al. 2001). Sequencing of the viral genome of IAPV discovered in both honey and bumble bees would be required to determine if we are actually comparing quantities of the same viral strain

and future research should sequence all discovered viral targets to determine differences in strains.

Though KBV and ABPV are part of the same complex as IAPV (Maori et al. 2007), each of these viruses was detected infrequently in this study. Similarly CBPV, another common virus of honey bees worldwide, was detected in only a small quantity of samples. Previous work by Desai et al. (2016) also showed that DWV, BQCV and IAPV were much more prevalent in honey bee populations sampled throughout Canada than were KBV or ABPV. However, regional differences in relative abundance of viruses do exist. A similar study in Great Britain found DWV and BQCV, as well as ABPV, to be widespread in wild bumble bee populations (McMahon et al. 2015). KBV and SBV have also previously been detected in bumble bees (Singh et al. 2010). It is worth noting, however, that in the few instances in which bumble bee samples tested positive for KBV and CBPV in this study, some of these positive detections were from HB-absent bees. Additionally, the only samples that tested positive for ABPV were from bumble bees. This suggests that with more intensive study, we would likely determine that these viruses have also spilled over into/from or are maintained in wild populations. In either case, it is clear that these viruses are not unique to honey bees. To better get an idea of the epidemiology of KBV, ABPV and CBPV on native pollinator communities, honey bees should be pre-screened for infection before attempting to discover these viruses in exposed bumble bee populations.

Intensity was examined by comparing relative concentrations of virus in 1 µg RNA samples from each specimen. It was proposed by Woolhouse et al. (2005) that viral titres should be higher in a novel host as more copies would likely be required to establish an

infection in an unfamiliar host cell. Similarily, a new host with no evolved barriers to infection would have no way of limiting virus replication. Therefore, if these viruses are currently spilling over from honey bee to native bumble bee populations it would be expected that the viral concentrations in their RNA would be higher than that in honey bees. This proved to be untrue for each of the four viruses that were found in significant prevalence amongst the samples (DWV, BQCV, SBV, IAPV). In no case, were viral titres higher in any *Bombus* species than in *A. mellifera*, and titres in *A. mellifera* of DWV, BQCV and IAPV were significantly higher than those found in some *Bombus* species. Furthermore, variation in viral titres detected within sample grouping was often very high, and though this combined with low sample sizes of some *Bombus* species limited statistical significance, average virus titres in honey bees were often a few orders of magnitude higher than those found in bumble bees (Appendix 1). Dolezal et al. (2016) found that viral titres for DWV, BQCV, SBV and IAPV were several orders of magnitude higher in honey bees from healthy colonies than those they detected in associated bumble bees captured in Iowa. McMahon et al. (2015), on the other hand, found that viral titres in sampled honey bees and bumble bees were similar for BQCV which is similar to what we found in HB-exposed sites. However, bumble bees from HB-absent sites had lower BQCV intensity than HB-exposed bumble bees. This could indicate either that bumble bees are not a novel host for these viruses and so do not exhibit characteristically higher titres, or that in some instances viral particles detected in samples were simply picked up from the environment and may not indicate an internal infection. A weakness of the study is that it does not test for the replicative or minus-strand of the RNA virus, which could tell us whether or not each virus is actually

replicating in the host. Further studies on viral intensity should focus on the minus-strand in order to use intensity to further inform our knowledge of the possibility of pathogen spillover.

Since different bumble bee species appear to be more prone to extinction risks than others, it is hypothesized that the different threats they face, such as pathogens, may affect them differently (Williams and Osborne 2009). There is currently little evidence that these viral pathogens have an effect on colony health for bumble bees, though there are some differences in prevalence and intensity of infection among the species captured in this study. This may suggest that viral infections affect *Bombus* species differently. Some species captured in this study were rare and not represented in both HB-exposed and HB-absent sites, however species that were more commonly captured exhibited similar trends between site types for the different viruses. For instance, *B. griseocollis* exhibited the lowest levels of prevalence for both DWV and SBV, and was also low in prevalence for IAPV and BQCV (where it was grouped with other bees). However, concentrations of DWV and BQCV detected in the few *B. griseocollis* from HB-exposed sites were among the highest, and were not comparatively low for SBV or IAPV either. This suggests that, at least compared to other *Bombus* species, *B. griseocollis* are relatively less experienced with these viruses. On the other hand, *B. ternarius* exhibits higher prevalence for each of the four viruses, but concentrations of BQCV are among the lowest for this species, and within HB-exposed sites *B. ternarius* has the lowest concentrations of DWV and IAPV, suggesting it may have more experience with infections with these viruses and may have built up some immunity to them. This also assumes that samples of each species in this study are a good representation of the species as a whole. One weakness of this study is the inability to determine which colony the bees that were captured for this study were from. Most

of these viruses are transmitted vertically as well as horizontally in honey bees (Chen et al. 2006b) such that a single colony infecting itself may not be representative of the species. It is possible bumble bees of any given species, like *B. griseocollis*, were only captured from a single colony. Additionally, not all bee species were represented at each of the three sites designated as HB-exposed or HB-absent. Whereas this would not obscure the results from pooled specimens it makes it difficult to determine with certainty whether or not a species is resistant to the virus. Additionally, viruses like DWV and SBV, which although carried by the adult workers, are most infective to the larval stages (Gauthier et al. 2007). Thus, determining DWV or SBV prevalence from samples of foraging adults may not accurately predict their prevalence or intensity in the population as a whole. Sampling all life stages directly from wild colonies would provide a better estimate of prevalence of a virus in a population, but was not possible to do within the resources available for this study.

Capture method seemed to have only minor effects on the estimation of virus prevalence in bee populations. Sweep netting captured slightly more DWV-infected bumble bees than bowls did at HB-exposed sites and DWV-infected bees that were captured in sweep nets also had slightly higher intensity of virus. This greater DWV intensity in sweep-net sampled bees could be due to them being easier to capture in a net, if for example, bees infected with a large amount of virus are less proficient at flight and more likely to be captured. Alternatively, if their ability to properly forage is compromised it could make it less likely to be caught in a bee bowl trap. Since there was no apparent sampling bias toward a particular treatment method our pooling of all bees was unlikely to have affected the overall conclusions.

Similarly timing of capture seemed to have little effect on viral prevalence without considering bee class, with the exception of IAPV which decreased in prevalence throughout the field season. When considering only bumble bees DWV, as well as BQCV in HB-absent bees, also tended to decrease as the summer progressed, with BQCV in HB-absent bees increasing in prevalence again toward the end of August. Previous data in the literature for honey bees indicates that viral titres often express seasonal changes with DWV and IAPV increasing in later fall months and BQCV and SBV peaking in midsummer and tapering off in the fall but little is know about patterns in wild pollinators (Tentcheva et al. 2004, Gauthier et al. 2007, Runckel et al. 2011, Dainat et al. 2012b). In this study, SBV intensity increased into fall and DWV intensity dipped in mid-summer. BQCV and IAPV intensities did not change significantly over the course of the field season. However, since these trends never differed between HB-exposed and HB-absent bumble bees it is unlikely to have biased assessment of treatment effects.

One last factor that was not considered in this study, was the effect that coinfection with several different viruses could have had on the prevalence and intensity of quantified virus. Co-infections are commonly documented in the literature (Shen et al. 2005b, Singh et al. 2010, Runckel et al. 2011, Desai et al. 2016) and are certainly seen in the samples tested in this study. For instance, Desai et al. (2016) showed a positive correlation between infection with BQCV and IAPV in honey bees. Similarly, infections with other pathogens such as *Nosema* may be correlated to viral infections, such as BQCV (Desai and Currie 2016). Further research, should analysze the correlation between infection with multiple pathogens as to its effect on conclusions made about differences in prevalence and intensity.

It is worth noting that honey bees used in this experiment are workers captured indiscriminately from sites containing experimental hives used for other research on pathogens and so it was expected that viral concentrations could be high. Infections in bees are exacerbated by reduced immune system function brought about by co-infection with other non-viral pathogens. Levels of DWV, for instance, are known to be associated with varroa mite infestations (Highfield et al. 2009, Locke et al. 2012). Therefore, in addition to the fact that viral titres in associated bumble bee populations are lower, it is unlikely that high viral titres found in some honey bee specimens are a result of 'pathogen spillback' (Daszak et al. 2000).

This is not the first study to look at pathogen spillover into native pollinators. Previous work looked at the spillover of non-viral gut parasites like *Nosema ceranae* from honey bees into wild bumble bees (Graystock et al. 2013a), and others such as *Apicystis bombi* (Arbetman et al. 2013), *Nosema bombi* and *Crithidia* spp. (Murray et al. 2013) from commercially-reared to wild bumble bees. In most of these cases these pathogens proved more infective to the new hosts. It was predicted that spillover of these bumble bee pathogens from greenhouses into the wild would occur rapidly (Otterstatter and Thomson 2008) and so we can speculate that the qualities of a virus would allow it to spread even more rapidly. Since work on this project began it has been demonstrated that associated bumble bees harbour the same strains of DWV as honey bees (Fürst et al. 2014) and that other viruses such as BQCV, SBV, KBV and IAPV were being detected in lower intensities in wild bees (Reynaldi et al. 2013, Ravoet et al. 2014, Guzman-Novoa et al. 2015, Dolezal et al. 2016, Tehel et al. 2016) and other arthropod species as well (Levitt et al. 2013, Lucia et al. 2014). No clear picture has emerged on the directionality of transmission of the viruses and the effect these infections have on wild bee hosts is still

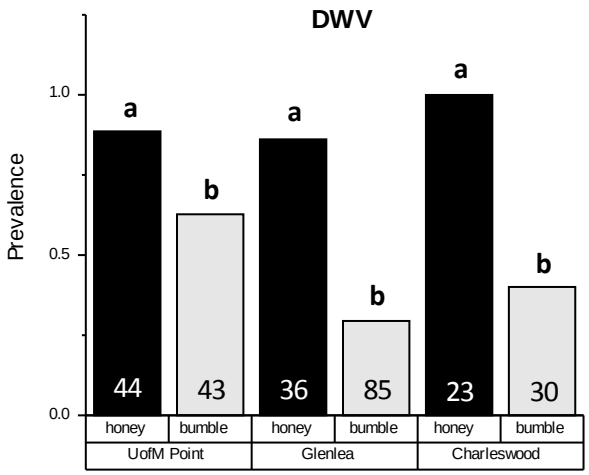
largely unknown. We do know that *Bombus atratus* in Argentina remain asymptomatic when infected with DWV, BQCV and SBV (Reynaldi et al. 2013). Additionally, experimental inoculation of *Megachile rotundata* Fabricius and *Colletes inaequalis* Say with a mixture of DWV, SBV, IAPV and BQCV doses that would be lethal to a honey bee had no measurable effect (Dolezal et al. 2016). This was the first study to sample for viruses in wild bees that were not concurrently associated with infected honey bees, and though it provides further evidence for pathogen spillover it still cannot conclusively state that these viruses originated in honey bees or that they have a negative effect on wild bee health. Future research should examine the effects infection with these RNA viruses have on the health of wild bee colonies.

In conclusion, prevalence of DWV, BQCV and SBV is higher in bumble bees associated with infected honey bees and intensity of BQCV infection is higher in bumble bees exposed to honey bees than in unexposed sites. DWV, BQCV, SBV and IAPV are never less prevalent or intense in honey bees hypothesized to be the original host. Overall this seems to suggest honey bees have an impact on the suite of viruses found in local bumble bee populations, but we still know nothing about how this could influence their fitness.

Table 2.1 - A list of the PCR primers used for virus detection in this study. Both the forward (F) and reverse (R) primers are included as well as the target size in base pairs (bp) and the author to which the creation of the primer pair is credited.

Primer sequences	Product size (bp)	Reference
DWV-F: CGAAACCAACTTCTGAGGAA DWV-R: GTGTTGATCCCTGAGGCTTA	174	Peng et al. (2011)
BQCV-F: CCTGTATTCATGCATCTCAGA BQCV-R: GCAACAAGAAGAAACGTAAACCAC	310	Chen personal communication
KBV-F: CCATACCTGCTGATAACC KBV-R: CTGAATAATACTGTGCGTATC	200	Locke et al. (2012)
SBV-F: TTGGAATTACGCATTCTCTG SBV-R: GCTCTAACCTCGCATCAAC	335	Locke et al. (2012)
IAPV-F: CCATGCCTGGCGATTCAC IAPV-R: CTGAATAATACTGTGCGTATC	203	Locke et al. (2012)
ABPV-F: TCATACCTGCCGATCAAG ABPV-R: CTGAATAATACTGTGCGTATC	197	Locke et al. (2012)
CBPV-F: CAACCTGCCTCAACACAG CBPV-R: AATCTGGCAAGGTTGACTGG	296	Locke et al. (2012)
Actin-F: AGGAATGGAAGCTTGCGGTA Actin-R: AATTTTCATGGTGGATGGTGC	181	Chen et al. (2005)

A)



B)

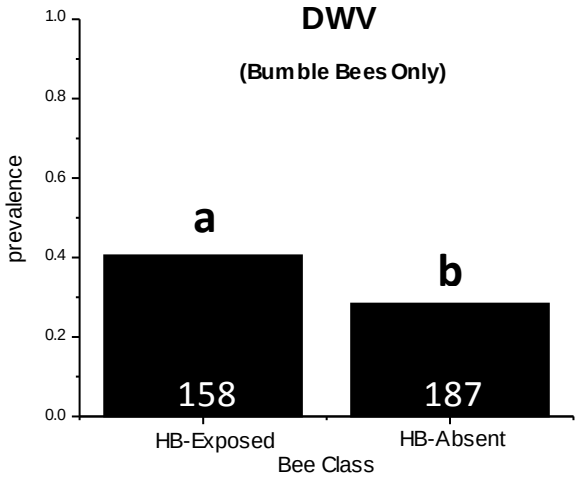


Fig. 2.1 – Comparisons of the prevalence of Deformed Wing Virus (DWV) in : A) honey bees (honey) and bumble bees (bumble) captured within sites where honey bees were present (HB-Exposed) and B) pooled samples of bumble bees captured within sites where honey bees were present (HB-Exposed) and where honey bees were absent (HB-Absent). Bars followed by the same letters indicate no difference between the prevalence of lettered groups within a figure ($P>0.05$). Numbers in bars indicate the sample size from which prevalence (proportion of infected bees) was determined.

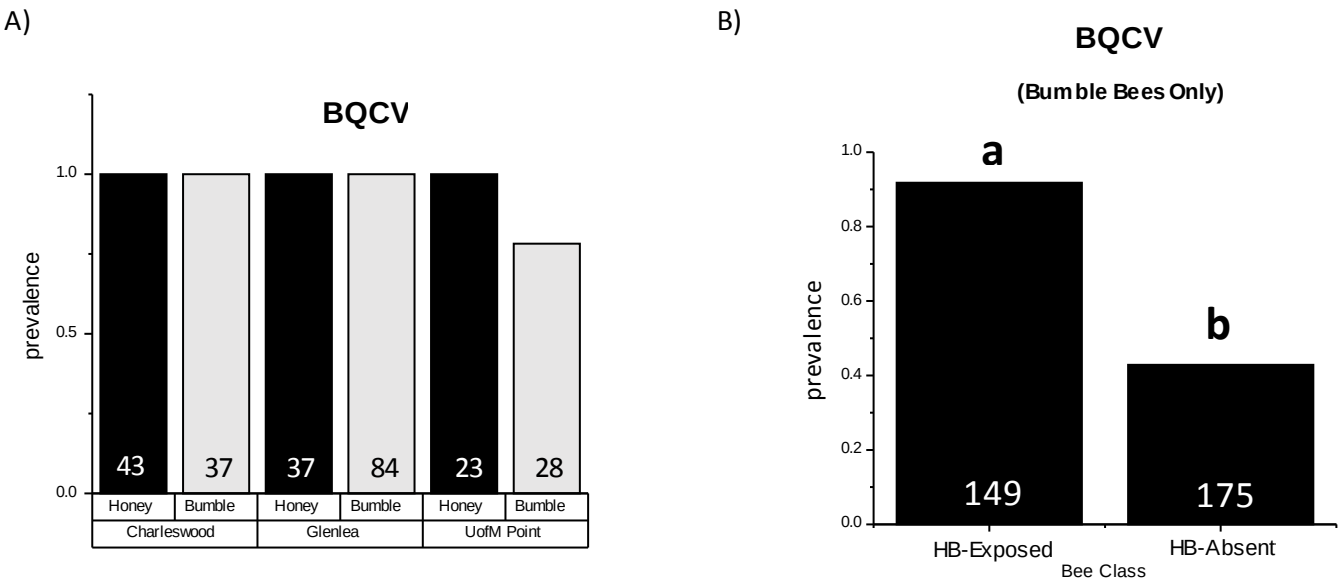
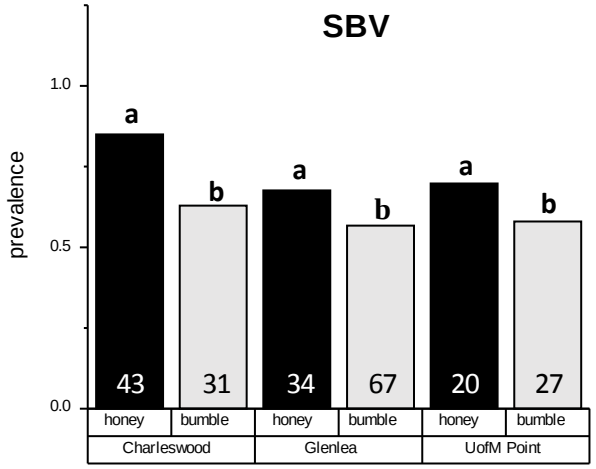


Fig. 2.2 – Comparisons of the prevalence of Black Queen Cell Virus (BQCV) in : A) honey bees (honey) and bumble bees (bumble) captured within sites where honey bees were present (HB-Exposed) and B) pooled samples of bumble bees captured within sites where honey bees were present (HB-Exposed) and where honey bees were absent (HB-Absent). Bars followed by the same letters indicate no difference between the prevalence of lettered groups within a figure (P>0.05) with absence of lettering indicating no statistical significance within a figure. Numbers in bars indicate the sample size from which prevalence (proportion of infected bees) was determined.

A)



B)

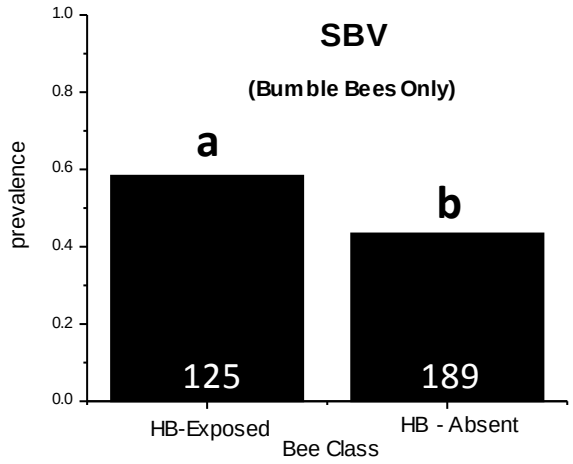


Fig. 2.3 – Comparisons of the prevalence of Sacbrood Virus (SBV) in : A) honey bees (honey) and bumble bees (bumble) captured within sites where honey bees were present (HB-Exposed) and B) pooled samples of bumble bees captured within sites where honey bees were present (HB-Exposed) and where honey bees were absent (HB-Absent). Bars followed by the same letters indicate no difference between the prevalence of lettered groups within a figure ($P>0.05$). Numbers in bars indicate the sample size from which prevalence (proportion of infected bees) was determined.

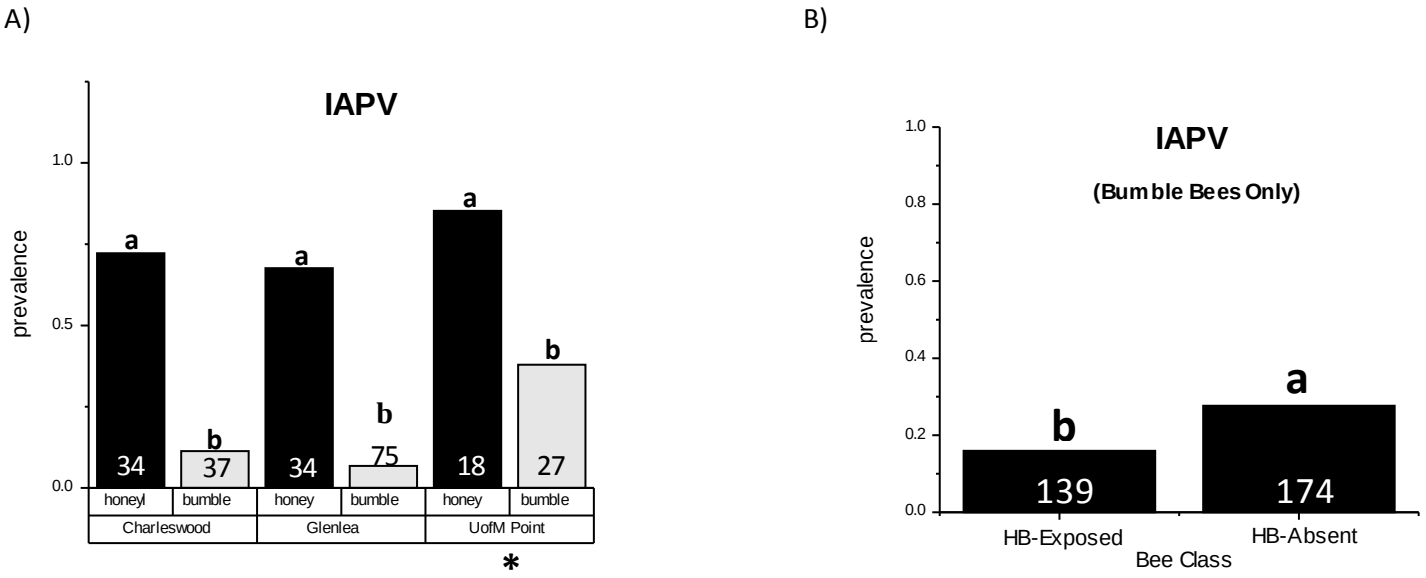
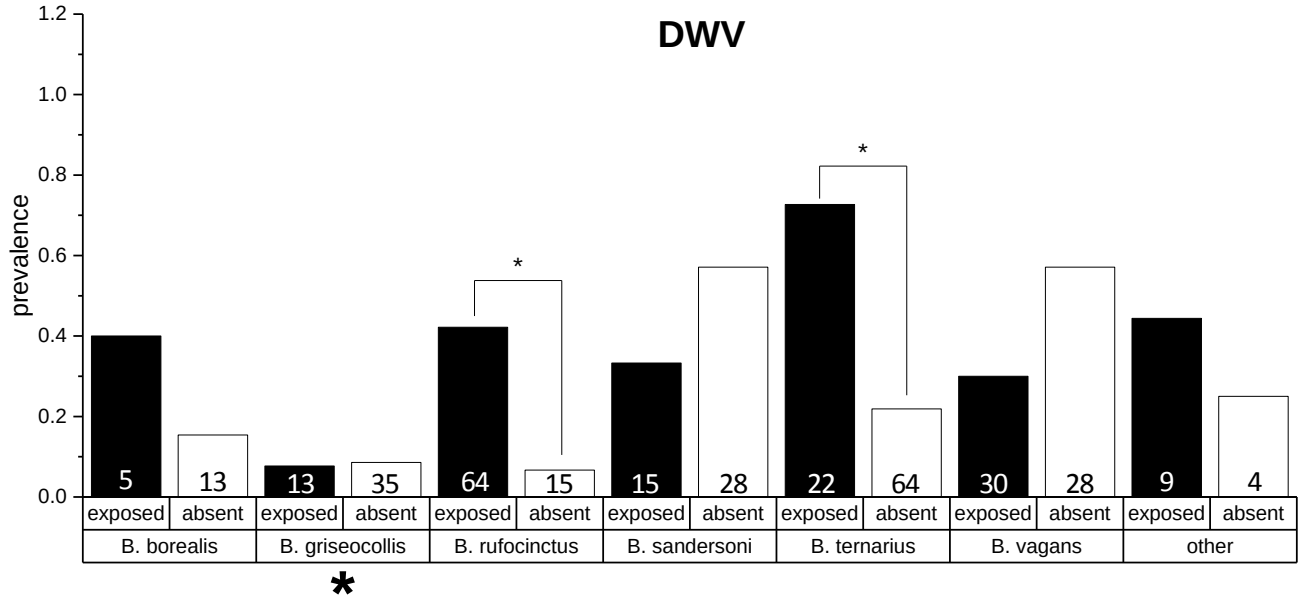
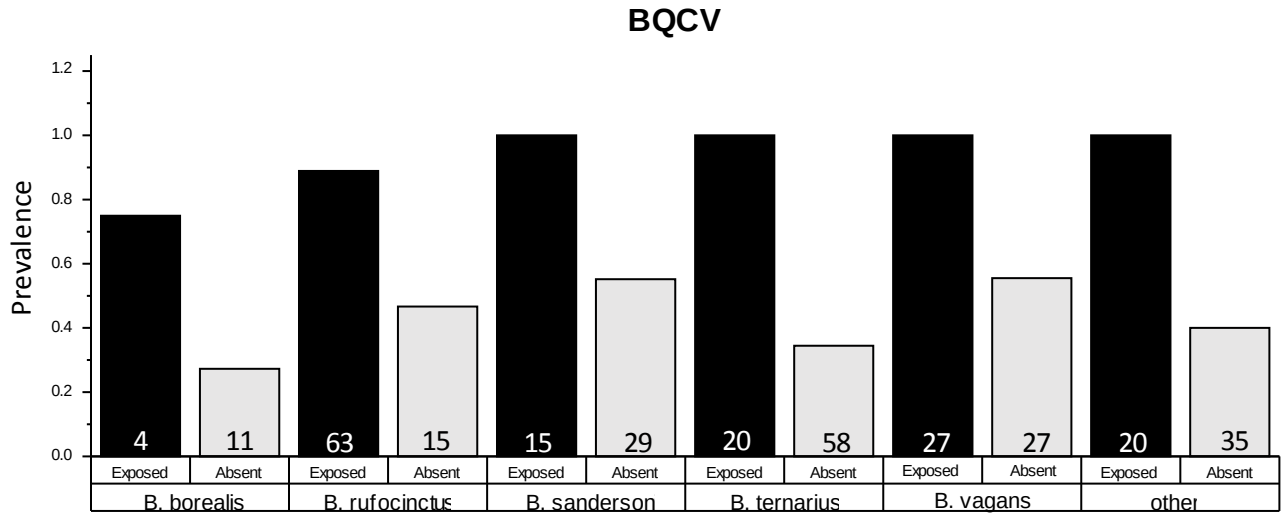


Fig. 2.4 – Comparisons of the prevalence of Israeli Acute Paralysis Virus (IAPV) in : A) honey bees (honey) and bumble bees (bumble) captured within sites where honey bees were present (HB-Exposed) and B) pooled samples of bumble bees captured within sites where honey bees were present (HB-Exposed) and where honey bees were absent (HB-Absent). Bars followed by the same letters indicate no difference between the prevalence of lettered groups within a figure (P>0.05). The * in A indicates that IAPV is more prevalent in bees captured from the U of M Point site. Numbers on bars indicate the sample size from which prevalence (proportion of infected bees) was determined.

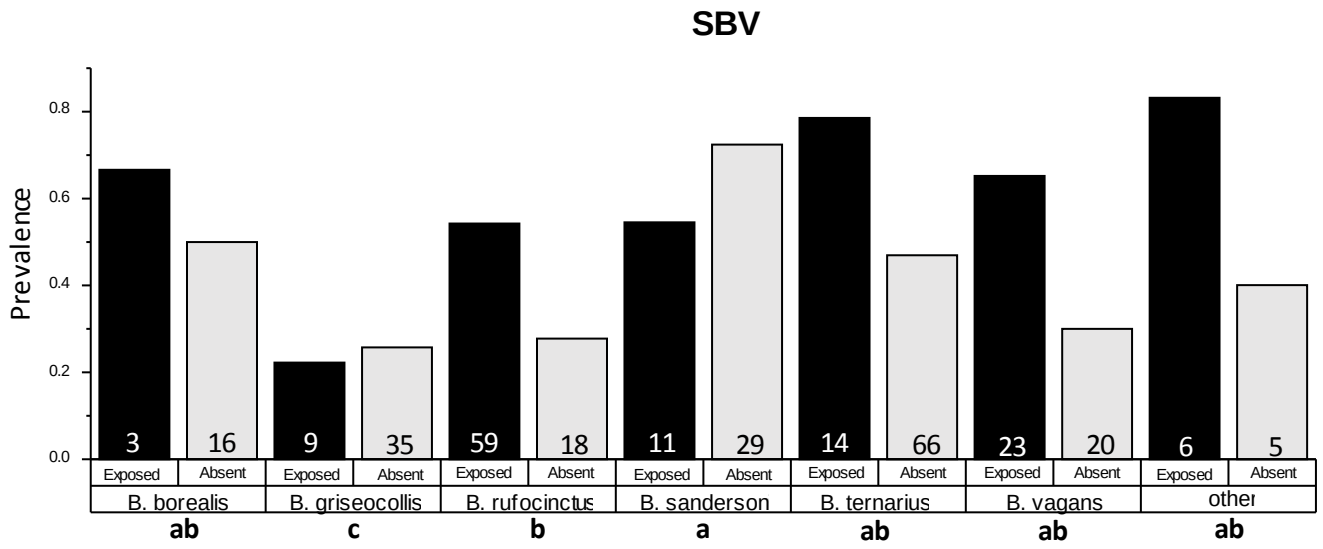
A)


DWV
prevalence
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
5
13
13
35
64
15
15
28
22
64
30
28
9
4
exposed
absent
B. borealis
B. griseocollis
B. rufocinctus
B. sandersoni
B. ternarius
B. vagans
other
*

B)


BQCV
Prevalence
1.2
1.0
0.8
0.6
0.4
0.2
0.0
4
11
63
15
15
29
20
58
27
27
20
35
Exposed
Absent
B. borealis
B. rufocinctus
B. sanderson
B. ternarius
B. vagans
other

C)


SBV
Prevalence
0.0
0.2
0.4
0.6
0.8
3
16
9
35
59
18
11
29
14
66
23
20
6
5
Exposed
Absent
B. borealis
B. griseocollis
B. rufocinctus
B. sanderson
B. ternarius
B. vagans
other
ab
c
b
a
ab
ab
ab

D)

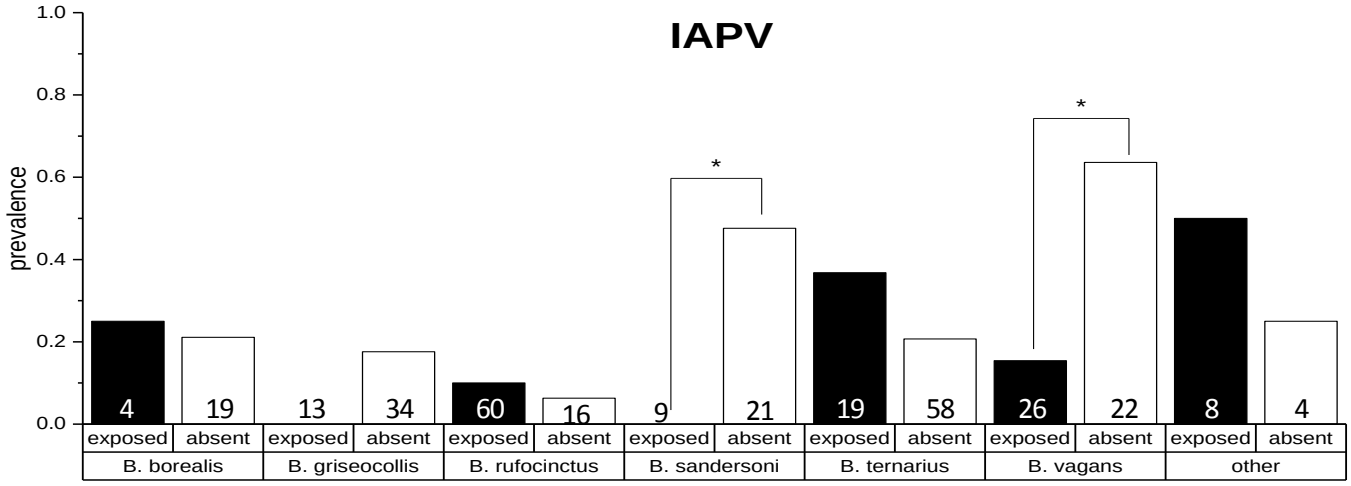

IAPV
prevalence
0.0
0.2
0.4
0.6
0.8
1.0
*
*
4
19
13
34
60
16
9
21
19
58
26
22
8
4
exposed
absent
B. borealis
B. griseocollis
B. rufocinctus
B. sandersoni
B. ternarius
B. vagans
other

Fig. 2.5 – Comparisons of the prevalence of A) Deformed Wing Virus (DWV), B) Black Queen Cell Virus (BQCV), C) Sacbrood Virus (SBV) and D) Israeli Acute Paralysis Virus (IAPV) in bumble bees captured in the presence (Exposed) and absence (Absent) of honey bees. Bars connected with an * indicate a significant difference in viral prevalence among bees of the same species between the type of site at which they were captured. A * below a particular species on the graph shows a significant difference in viral prevalence in that particular species from all others. In the graph for SBV prevalence (C) species that have letters below the graph that are the same indicate prevalence is not significantly different (P>0.05). Differences are examined at the species level, with the 'other' category including pooled numbers of rare species (<8 captured) including *Bombus fervidus* (n=3), *B. fernaldae* (n=1), *B. terricola* (n=2), *B. perplexus* (n=2) and *B. bimaculatus* (n=7) (*B. griseocollis was* also pooled for testing BQCV prevalence). Numbers on the bars indicate the sample size from which prevalence was determined.

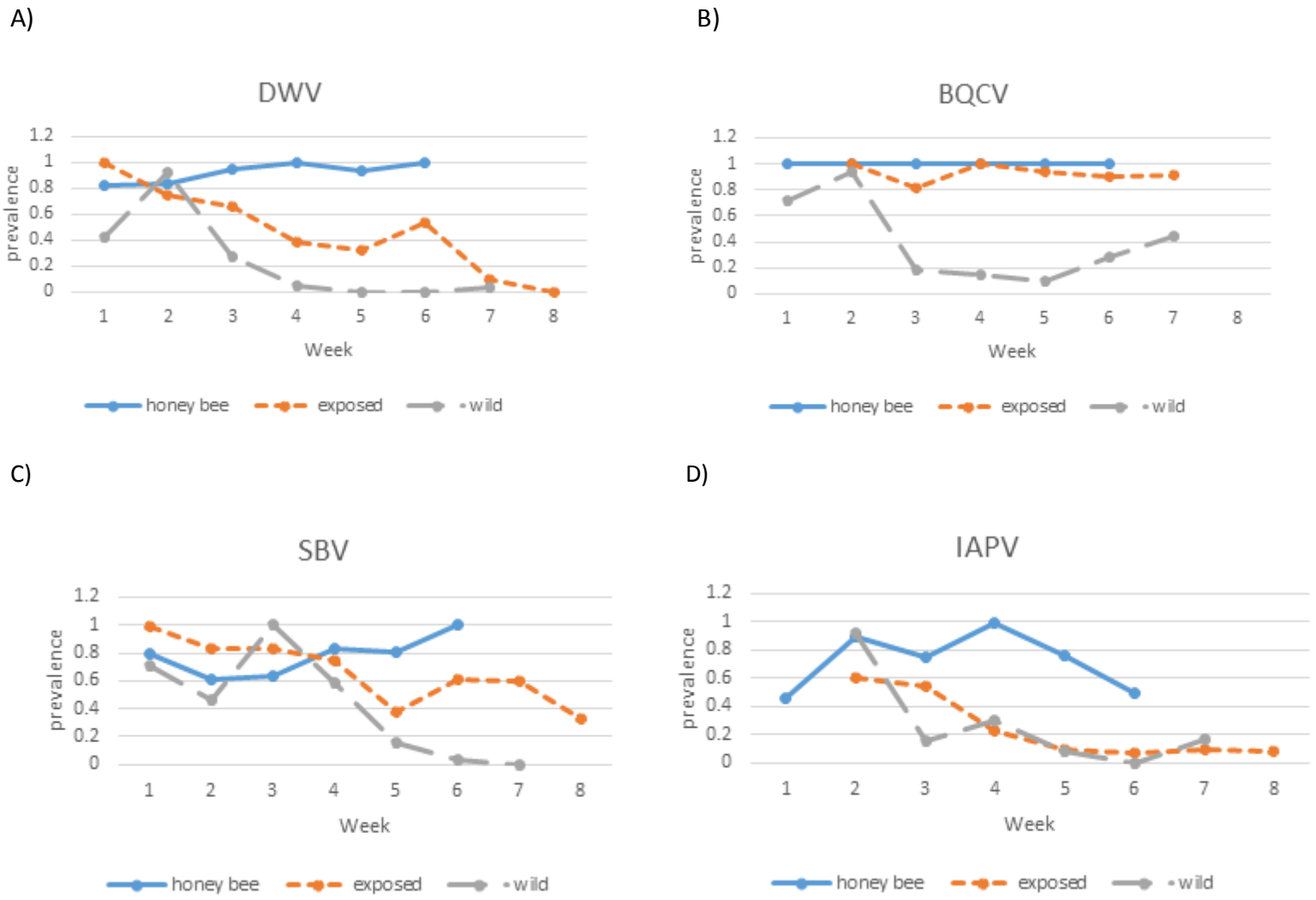
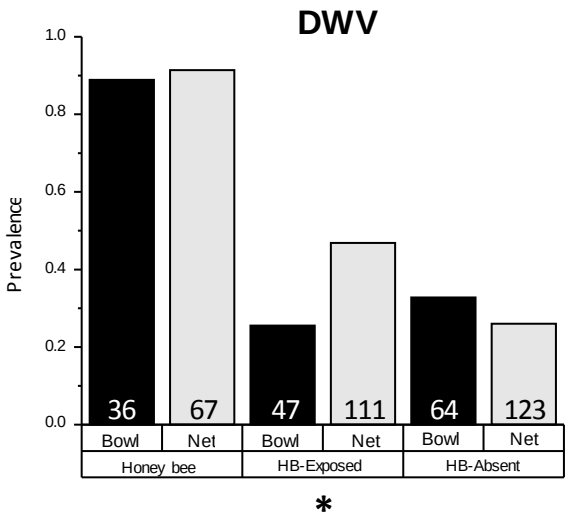
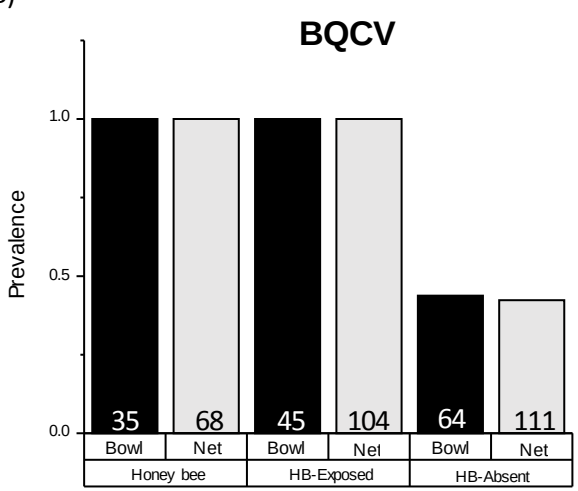


Fig. 2.6 – Seasonal prevalence of A) Deformed Wing Virus (DWV), B) Black Queen Cell Virus (BQCV), C) Sacbrood Virus (SBV) and D) Israeli Acute Paralysis Virus (IAPV) in honey bees and bumble bees caught in the presence (Exposed) and absence (wild) of honey bees captured each week through the months of July and August 2012. Note that some sample types do not appear in each week of sampling. Statistics are described in the results.

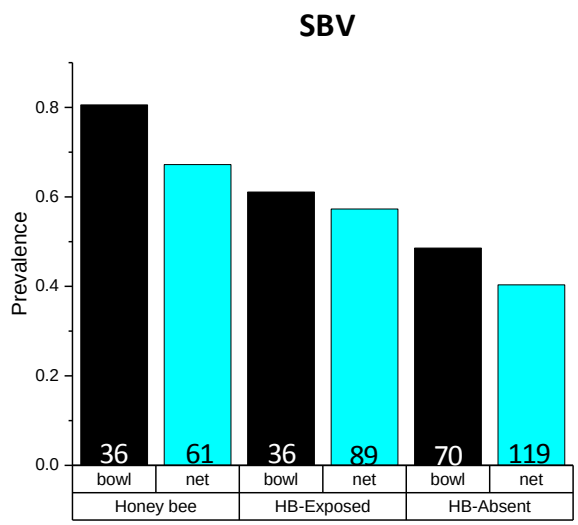
A)


DWV
Prevalence
1.0
0.8
0.6
0.4
0.2
0.0
36
67
47
111
64
123
Bowl
Net
Bowl
Net
Bowl
Net
Honey bee
HB-Exposed
HB-Absent
*

B)


BQCV
Prevalence
1.0
0.5
0.0
35
68
45
104
64
111
Bowl
Net
Bowl
Net
Bowl
Net
Honey bee
HB-Exposed
HB-Absent

C)


SBV
Prevalence
0.8
0.6
0.4
0.2
0.0
36
61
36
89
70
119
bowl
net
bowl
net
bowl
net
Honey bee
HB-Exposed
HB-Absent

D)

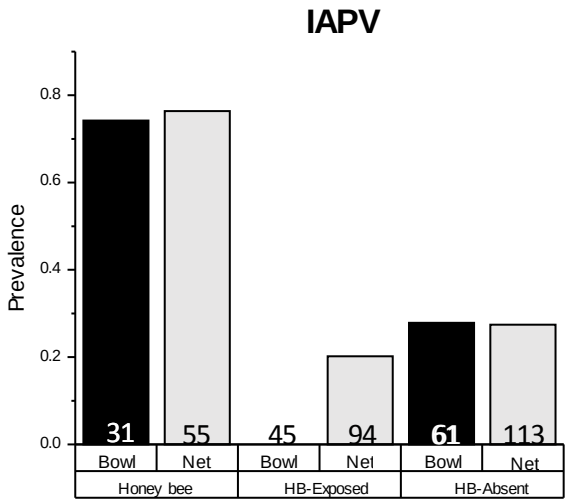

IAPV
Prevalence
0.8
0.6
0.4
0.2
0.0
31
55
45
94
61
113
Bowl
Net
Bowl
Net
Bowl
Net
Honey bee
HB-Exposed
HB-Absent

Fig. 2.7 –The effect of capture method on the prevalence of RNA viruses in the different treatment combinations for this experiment. Virus prevalence in honey bees (Honey bee) and bumble bees both in the presence (HB-Exposed) and absence (HB-Absent) of honey bees are compared between the two capture methods used; passive bee bowl trapping (Bowl) and active and opportunistic sweep netting (Net). The prevalence of four different viruses are examined: A) Deformed Wing Virus (DWV), B) Black Queen Cell Virus (BQCV), C) Sacbrood Virus (SBV) and D) Israeli Acute Paralysis Virus (IAPV). The * below A indicates that the prevalence of DWV was higher in bumble bees caught in a net than in a bowl at HB-exposed sites.

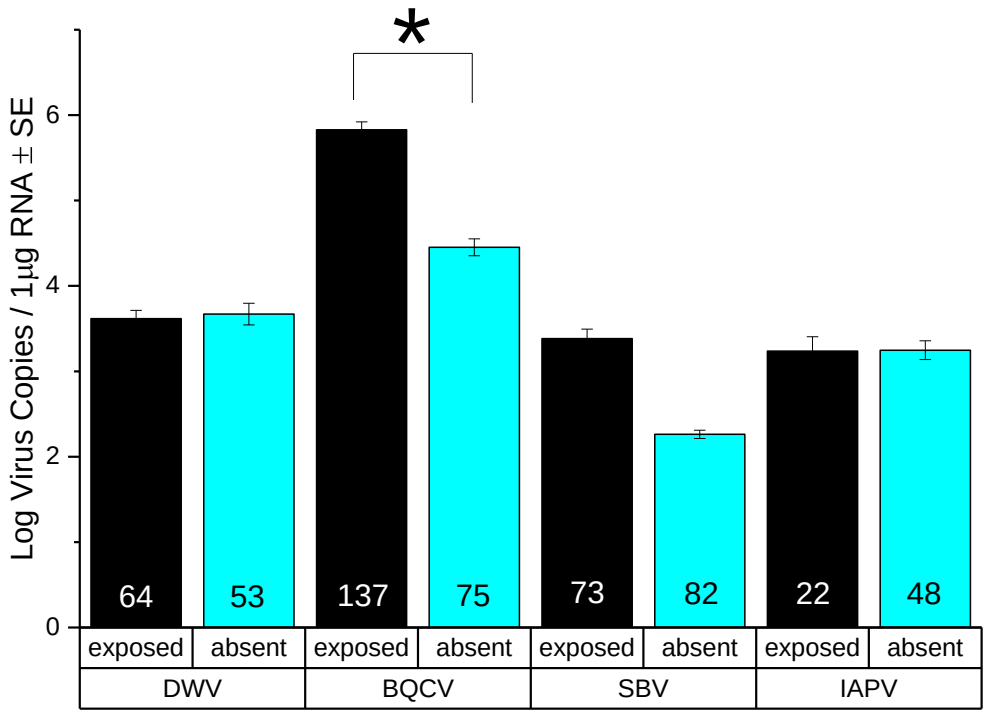


Fig. 2.8 –Effect of two bee site exposure classes (exposed = bumble bees captured on sites where honey bees were present; and absent = bumble bees captured on sites where honey bees were deemed absent) on average intensity of infection for 4 viruses. The number of copies of detected virus per µg of RNA ranged several orders of magnitude and so were log-transformed for ease of interpretation and to fit the assumptions of an ANOVA. The * indicates BQCV was the only virus that showed significant differences in intensity of infection between the two groups of bees. Numbers on the columns represent the sample sizes from which average intensity was calculated.

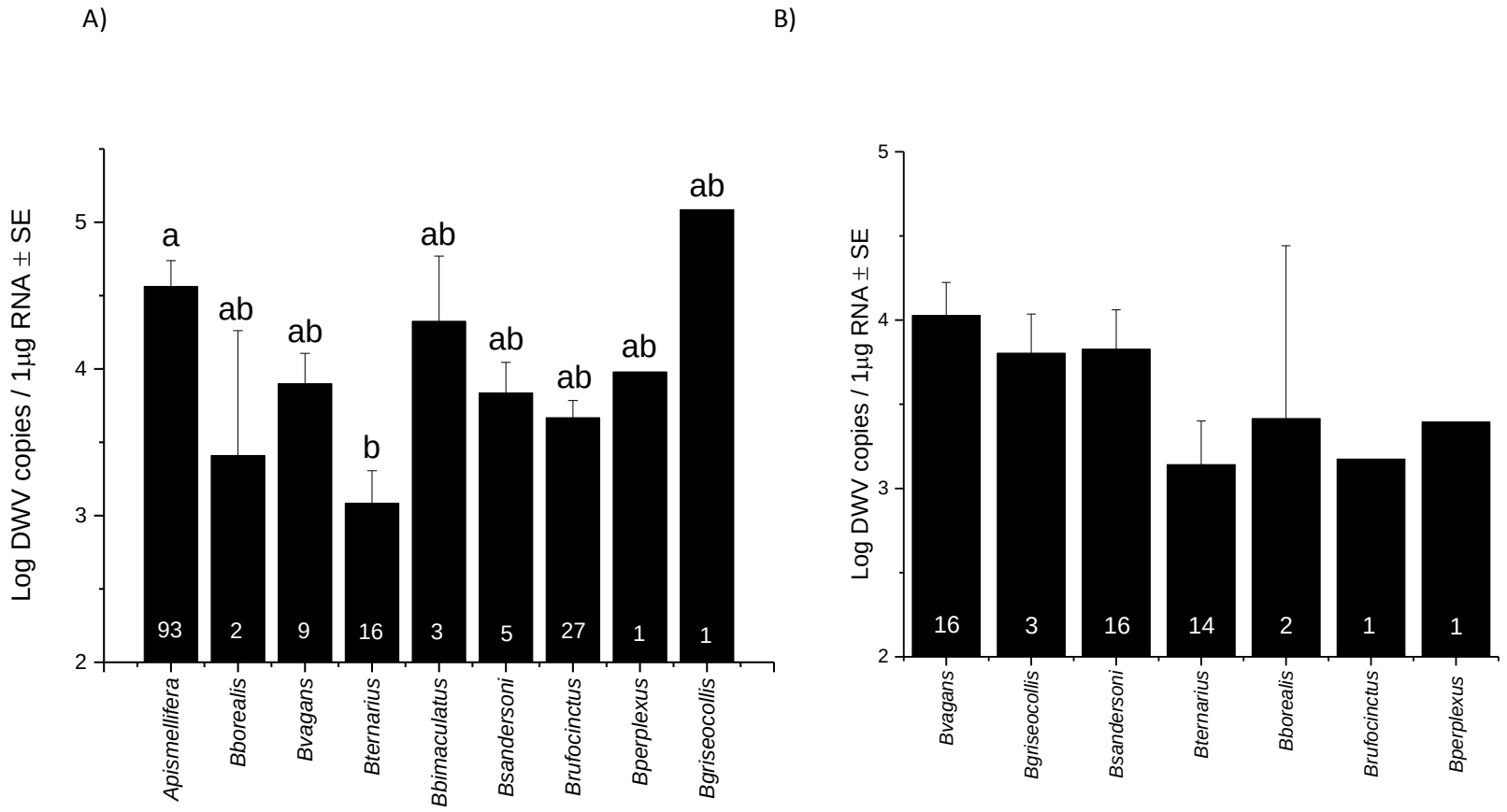


Fig. 2.9 – Comparison of intensity of infection with Deformed Wing Virus (DWV) in all captured bee species from within: A) sites where honey bees are present and B) sites where honey bees were absent. The number of copies of detected virus per 2µg of RNA ranged several orders of magnitude and so were log-transformed for ease of interpretation and to fit the assumptions of an ANOVA. Letters above the bars that are the same indicate bee species that are not significantly different from each other (P>0.05). There were no significant differences in intensity between bumble bee species from those captured at sites where honey bees were absent. White numbers within the bars are the s ample size of bees from which the average intensity was determined.

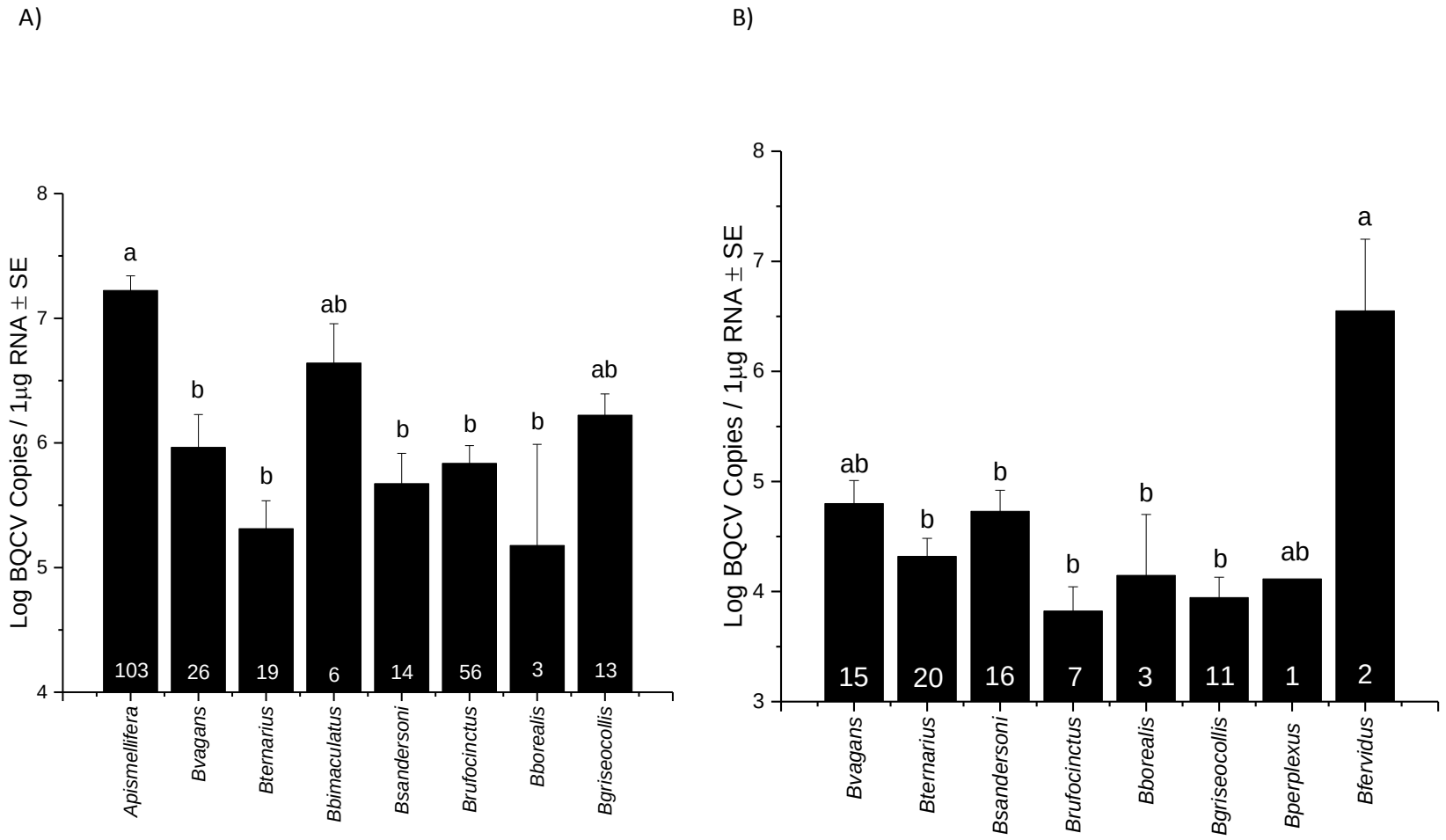


Fig. 2.10 – Comparison of intensity of infection with Black Queen Cell Virus (BQCV) in all captured bee species from within: A) sites where honey bees are present and B) sites where honey bees were absent. The number of copies of detected virus per µg of RNA ranged several orders of magnitude and so were log-transformed for ease of interpretation and to fit the assumptions of an ANOVA. Letters above the bars that are the same indicate bee species that are not significantly different from each other (P>0.05). White numbers within the bars are the sample size of bees from which the average intensity was determined.

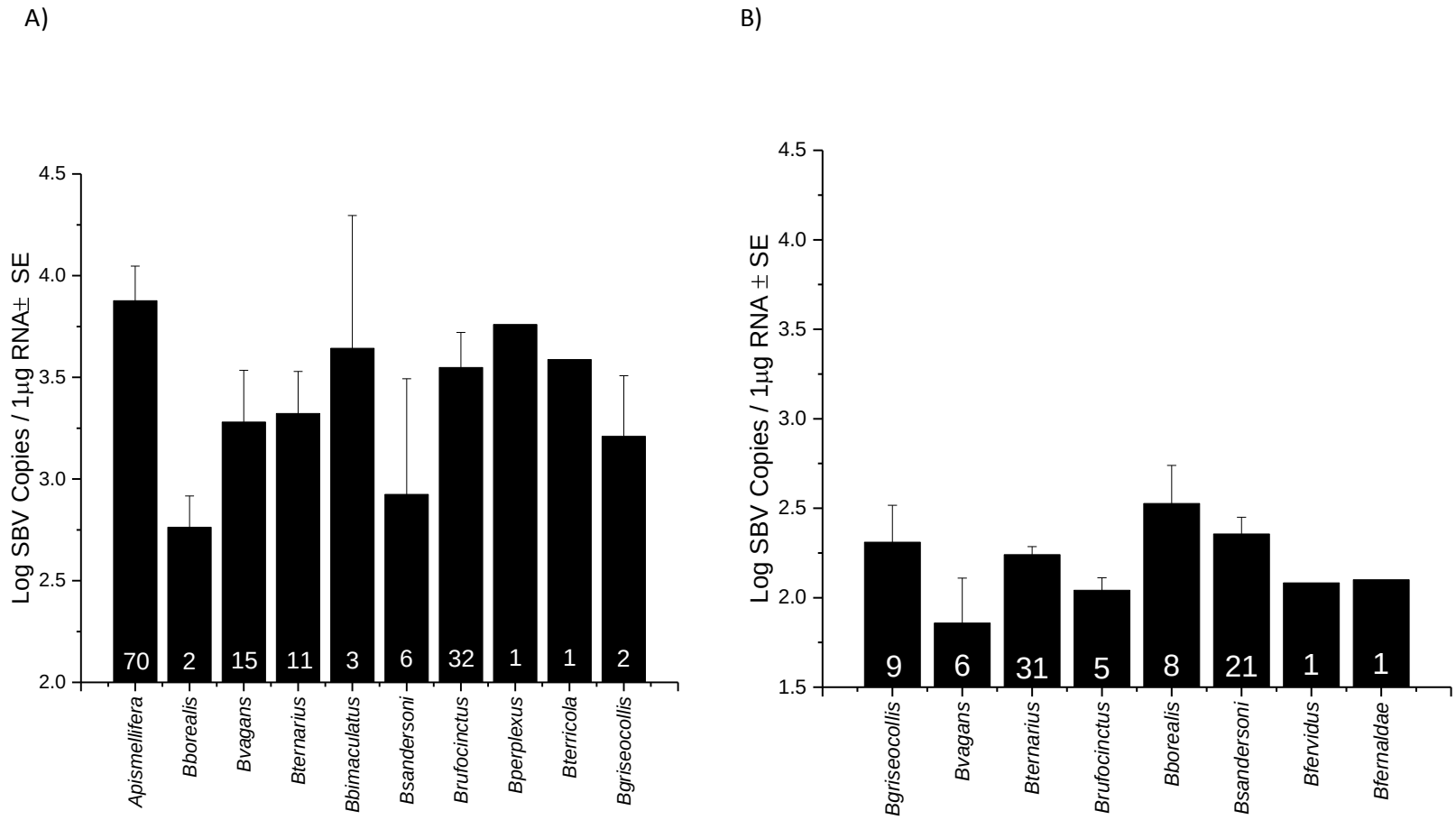


Fig. 2.11 – Comparison of intensity of infection with Sacbrood Virus (SBV) in all captured bee species from within: A) sites where honey bees are present and B) sites where honey bees were absent. The number of copies of detected virus per µg of RNA ranged several orders of magnitude and so were log-transformed for ease of interpretation and to fit the assumptions of an ANOVA. Letters above the bars that are the same indicate bee species that are not significantly different from each other (P>0.05). White numbers within the bars are the sample size of bees from which the average intensity was determined

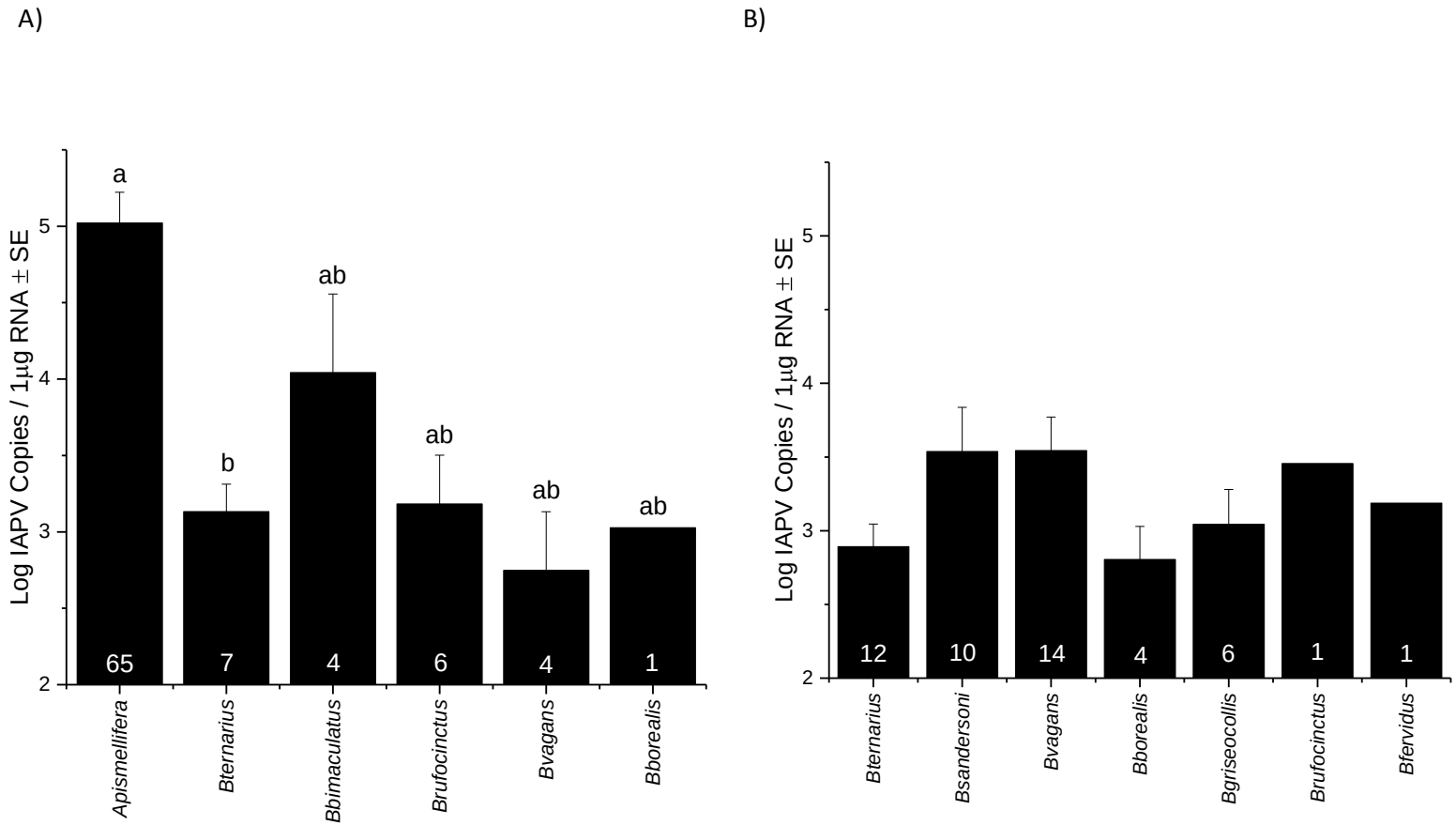


Fig. 2.12 – Comparison of intensity of infection with Israeli Acute Paralysis Virus (IAPV) in all captured bee species from within: A) sites where honey bees are present and B) sites where honey bees were absent. The number of copies of detected virus per 2 µg of RNA ranged several orders of magnitude and so were log-transformed for ease of interpretation and to fit the assumptions of an ANOVA. Letters above the bars that are the same indicate bee species that are not significantly different from each other (P>0.05). There were no significant differences in intensity between bumble bee species from those captured at sites where honey bees were absent. White numbers within the bars are the sample size of bees from which the average intensity was determined.

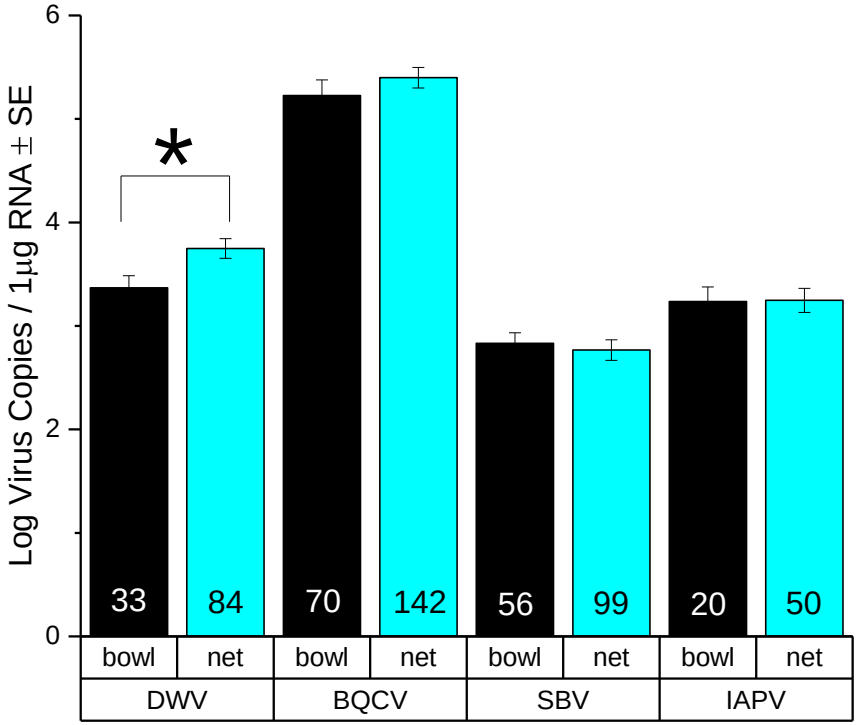
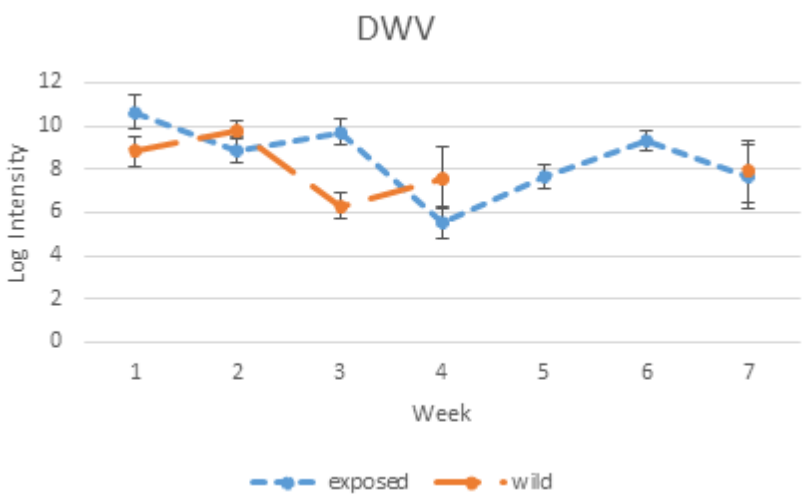
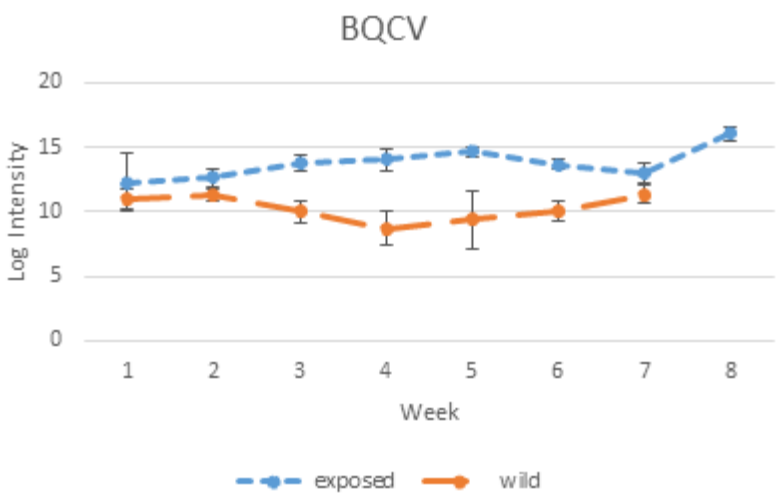


Fig. 2.13 – Effect of two sampling methods on average intensity of infection with 4 viruses between groups of bumble bees (bowl = captured using the passive bee bowl method; net = captured with active and opportunistic sweep netting). The number of copies of virus per µg of RNA ranged several orders of magnitude and so were log-transformed for ease of interpretation and to fit the assumptions of an ANOVA. The * indicates DWV was the only virus that showed significant differences in intensity of infection between the two capture methods for bumble bees. The numbers in the columns represent the sample sizes from which average intensities were calculated.

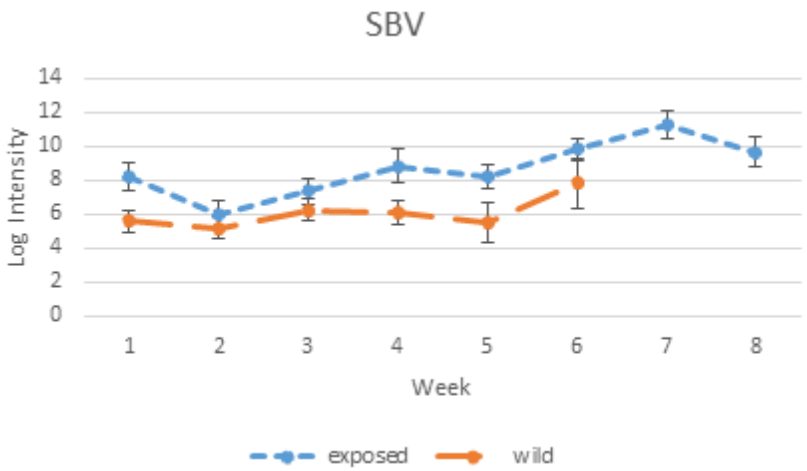
A)


DWV
Log Intensity
0
2
4
6
8
10
12
1
2
3
4
5
6
7
Week
exposed
wild

B)


BQCV
Log Intensity
0
5
10
15
20
1
2
3
4
5
6
7
8
Week
exposed
wild

C)


SBV
Log Intensity
0
2
4
6
8
10
12
14
1
2
3
4
5
6
7
8
Week
exposed
wild

D)

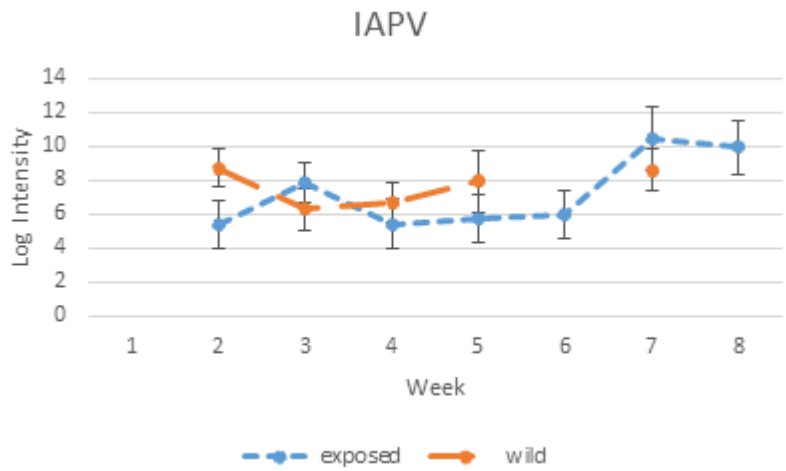

IAPV
Log Intensity
0
2
4
6
8
10
12
14
1
2
3
4
5
6
7
8
Week
exposed
wild

Fig. 2.14 – Effect of seasonal variation on average intensity of infection with 4 different viruses: A) Deformed Wing Virus (DWV), B) Black Queen Cell Virus (BQCV), C) Sacbrood Virus (SBV) and D) Israeli Acute Paralysis Virus (IAPV). Comparisons within each figure are be tween two groups of sampled bumble bees ('exposed' = captured on sites where honey bees were present; 'wild' = captured on sites where honey bees were deemed absent). The number of copies of detected virus per µg of RNA ranged several orders of magnitude and so were log-transformed for ease of interpretation and to fit the assumptions of an ANOVA. (NOTE: lines do not always extend across all 8 weeks, due to missing datapoints where no infected bumble bees were captured in a given week. Statistics are described in the results.

<u>Chapter 2:</u> Effects of sampling and storage techniques on RNA virus concentrations in honey bee (*Apis mellifera* L.) and alfalfa leafcutting bee (*Megachile rotundata* Fabricus) samples.

Abstract

Recent declines in managed honey bee populations and native bee species ranges has sparked a wealth of scientific research into the cause. One particularly promising candidate is a suite of approximately 24 distinct RNA viruses that are known to infect honey bees, many of which have been characterized in bumble bees and other bee species as well. In order to determine their possible contribution to endangerment risk of native bee species, studies must be able to accurately determine the level of infection in field caught specimens. This study looked at the efficacy of several combinations of storage medium, temperature and the effect of elapsed time until storage, on the relative preservation of viral and host RNA. Additionally, this study investigated the commonly used bee bowl trap as a possible contamination source that could generate false positive infection results when used for these kinds of studies due to infected bees sharing the bowl with uninfected ones. It was determined that storage at -80^{o}C better preserved RNA over storage at -20^{o}C and that delaying storage up to 48 hours had little effect on the degradation of viral RNA without considering other factors. Storage in either 95% ethanol, RNAlater^{TM} or nothing caused differential levels of preservation of viral and host RNA. Host β-actin mRNA molecules were better preserved in ethanol, whereas under certain conditions DWV RNA molecules were better preserved by RNAlater^{TM}. BQCV RNA molecules did not differ in relative preservation under different storage treatments. Findings suggest that the

use of no storage medium at -80°C may be preferential for relative preservation of host and virus RNA. Alfalfa leafcutting bees that shared bee bowls with honey bees infected with DWV and BQCV never tested positive for DWV. Experimental and control leafcutting bees both tested positive for BQCV infection, but upon closer inspection positive detections in bowls shared with honey bees were likely the cause of a BQCV analog replicated by the primers used. Therefore, bee bowls are deemed a viable method of capture for virus research in field-collected specimens.

Introduction

Bees are important both economically and ecologically for the pollination services they provide (Meffe 1998, Memmott et al. 2004, Goulson et al. 2008, Ellis et al. 2010). Recently reported declines in both honey bee and wild bee species worldwide have largely been associated with high levels of mites, bacterial and fungal parasites and RNA viruses (Cox-Foster et al. 2007, Williams and Osborne 2009, Winfree 2010, Cornman et al. 2012, Fürst et al. 2014) although pesticides, climate change and loss of resource diversity are also cited as potential contributors (Evans et al. 2009, Potts et al. 2010). The role that viruses play has recently become an area of intense study. There are currently more than 24 distinct viral species characterized in honey bees that could be implicated in both honey bee and wild bee disappearances (de Miranda et al. 2013). These viruses when present typically exist as latent, low-level infections within colonies of social bees (Bailey 1967, Bailey and Woods 1974, Dall 1985, Chen et al. 2006b, Genersch and Aubert 2010, de Miranda et al. 2013). Previously

reported prevalence of most viruses in honey bee colonies was low until the use of more sensitive techniques such as qPCR. Since then, much research has been conducted using these techniques to study their spread and virulence amongst honey bees, bumble bees and other arthropod vectors.

Transmission and replication of these viruses is not yet fully understood, particularly when viruses are introduced into new host species such as bumble bees. Within honey bee colonies RNA viruses can spread vertically from parent(s) to offspring (Shen et al. 2005a, Chen et al. 2006a, Chen et al. 2006b, De Miranda and Fries 2008) and horizontally through bee to bee contact, contaminated food (Shen et al. 2005a, Chen et al. 2006a), feces (Hung 2000, Chen et al. 2006b, Ribiere et al. 2007) and mite vectors (Ball and Allen 1988, Bowen-Walker et al. 1999, Nordström 2003, Chen et al. 2004, Shen et al. 2005b, Genersch and Aubert 2010, Neumann et al. 2012). Horizontal spread of virus into bumble bee hosts occurs in part, through shared flower use for at least one pathogen, Israeli Acute Paralysis Virus (IAPV) (Singh et al. 2010), and other viruses are hypothesized to also be spread through mutual insect pests, such as ants (*Camponotus vagus* and *Formica rufa*), small hive beetles (*Aethina tumida* Murray [Coleoptera: Nitidulidae]) and wax moth larvae (*Galleria mellonela* Fabricius [Lepidoptera : Pyralidae]) (Celle et al. 2008, Eyer et al. 2009, Triyasut et al. 2015). However, research in this area is lacking. Without knowing the true degree of transmission potential for these viruses, through direct or indirect mechanisms, we must be careful in collecting specimens for qPCR analysis to guard against incidental contamination that may lead to false positives.

Despite the popularity of using quantitative PCR to study these viruses, most research has sampled lab-based research colonies and very little information is known for how to

properly capture and store field-caught specimens for qPCR quantification in order to prevent virus degradation. In a study by Chen et al. (2007) it was shown that storing bees at -80^{o}C best preserved the integrity of the viral RNA. They also showed that -20^{o}C worked as well as storing sliced or sectioned pieces of the bee in RNALater solution (Ambion) at 4^{o}C for preserving the virus' integrity, whereas the common practice of storing specimens in 70% ethanol typically used for other honey bee parasites and pathogens resulted in high levels of degradation over time.

Honey bee virus particles, are rather hardy in general, possibly even more so than the host's mRNA (Dainat et al. 2011). Acute Bee Paralysis Virus (ABPV) particles can remain viable in dried fecal material for months (Bailey and Gibbs 1964), and other viruses have persisted in contaminated food for the same amount of time (Singh et al. 2010). DWV and BQCV can still be detected in specimens kept at room temperature for five days (Dainat et al. 2011), however there would be high levels of RNA or DNA degradation in all these instances, which does not bode well for a quantitative study, especially when using the relative CT method. Additionally, when working with field-collected specimens one must be assured that their sampling and storage equipment remains uncontaminated when collecting new specimens and does not cause cross contamination of samples. A potential source of virus cross contamination could occur when bees are collected in bee bowls and remain soaking in the same fluid for periods of several hours. The use of bee bowls is a standard pollinator sampling technique, that involves passive pan trapping of pollinating insects using the blue, yellow and white coloured plastic bowls and the scent of Dawn^{TM} original dish soap as attractants (NSERC-CANPOLIN 2009).

Assuming that sweep net sampling and immediately placing the field-caught specimens on dry ice, and storing them in a freezer at -80^{o}C upon arrival to the lab would provide the best storage conditions, the objectives of this study were to look specifically at whether or not different transportation times, storage media and freezer temperatures have any effect on the quantity of host and viral RNA particles detected in captured honey bees. This study also examines the potential for bee bowl sampling to cause cross contamination between wild caught specimens, by determining whether or not virus particles can spread from an infected honey bee to uninfected alfalfa leafcutting bees captured by the same trap. This is important information to have in studies of virus ecology in order to ensure accurate quantification of viruses and guard against false positive results when using standard ecological sampling techniques.

Materials and methods

Storage experiment

A sample of roughly 270 honey bees was collected from a colony at the U of M Point site known to be infected with DWV and stored on dry ice in order to freeze-kill them before bringing the sample back to the lab. The cooler containing the sample was left in the lab at room temperature for 8 hours before 90 bees were removed and randomly assigned to be stored in triplicate to a 1.5 ml Eppendorf tube to which was added 95% ethanol, RNAlater™ or nothing. This resulted in ten tubes for each storage medium treatment, that were then

randomly assigned a storage temperature, such that half of them were then stored at -20°C and half of them at -80°C. This process was repeated after 24 hours and 48 hours, during which time no additional dry ice was added to the cooler, such that before the end of the first 24 hours it had all dissipated. This was done to simulate varying lengths of delay to return field-caught specimens from the field. In this way, 15 bees were randomly assigned to each possible combination of storage criteria (ie. storage medium, storage temperature and time until storage) for a sample size large enough to guard against inherent variability in viral titres among bees from the same colony.

Bee bowl experiment

Alfalfa leafcutting bees (*Megachile rotunda* Fabricus) served as an uninfected bee source in this experiment, as they had not been shown to carry the RNA viruses in nature at the time the study was started. Cocoons were stored individually in 24-cell culture plates and placed in an incubator at 30ºC until the time of the experiment. Once hatched, newly emerged adults were fed on sugar water by providing it on pieces of syrup-soaked paper towel. Virus infected honey bees for the experiment came from the same infected hive used in the storage experiment.

Forty plastic bowls 2/3 filled with soapy water were spread out evenly in a bee flight room (NSERC-CANPOLIN 2009) kept at 25ºC for seven hours to simulate an average sampling day in the summer. Each bowl was randomly assigned one of five treatments (8 replicates of each):

1) three honey bees and a living alfalfa leafcutting bee (AE – alive experimental)

2) three honey bees and a dead alfalfa leafcutting bee (DE – dead experimental)

3) three honey bees (HC – honey bee control)

4) a living alfalfa leafcutting bee (LC – leafcutting bee control)

5) a dead alfafa leafcutting bee (DLC – dead leafcutting bee control)

As it was likely that, based on experience, in realistic field conditions a bowl-captured leafcutting bee would share the bowl with more than a single honey bee, honey bees were added to each bowl in triplicate. Dead alfalfa leafcutting bees were tested to see if a non-living host (ie. pre-drowned before the addition of infected bees) would accumulate virus particles at the same rate as a host that was alive and actively metabolising before the addition of infected bees.

A second trial was run to test if a single infected bee is enough to transmit the virus to other bees in the bowl. Each treatment consisted of only two bees in the bowl: an infected honey bee with a living alfalfa leafcutting bee (E - experimental) or a control group containing either two honey bees (HC – honey bee control) or two living alfalfa leafcutting bees (LC – leafcutting bee control). Each treatment for this trial was replicated ten times.

Bees from these experiments were stored individually (honey bees were stored together only in the case of those added to bowls in triplicate) in 1.5 ml Eppendorf tubes at -80ºC to await processing for RNA extraction.

RNA extraction

All samples in the eight hour treatment group were submerged in liquid nitrogen and crushed into a fine powder using a mortar and pestle. Between 15 and 35 mg of this powder was used for extraction. All other samples were homogenized directly in 1 ml of lysis buffer for honey bees or 600 µl for leaf-cutter bees (100µl buffer RLT [Qiagen]: 1 µl β-mercaptoethanol [Sigma-Aldritch]) using a Retsch MM400 bead homogenizer. Five metal beads were used per honey bee and 1-3 per leafcutter bee, and the machine was set to 30 cycles/sec for 3-4 10 min. runs, rotating samples between runs to ensure equal homogenization. RNA extraction from the homogenized product was performed using an RNeasy extraction kit (Qiagen, Valencia, Ont., Can.) according to the included protocols and mixing 1:100 β-mercaptoethanol with Buffer RLT for tissue lysis. Twenty to 25µl of molecular grade water was used to elute the sample from the column in order to maximize RNA concentration. Concentration of extracted RNA samples was determined by measuring the optical density using a ThermoScientific NanoDrop 2000 spectrophotometer.

cDNA synthesis

Extracted honey bee RNA was converted to cDNA by adding oligo dT (0.025 µg/µl; Invitrogen) and 1µl dNTP mix (0.5mM; Promega) as non-specific primers to a 1 µg/ml concentration of the RNA sample (or less depending on level of degradation in samples from the storage experiment). A maximum of 10 µl of elution was used in cDNA synthesis, but all samples were standardized to 1 µg/µl by calculating the dilution factor. This mixture was then annealed through incubation at 65ºC for 5 min. followed by 5 min. on ice to halt the reaction.

Four µl of 5x first strand buffer (0.2M; Invitrogen), 2µl 0.01M DTT (Invitrogen) and 1 µl RNaseout (2U/µl; Invitrogen) were then added to the mixture and incubated at 37ºC for 2 min, after which point 1 µl M-MLV (10U/µl; Invitrogen) was added to make a total volume of 20 µl. This final mixture was elongated through incubation at 37ºC for 1 hour followed by a 15 min. denaturation step at 70ºC.

RNA extraction of alfalfa leafcutting bees often yielded concentrations too low for cDNA synthesis using the previous method. For these samples a Bio-Rad iScript™ Reverse Transcription Supermix for RT-qPCR kit was used. Dilution factors were again calculated to standardize all results to 1 µg/µl and high actin CT values were accepted for diluted alfalfa leafcutting bee samples.

RT-qPCR

Each cDNA sample was analyzed for copies of two different RNA viral targets: Deformed Wing Virus (DWV-174; accession #: KF378605.1) and Black Queen Cell Virus (BQCV-310; AF183905.1) using a CFX96 (BioRad) real-time qPCR machine to perform absolute qPCR reactions. Additionally, copies of β-actin mRNA were quantified for comparison in the storage experiment samples (*see* Table 3.1 *for a list of forward and reverse primers used to detect targets*).

Ninety-six-well optical plates were prepared with 20 µl volumes made up of 0.5M 2xpower SYBR Green PCR Master Mix (Bio Rad), a mixture of the appropriate forward and reverse virus primers (0.05M), the appropriate cDNA sample (0.01M) and 0.4M ddH_20. A plate held a maximum of twenty-two samples, each run in triplicate with an additional positive

control involving actin amplification (Actin-181; table 3.1). Wells were also set aside on each plate for negative primer and no template controls as well as five concentrations for a standard curve (selected based on expected gene copies to be found in samples, and rerun if gene copy numbers amplified outside of the standard curve). Standard curves were produced from ten-fold serial dilutions of synthesized target sequences (GBLOCKS^{TM}) beginning with a sample known to contain 10^9 gene copies. Plates were run on the following program: 95ºC for 10 min. followed by 40 cycles of 95ºC for 15s, 55ºC for 30s and 72ºC for 30s followed by a disassociation step that produced a melt curve. If the melt curve was not represented by a single sharp peak the sample was discarded from analysis, as this shows something other than the target sequence was amplified. Copies of the viral sequence were replicated from the sample over the course of the amplification program and translated into a real-time curve of the quantity of virus found over time. From this curve a CT, or cycle threshold, was taken for each sample. CT values were compared to the standard curve in order to estimate a number of viral copies in each sample for each virus tested. Each virus tested for a given set of samples was run on a separate plate.

Statistical analysis

Data from 8-hour treatment groups were analyzed separately from the 24 and 48-hour samples because they were extracted using an alternate homogenization method which could confound the results. For the 8-hour study the data were analysed using a two way ANOVA (PROC GLM program, SAS 9.3) with storage medium, temperature and their interaction as terms in the model and the remainder of the data was run as a 3 way ANOVA with storage

medium, temperature and time as factors. The variables compared consisted of total concentration of RNA produced by the extraction protocol, DWV and BQCV copies produced by the PCR reaction and average CT values produced by the PCR reaction for the β-actin control target sequence. Lastly, differences between the viral and actin control CT values were compared for both DWV and BQCV using a 2^-ΔCT method similar to that proposed by Pfaffl (2004) for relative PCR to account for the replicative nature of a polymerase chain reaction (PCR). Since each sample underwent PCR separately for each of the two viruses, an average of the two actin CTs produced for each sample was used for the statistical analysis. Copy numbers calculated from the PCR analysis were log-transformed for further analysis after a Bartlett's homogeneity of variance test was applied to the data indicating if it violated the assumptions of an ANOVA. Data for the bee bowl experiment were analysed as a two way ANOVA (Proc Mixed, SAS 9.3) using species within bowls as a random factor in the model.

RESULTS

Storage Experiment – 8-hours

When specimens were stored 8-hours after sampling and later processed for RNA extraction using the mortar and pestle crush method, the total concentration of RNA extracted was affected by both the storage temperature ($F=6.14$, $df=1,88$ $p=0.0032$) (Fig 3.3A) and storage medium ($F=7.5$, $df=2,87$ $p=0.0075$) (Fig 3.3B). Similar trends in the relative effectiveness of each storage medium were observed at both -20^{o}C and -80^{o}C as indicated by the lack of interaction between temperature and storage media ($F=1.71$, $df=2,84$ $p=0.1877$). RNA

concentration was consistently higher when stored at -80°C than at -20°C (Fig. 3.3A). Storage in ethanol yielded significantly higher RNA concentrations in 8-hour extractions than storage in RNAlater™, though neither differed from concentrations produced from specimens not stored in any media (Fig. 3.3B).

When considering only β-actin RNA, which was used as an internal control for RNA degradation, a strong interactive effect between storage medium and temperature was found ($F=50.98$, $df=2,80$ $p<0.0001$) (Fig 3.3C). When specimens were stored at -20°C, the relative actin concentration in bees was higher when stored in ethanol than when stored in RNAlater™ or nothing (as indicated by a lower cycle threshold, CT -- the number of PCR cycles required to reach a detectable amount of RNA). This is consistent with previous results showing higher overall concentrations of RNA in ethanol than in RNAlater™ (Fig. 3.3B). However, when stored at -80°C ethanol preserved less actin than either RNAlater™ or no medium (the actin CT was higher for ethanol than RNAlater™ or no medium). Similarly, there were more copies of the target β-actin sequence stored in ethanol when it was kept at -20°C than -80°C. When stored at -80°C actin concentrations were similar to those at -20°C when stored in RNAlater™ or no storage medium (Fig. 3.3C).

In regards to the target sequence for DWV, only temperature affected the preservation of DWV ($F=10.24$, $df=1,84$ $p=0.002$), with storage of samples in -80°C resulting in higher concentrations of this virus being detected than at -20°C (Fig. 3.3D). There was no effect of storage medium ($F=2.79$, $df=2,83$ $p=0.0674$) and no interaction between temperature and storage medium ($F=1.86$, $df=2,80$ $p=0.163$). Lastly, when comparing the relative CT values between DWV and the reference actin gene there was no significant effect of medium ($F=2.13$,

df=2,83 p=0.1259), temperature (F=0.00, df=1,84 p=0.9461) or any combination of media and temperatures on differences in their preservation (F=0.54, df=2, 80 p=0.5859).

For BQCV storage, after 8 hours neither temperature (F=0.11, df=1,84 p=0.7434) nor storage medium (F=0.65, df=2,83 p=0.5249), affected relative preservation of the virus and there were no interactions between these factors (F=1.26, df=2,80 0.2901).

However, when comparing the CT values of actin and BQCV within bees, there was a significant interaction between storage medium and temperature (F=12.95, df=2,80 p<.0001) on differences in the preservation of the two target sequences. The use of RNAlater™ at -20°C led to less difference in preservation of actin and BQCV than when using ethanol at the same temperature. However, relatively more actin was preserved when storage was at -80°C using RNAlater™, and less when using ethanol (Fig. 3.4), which agrees with the findings from previous analyses.

Storage Experiment -24 vs 48 hours

When specimens were stored 24 or 48 hours after capture and later processed using a bead homogenization crush method, the trends were not always consistent with what was found using the 8-hour data under the mortar and pestle crush method. Though there was an overall effect of medium (F=5.74, df=2,177, p=0.0038), this was confounded by interactive effects with time (F=5.63, df=2, 174, p=0.0043) (Fig. 3.5A) and temperature (F=7.55, df=2,174, p=0.0007) (Fig. 3.5B). Similarly, there was an overall effect of temperature (F=10.26, df=1,178, p=0.0016), that is confounded by an interaction with medium. Time alone had no effect on the

total concentration of RNA (F=0, df=1,178, p=0.9857), nor was there any interaction between time and temperature (F=1.28, df=1,176, p=0.2589) or all 3 factors combined (F=3.02, df=2,168, p=0.0515). Samples stored after 48 hours in ethanol yielded significantly lower concentrations of RNA than when stored in RNAlater™ or nothing. Concentrations of RNA stored in ethanol after only 24 hours were not significantly lower than those stored in other storage media but also were not higher than those stored in ethanol after 48 hours (Fig. 3.5A). In regards to storage temperature samples stored in ethanol at -20°C yielded lower concentrations of RNA than all other treatments, with other combinations of storage media or temperature showing similar concentrations to each other, including ethanol when the storage temperature was -80°C (Fig. 3.5B).

When comparing only the preservation of β-actin using CT values generated with each sample, there was still an interaction between storage medium and time (F=8.18, df=2,174, p=0.0004) (Fig. 3.6A), as well as storage medium and temperature (F=34.32, df=2,174, p<0.0001)(Fig. 3.6B), but additionally there was an interaction between time and the temperature specimens were stored at (F=10.19, df=1,176 , p=0.0017) (Fig. 3.6C). However, there was no interaction between all three factors (F=2.04, df=2,168, p=0.133). Though elapsed time prior to storage was not a factor when comparing only specimens stored in ethanol, those stored in ethanol after 48 hours had a lower cycle threshold for actin than specimens stored either in RNAlater™ or nothing, suggesting that the proportion of β-actin to other RNA types in the sample is highest when stored in ethanol after 48 hours. Within mediums, only RNAlater™ showed a significant effect of time until storage, with those stored after 48 hours having a higher cycle threshold on average than samples stored for 24 hours (Fig. 3.6A). As was seen

when looking at the 8-hour storage experiment, when specimens were stored at -20°C, ethanol preserved a higher proportion of actin in the RNA sample (as evidenced by the lower CT on average) than at -80°C. When samples were stored at -80°C there was no difference in average actin CT values. When looking within mediums CT values were lower at -80°C when specimens were stored in either RNAlater™ or nothing, but higher when stored in ethanol (Fig. 3.6B). Lastly, specimens stored in -20°C had lower actin CTs if they were stored for less time (ie. 24 hours after capture), but when specimens were stored at -80°C, time until storage did not matter. Additionally, when specimens were stored after 48 hours actin CTs were lower at -80°C than at -20°C, but when stored 24 hours after capture storage temperature was not important (Fig. 3.6C).

The relative preservation of DWV was affected by an interaction between temperature and both storage medium ($F=6.25$, $df=2,174$ $p=0.0024$) and time ($F=5.41$, $df=1,176$, $p=0.0212$). There was little difference among temperatures and mediums used on the amount of DWV extracted. Fewer DWV copies were found in specimens stored in ethanol at -20°C than from specimens stored in nothing at -80°C. All other treatment combinations had similar concentrations of the virus (Fig. 3.7A). However, when looking at time until storage, it was observed that if specimens were stored at a -20°C only 24 hours after capture proportionately less DWV was extracted than at colder temperatures or when stored later at 48 hours after capture (Fig. 3.7B).

DWV, also showed some differences in relative CT values with actin when medium and temperature were considered together ($F=6.76$, $df=2, 174$, $p=0.0015$). At 20°C specimens stored in ethanol produced the most similar CT values for actin and DWV, which may suggest

they were preserved equally as well under these conditions provided they began at similar amounts. When specimens were stored in ethanol at -80^{o}C, both the CT values of DWV decreased and the CT values of actin increased, resulting in much larger differences, where these effects were not seen with other storage media (ie. RNAlater^{TM} or nothing) (Fig. 3.8).

As was observed in the 8-hour data, there was no effect of medium (F=0.77, df=2, 177 p=0.4663) or temperature (F=1.8, df=1, 178 p=0.182) on the preservation of BQCV. Additionally, there was no effect of time (F=1.02, df=1, 178 p=0.3143) nor any interactive effects between any (medium and time [F=0.57, df=2, 174 p=0.5672]; medium and temperature [F=1.03, df=2, 174 p=0.3607]; time and temperature [F=0.5, df=1, 176 p=0.4794]) or all treatment factors (F=0.17, df=2, 168 p=0.8426), suggesting none of our experimental treatments had any effect on the amount of BQCV extracted from stored specimens.

Likewise, there were no treatment combinations that altered the relative difference between BQCV and actin sequence preservation, unlike what was observed in the 8-hour specimens (Fig. 3.4). When comparing the CT values of actin and BQCV on a per sample basis, on average they were not different regardless of medium (F=1.6, df=2, 177 p=0.2051), temperature (F=0.81, df=1, 178 p=0.3702) or time (F=0.05, df=1, 178 p=0.8260). Neither was there any effect with a combination of any (medium and temperature [F=2.59, df=2, 174 p=0.0777]; medium and time [F=0.97, df=2, 174 p=0.3815]; temperature and time [F=0.08, df=1, 176 p=0.7753]) or all of these factors (F=1.41, df=2, 168 p=0.2479).

Bee Bowl Experiments

Trial 1

Looking first at the prevalence of DWV, none of the 32 leafcutting bees used in this trial tested positive for the virus, whereas a majority of honey bees did (n=57/69) (Table 3.2). Therefore, when examining viral intensity across all treatment groups there was a strong effect of species ($F=27.06$, $df=1, 99$ $p<0.0001$) as a result of DWV infection being unique to honey bees, and no effect of treatment ($F= 0.27$, $df=4, 96$, $p=0.8965$) nor was there any treatment by species interaction ($F=1.35$ $df=1, 91$ $p=0.2513$).

More than half of the leafcutting bees tested positive for BQCV (n=18/26), and honey bees also showed high prevalence of infection with this virus (n=61/69) (Table 3.2). Although prevalence was higher in honey bees overall than in leafcutter bees ($\chi^2 = 4.96$, $df =1$, $p<0.05$), prevalence in both living ($\chi^2 = 1.95$, $df = 1$, $P>0.05$) and dead ($\chi^2 = 2.84$, $df = 1$, $P>0.05$) leafcutting bees that shared bowls with honey bees was not significantly different. Likewise, when comparing only the leafcutter bees across the various treatment groups BQCV was not more prevalent in living ($\chi^2 = 0.74$, $df = 1$, $p>0.05$) or dead ($\chi^2 = 0.68$, $df = 1$, $p>0.05$) leafcutting bees that shared a bowl with honey bees than those that did not. Additionally, whether the leafcutting bee was living or dead when introduced to the bowl seemed to have no influence on BQCV prevalence ($\chi^2 = 1.76$, $df = 1$, $p>0.05$). Intensity of BQCV showed a significant effect of treatment ($F=4.93$ $df=4, 96$ $p=0.0022$), but not of species ($F=0.1$ $df=1, 99$ $p=0.7576$) and there was no effect of treatment on species ($F=1.46$ $df=1, 91$ $p=0.2338$). BQCV viral intensity was

highest on average in dead leafcutting bees applied singly to a bowl as a control (DLC) and lowest in living leafcutting bees applied singly to a bowl as a control (LC) (Fig. 3.7).

Trial 2

Similarly, none of the leafcutting bees used in the second trial tested positive for DWV (n=0/28), and all of the honey bees used tested positive (n=28/28) (Table 3.3). As expected there was a strong effect of species on viral intensity ($F=224.76$ df=1, 54 $p<0.0001$) with the infection remaining unique to honey bees, and no effect of treatment on viral intensity ($F=0.48$ df=2, 55 $p=0.6248$).

In the second trial, BQCV was more prevalent in honey bees than leafcutting bees ($\chi^2 = 29.89$, df = 1, $p<0.05$). Prevalence did not differ between the control and experimental groups for either the leafcutting ($\chi^2 = 0.89$, df = 1, $p>0.05$) or honey bees ($\chi^2 = 0.48$, df =1, $p>0.05$) (Table 3.3). For BQCV intensity there was also a significant effect of species (honey bee = 50026 ± 28070 BQCV copies/µg RNA; leafcutter bee = 4772 ± 2796 BQCV copies/µg RNA) ($F=11.7$, df=1, 54 $p=0.0016$) but not of treatment ($F=0.07$, df=2, 53 $p=0.9345$).

Discussion

This study examined the effect of different storage methods on the preservation of RNA, and RNA viruses in particular. Honey bee samples known to harbour DWV and BQCV infections were stored at either -20°C or -80°C, with -80°C usually being best for preserving RNA

concentrations. However, I showed that storage media (95% ethanol or RNAlater™ or nothing) and storage time before freezing showed interactions with temperature that could bias estimates of the relative concentrations of viral to host RNA. This study also looked at the potential for bee bowls, a common passive trapping method for capturing bees, to cause cross contamination of virus in bees within the same bowls. Two trials were conducted that placed uninfected alfalfa leafcutting bees in a bowl with honey bees known to be infested with DWV and BQCV. We found no instances where DWV was found in leafcutting bees when sharing a bowl with honey bees infected with DWV. Although BQCV was found in leafcutting bees sharing bowls with honey bees it seems likely that this is not indicative of transfer within bowls as the leafcutting bees were previously infected and because it is likey that these bees harboured a separate strain of BQCV that cross reacted with our primer. Because cross contamination of bees did not occur in the bee bowl environment, this was shown to be a viable capture method for further virus research.

When studying RNA viruses in the lab it is necessary to be able to extract a suitable concentration of intact RNA from the sample, of which the virus is a part. As a result of this comparative degredation of host RNA molecules will bias your conclusions about viral load in the specimens. Overall concentration of RNA was always better preserved at -80^{o}C or at least equivalent to storage at -20^{o}C, as other studies have also shown (Chen et al. 2007). This occurred regardless of whether specimens were ground in liquid nitogen and stored after 8 hours or bead homonginized in buffer and stored after 24 or 48 hours. When specimens were stored 8 hours after capture using the liquid nitrogen method, storage in ethanol yielded the highest overall concentrations of RNA. In contrast, for samples bead homonginized 48 hours

after capture, storage in ethanol yielded the lowest concentrations of overall RNA. Some of this variation may have been due to differences in the crushing method. However, when using bead homoginization 24 hours after storage, RNA concentrations were roughly equal regardless of storage medium. Additionally, temperature at which specimens were stored only mattered at -20^{o}C, where RNA preservation was worst in ethanol. In a previous study by Chen et al. (2007), ethanol resulted in the poorest RNA yields although this may be due to their use of a 70% concentration, which is typically used for denaturing proteins and disinfection. In contrast, our study used 95% ethanol which appeared to be acceptable under some temperature time combinations.

More can be determined about which storage methods are best when we look at the effect different treatments had on the relative preservation of β-actin, a host mRNA that is often used as a reference gene in qPCR studies. Since a standardized amount of 1 µg of a sample of RNA is used in cDNA synthesis, lower actin cycle thresholds (CTs) in samples stored in ethanol at -20^{o}C (as well as at -80^{o}C in all specimens stored after 24/48 hours) would be indicative of a larger proportion of β-actin compared to other RNA molecules. Furthermore, 8 hours after capture, actin CTs were lower when stored in ethanol at -20^{o}C, suggesting that ethanol is relatively good at preserving actin compared to other RNA molecules that had not degraded in the 8 hours before storage at -20^{o}C. Storing specimens in RNAlater^{TM} or nothing on the other hand, resulted in CTs that were higher when specimens were stored at only -20^{o}C compared to -80^{o}C suggesting poor preservation of actin. Additionally, CTs for RNAlater^{TM} were higher when 48 hours elapsed before storage than for 24 hours and RNAlater^{TM}. All of this suggests that actin degrades more at higher temperatures and if more time elapses before

storage and that using ethanol preserves actin well, but not necessarily other associated RNA molecules.

The RNA molecules of the most interest in this study, however, were those of the viruses DWV and BQCV, both of which appeared to be comparatively resistant to degradation. In general, DWV was preserved better at -80°C, though when RNAlater™ was used on specimens stored after 24/48 hours copy numbers did not differ from samples stored at -20°C. As with actin this lack of difference between -20°C and -80°C for samples stored in RNAlater™ may simply be a result of DWV degrading relatively slower than other RNA molecules when RNAlater™ is not used. If this is true it would mean RNAlater™ preserves DWV better than ethanol or no storage medium as the same trend was not observed when they were used. Similarly, fewer copies of DWV were found in specimens stored after 24 hours at -20°C than at 48 hours, which may indicate more actin or other RNA molecules made up the sample in this treatment, although this would mean these same RNA molecules preserved less well than DWV after 48 hours or when stored at -80°C. BQCV copies on the other hand were unaffected by any treatment combination of temperature, storage medium or time until storage. If the assumptions we made previously for relative degradation of actin and DWV within the 1µg of RNA making up the sample are true, this would indicate that BQCV preserves no more or less well than the other host mRNAs in the sample. Suggesting any treatment combination would be suitable for a study detecting BQCV in bee samples.

However, when doing PCR work it is critical to be mindful of your reference gene (in this case β-actin) as well as your target, and so differences in the preservation of each were also compared as part of this study. Specimens stored after 8 hours showed no differences in the

relative preservation of actin and DWV with treatment type, but at 24/48 hours when specimens were stored in ethanol at -80^{o}C the relative CT of DWV decreased whereas that of actin increased meaning DWV was relatively more preserved for this treatment. For BQCV on the other hand, there were no differences in relative CTs for 24/48 hour specimens, but those stored after 8 hours showed significant effects of medium used. Actin and BQCV CT values were less different at -20^{o}C when specimens were stored in RNAlater™ than when they were stored in ethanol. This makes sense given the previous evidence that ethanol preserves relatively more actin, however this trend shifts when specimens were stored at -80^{o}C where actin is relatively better preserved by RNAlater™. This suggests that it is best to use no storage media if relatively equal preservation of actin and virus RNA is your goal.

Previous work by Chen et al. (2007) showed that RNAlater™ was more effective if used in sliced or homogenized tissue that allowed it to penetrate the cuticle and bathe virus-rich tissues directly. In this way virus preservation was achieved even at 4^{o}C for up to a week. Other studies also show that viral RNA is less prone to degradation than host mRNAs and can be detected in samples stored at 4^{o}C after 5 days even though other RNA molecules have degraded (Dainat et al. 2011). This may account for some of the differences seen in this experiment.

This study also examined the potential of bee bowls as a contamination source between infected and unifected bees. Across both trials none of the uninfected alfalfa leafcutting bees tested positive for DWV suggesting neither infection nor false-positives from accumulation of virus particles in the bowl are possible. With BQCV on the other hand, both experimental and control leafcutting bees tested positive for the virus. When intensity within leafcutting bees was

examined it was shown that they were significantly higher in the dead than in the living controls. This apparently higher level of BQCV in dead bees is likely due to other host RNA molecules degrading at a more rapid rate after death than the virus (as was evidenced in the storage experiments), leading to a larger representation of BQCV in the sample used in PCR amplification. The detection of BQCV that was in leafcutting bees sharing bowls with honey bees likely did not originate from the honey bees. Upon closer examination of the melting curves it was determined that the BQCV detected in leafcutting bees had a unique peak from that detected in honey bees (Fig. 3.8). A pure PCR product should produce a distinct peak (Pfaffl 2004), but it is clear that these two reactions have produced distinct products and since the primers are detecting both products neither is present together in one specimen. It is likely we are detecting a different viral strain that maybe unique to leafcutting bees. Therefore, there is no clear evidence in this study that BQCV was transmitted between honey and leafcutting bees within the bee bowl environment.

In conclusion, strict and exact storage techniques are not required for the study of RNA virus prevalence as DWV and BQCV were detectable when stored in nothing, ethanol or RNAlater, at -20^{o}C or -80^{o}C up to 48 hours after capture. However, the results of this study showed that storage should occur as soon as possible upon return from the field at -80^{o}C when needing to quantify viral titres in your specimens or compare them to a reference gene such as β-actin. We found that DWV and BQCV differed in terms of their stability relative to other RNA in the samples and thus consideration of storage conditions is important as it can affect accuracy in quantifying these viruses. Additionally, this study indicates that storage in no medium may be better than using RNA-Later or 95% ethanol for relative comparisons of your

RNA targets in intact specimens providing they are stored at -80^{o}C. Lastly, I found that the passive method of bee bowl trapping did not result in cross contamination and thus remains a viable option for studies involving bee viruses.

Table 3.1 - A list of the PCR primers used for virus detection in this study. Both the forward (F) and reverse (R) primers are included as well as the target size in base pairs (bp) and the author to which the creation of the primer pair is credited.

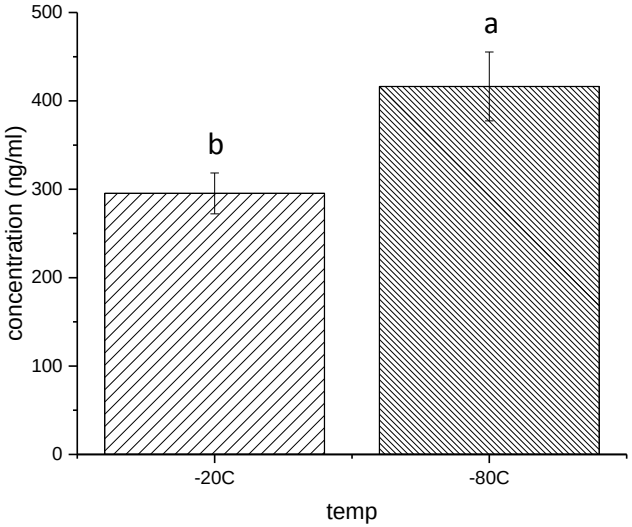
Primer sequences	Product size (bp)	Reference
DWV-F: CGAAACCAACTTCTGAGGAA DWV-R: GTGTTGATCCCTGAGGCTTA	174	Peng et al. (2011)
BQCV-F: CCTGTATTCATGCATCTCAGA BQCV-R: GCAACAAGAAGAAACGTAAACCAC	310	Chen personal communication
Actin-F: AGGAATGGAAGCTTGCGGTA Actin-R: AATTTTCATGGTGGATGGTGC	181	Chen et al. (2005)

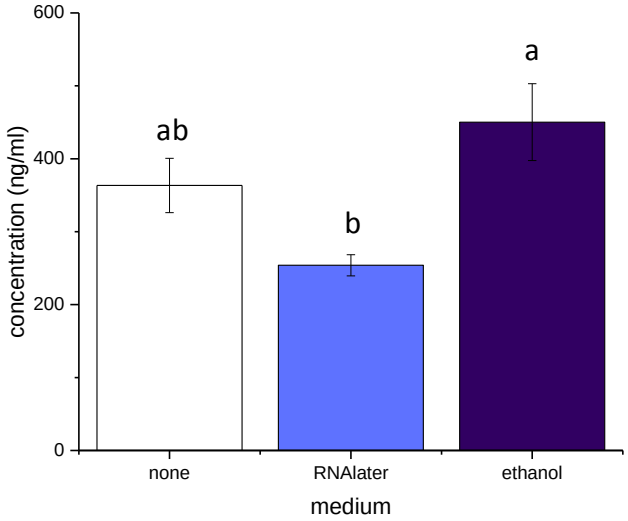
Table 3.2 – Prevalence of DWV and BQCV infection among bees confined within bee bowls for 7 hours in the following treatment groups: **AE** = Alive Experimental (a single living leafcutting bee sharing a bowl with 3 honey bees); **DE** = Dead Experimental (a single dead leafcutting bee sharing a bowl with three honey bees) ; **LC** = Leafcutter Control (a single living leafcutting bee in a bowl); **DLC** = Dead Leafcutter Control (a single dead leafcutting bee in a bowl); **HC** = Honey bee Control (three honey bees in a bowl). Each treatment was equally represented by eight bowls in what is described as ***trial 1*** of the bee bowl experiment. *Note*: the table only includes those specimens for which the full process of PCR was successful and as such some are missing from what was quoted in the original experimental design.

		leacutting bees				*honey bees*		
		AE	**LC**	**DE**	**DLC**	**AE**	**DE**	**HC**
DWV	N=	8	8	8	8	23	22	24
	% infected	0	0	0	0	91.3	72.7	83.3
	% uninfected	100	100	100	100	8.7	27.3	16.7
BQCV	N=	7	6	8	5	23	22	24
	% infected	57.1	33.3	87.5	100	82.6	100	83.3
	% uninfected	42.9	66.7	12.5	0	17.4	0	16.7

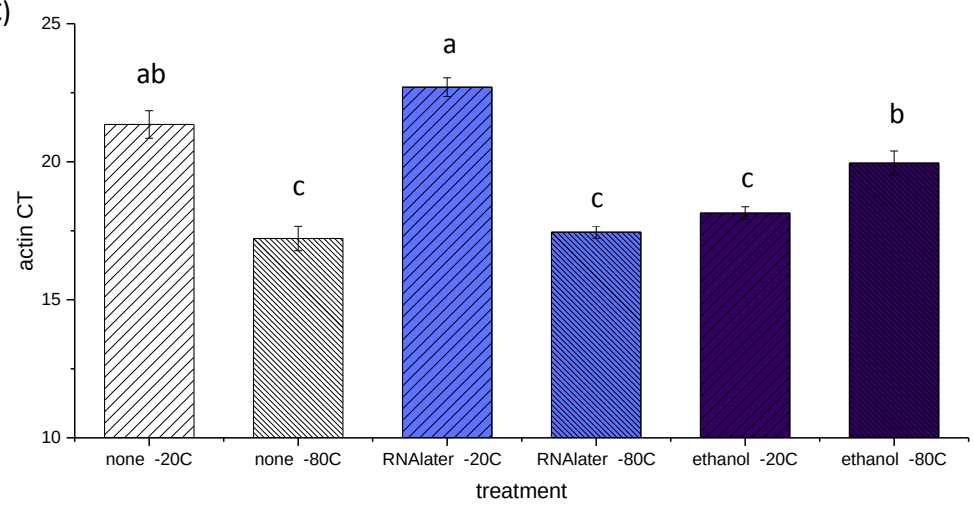
Table 3.3 - Prevalence of DWV and BQCV infection among the bees confined within bee bowls for 7 hours in the following treatment groups: **E** = Experimental (a single leafcutting bee *and* a single honey bee sharing a bowl; **C** = Control (two leafcutting bees *or* two honey bees within a bee bowl). There 10 each of the control bowls for each species and 10 total experimental bowls included in what is described as ***trial 2*** of the bee bowl experiment. *Note*: the table only includes those specimens for which the full process of PCR was successful and as such some are missing from what was quoted in the original experimental design.

		leafcutting bees		*honey bees*	
		E	**C**	**E**	**C**
DWV	N=	10	18	8	20
	% infected	0	0	100	100
	% uninfected	100	100	0	0
BQCV	N=	10	16	8	20
	% infected	10	25	87.5	95
	% uninfected	90	75	12.5	5


500
400
300
200
100
0
concentration (ng/ml)
b
a
-20C
-80C
temp


600
400
200
0
concentration (ng/ml)
ab
b
a
none
RNAlater
ethanol
medium

C)


25
20
15
10
actin CT
ab
c
a
c
c
b
none -20C
none -80C
RNAlater -20C
RNAlater -80C
ethanol -20C
ethanol -80C
treatment

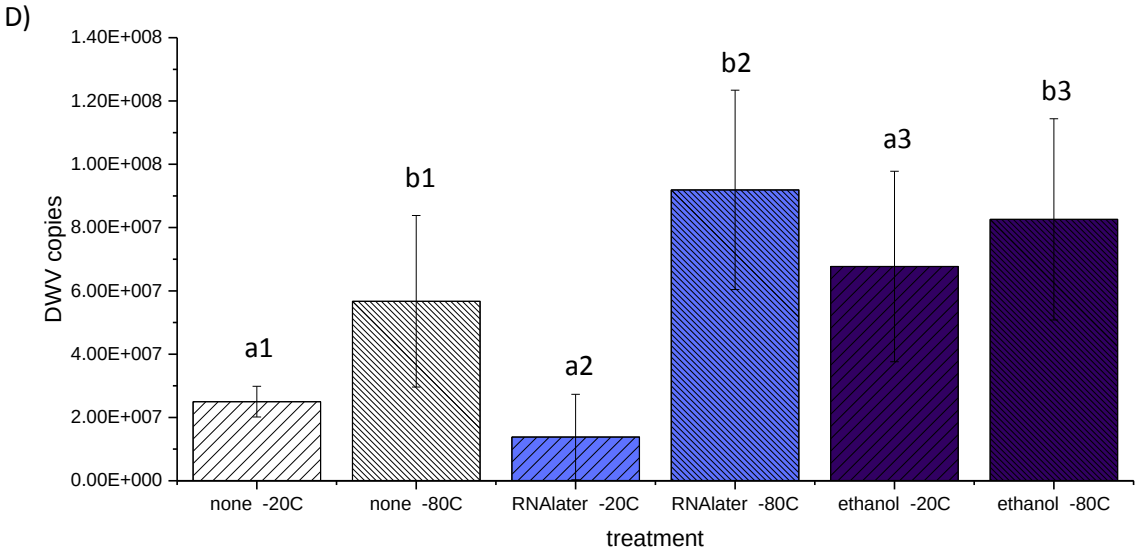


Fig. 3.1 – Compilation of data from the storage experiment that was generated from specimens crushed for RNA extraction 8hr after capture using a mortar and pestle. A) The effect of storage in two different freezers at different temperatures (-20°C and -80°C) on the total concentration of RNA extracted (ng/ml) from the honey bee sample. B) The effect of storing honey bee samples whole in nothing, RNAlater™ or ethanol on the total concentration of RNA extracted (ng/ml) from each sample. C) The effect that storage temperature has on the efficacy of three storage media (nothing, RNAlater™, ethanol) to preserve actin RNA used as a reference in PCR reactions. Actin CT values represent the number of PCR cycles required to replicate the target sequence to a level that is detectable above a background threshold, such that low CT values indicate a larger amount of the target sequence in the initial sample. D) The effect that storage temperature had on the efficacy of three storage media (nothing, RNAlater™, ethanol) to preserve DWV RNA indicated by total copies of the target sequence for the virus detected after the PCR process. Within all three storage media, more copies of DWV were preserved when honey bees were stored at -80°C. Standard errors are indicated by the bars on each of the columns. Numbered letters above the bars that are the same indicate that means are significantly different from each other when considering each storage medium separately (P>0.05).

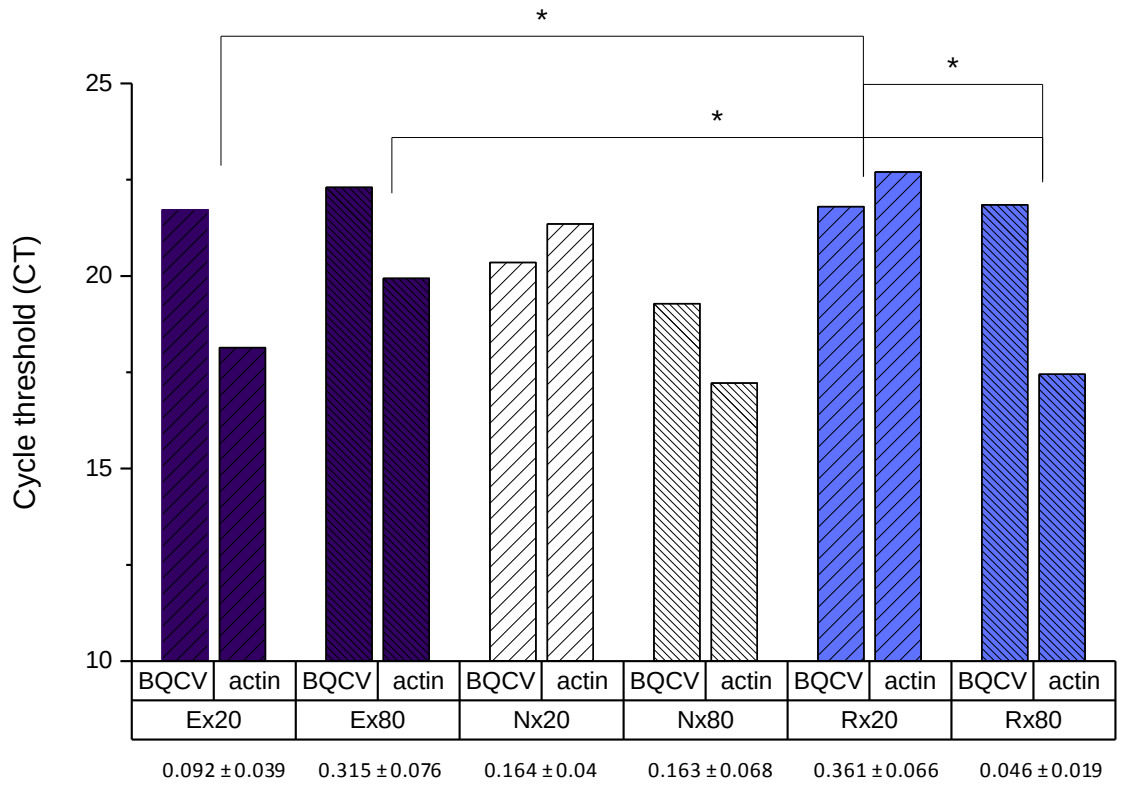
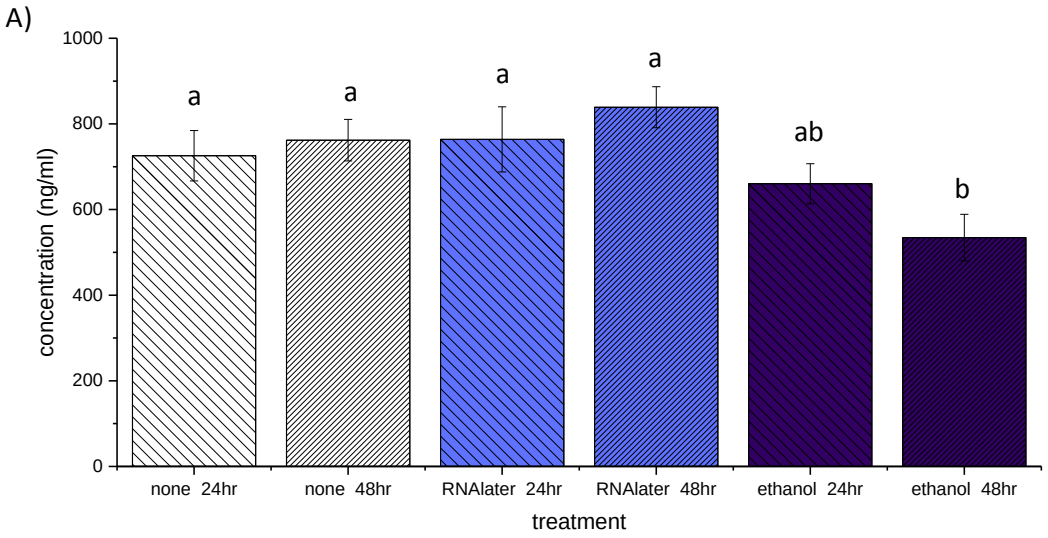


Fig. 3.2 – A bar graph depicting the relative difference between the average cycle threshold (CT) required to detect actin and BQCV in RNA extracted from honey bees stored under a variety of treatments (Ex20 = in ethanol at -20°C; Ex80 = in ethanol at -80°C; Nx20 = in nothing at -20°C; Nx80 = in nothing at -80°C; Rx20 = in RNAlater™ at -20°C; Rx80 = in RNAlater™ at -80°C). Sets of columns connected by an asterisk showed significant differences in their average relative CT values when analyzing the data using a one-way ANOVA. This dataset is made up of honey bees stored 8 hours after capture and crushed for extraction using the mortar and pestle method. Values below the graph are the average differences adjusted by the 2^-ΔCT method (as well as the standard error) that were used in the statistical comparison.


A)
1000
800
600
400
200
0
concentration (ng/ml)
a
a
a
a
ab
b
none 24hr
none 48hr
RNAlater 24hr
RNAlater 48hr
ethanol 24hr
ethanol 48hr
treatment

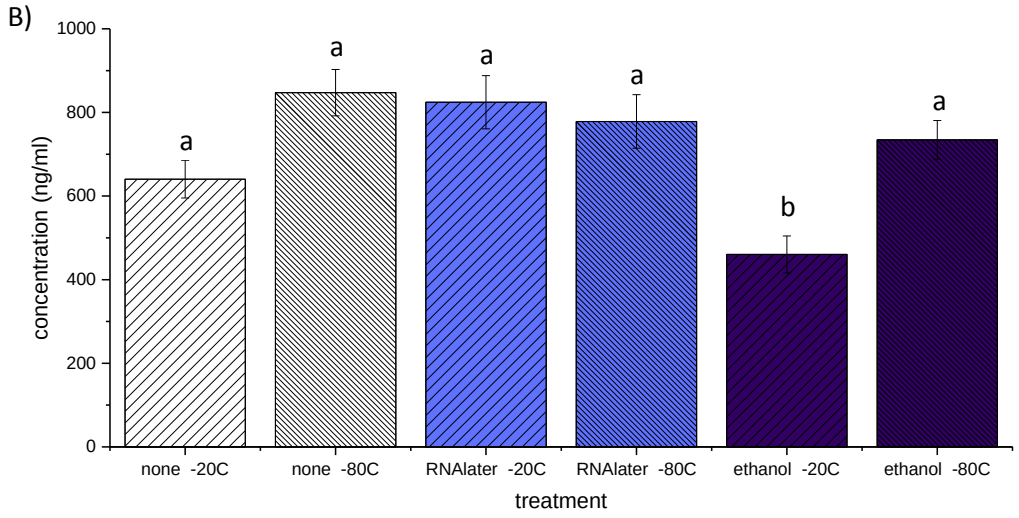
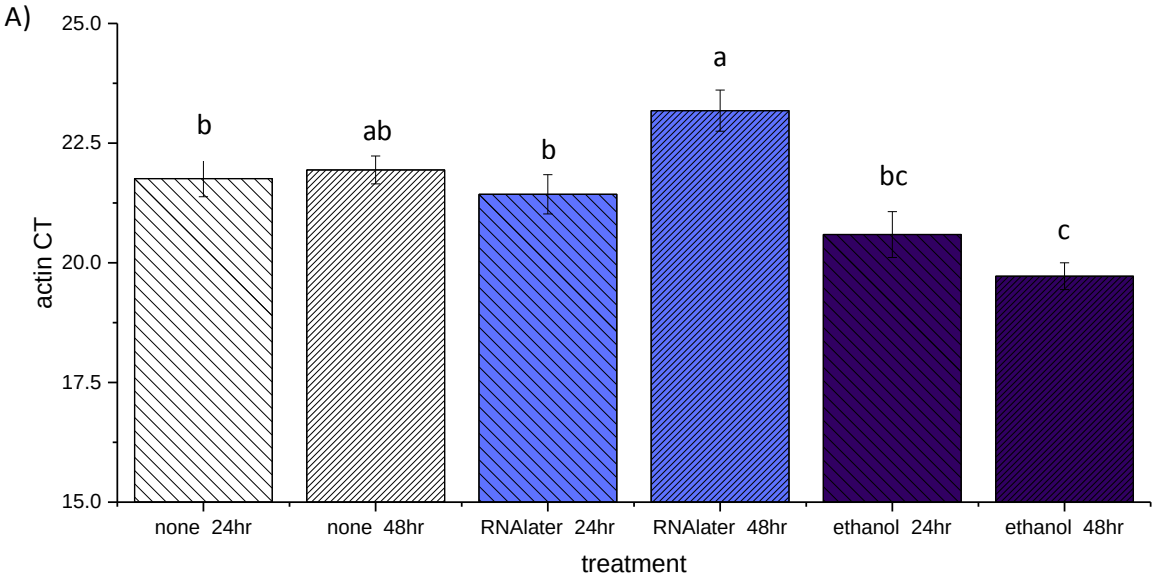
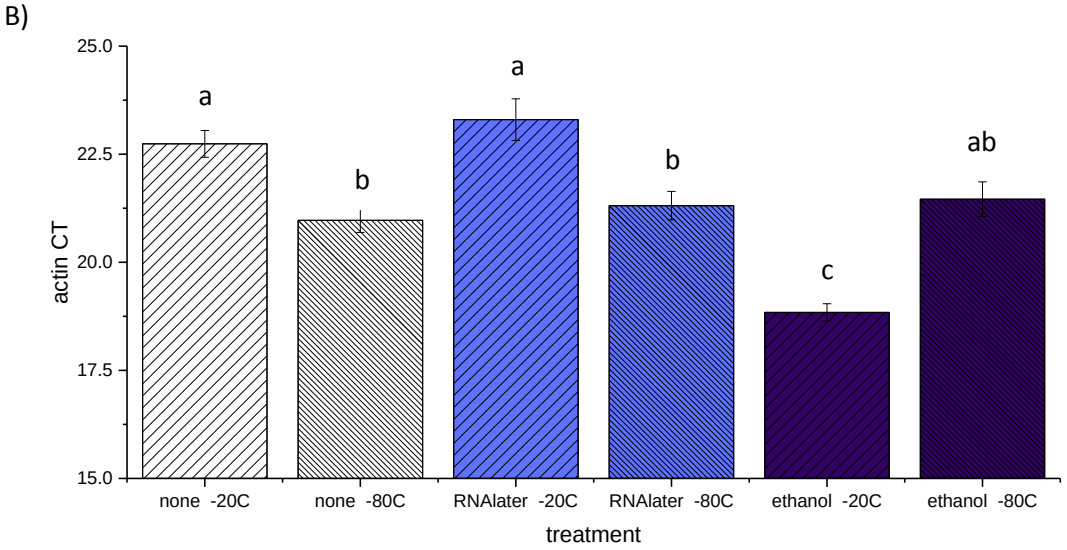

B)
1000
800
600
400
200
0
concentration (ng/ml)
a
a
a
a
b
a
none -20C
none -80C
RNAlater -20C
RNAlater -80C
ethanol -20C
ethanol -80C
treatment

Fig. 3.3 – Two bar graphs depicting the effect that both: A) time until storage (either 24 or 48 hours) and B) temperature (-20°C and -80°C) had on the efficacy of three different storage media (nothing, RNAlater™, ethanol) to preserve RNA in honey bees, indicated by the average RNA concentration (ng/ml) found in extractions. Standard error are indicated by the bars on each of the columns. Letters above the bars that are the same indicate groups that are not statistically different from each other (P>0,05).


A)
25.0
22.5
20.0
17.5
15.0
actin CT
b
ab
b
a
bc
c
none 24hr
none 48hr
RNAlater 24hr
RNAlater 48hr
ethanol 24hr
ethanol 48hr
treatment


B)
25.0
22.5
20.0
17.5
15.0
actin CT
a
b
a
b
c
ab
none -20C
none -80C
RNAlater -20C
RNAlater -80C
ethanol -20C
ethanol -80C
treatment

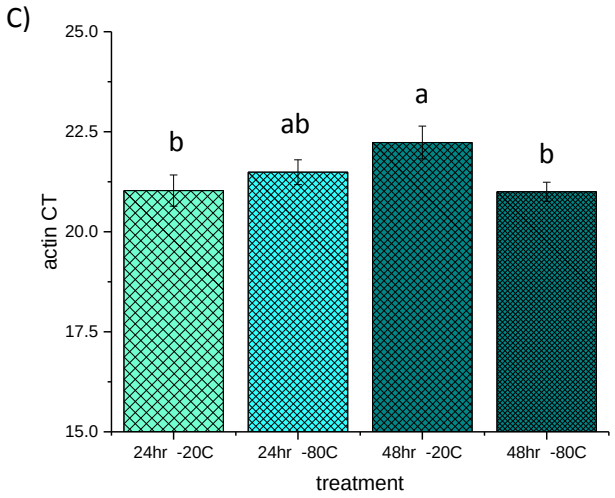
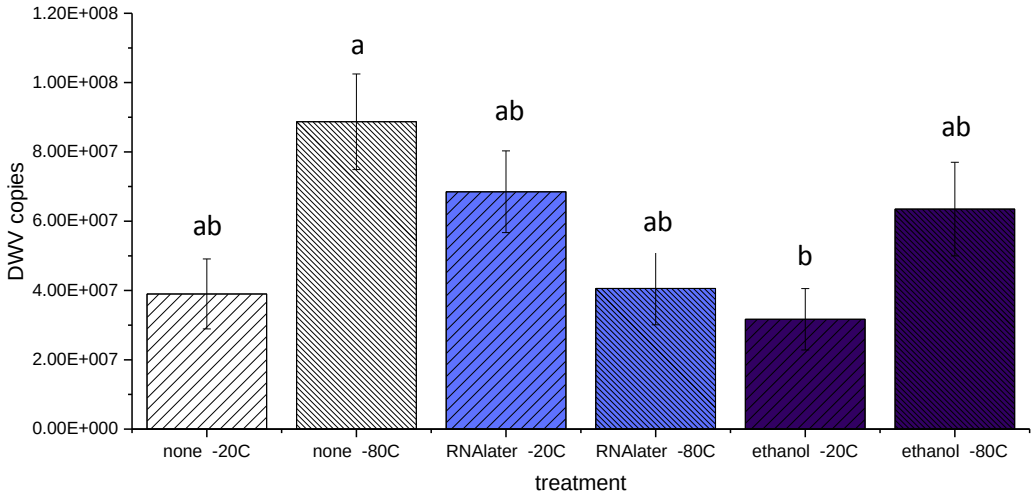


Fig. 3.4 – Three bar graphs depicting the effect that both: A) time until storage (24 or 48 hours) and B) storage temperature (-20°C and -80°C) have on the efficacy of three storage media (nothing, RNAlater™, ethanol) in preserving actin RNA. C) Additionally it is shown that there is an effect of the time until storage on the efficacy of two different temperatures in preserving actin RNA. Preservation is indicated by a cycle threshold (CT) value that represents the number of PCR cycles required to duplicate enough of the RNA target for it to be detected above a threshold level, such that a low CT value indicates higher levels of the target sequence in the initial sample. Standard errors are indicated by the bars on each of the columns. Letters above the bars that are the same indicate means that are not statistically different from each other ($P>0.05$).

A)


1.20E+008
1.00E+008
8.00E+007
6.00E+007
4.00E+007
2.00E+007
0.00E+000
DWV copies
a
ab
ab
ab
b
ab
none -20C
none -80C
RNAlater -20C
RNAlater -80C
ethanol -20C
ethanol -80C
treatment

B)

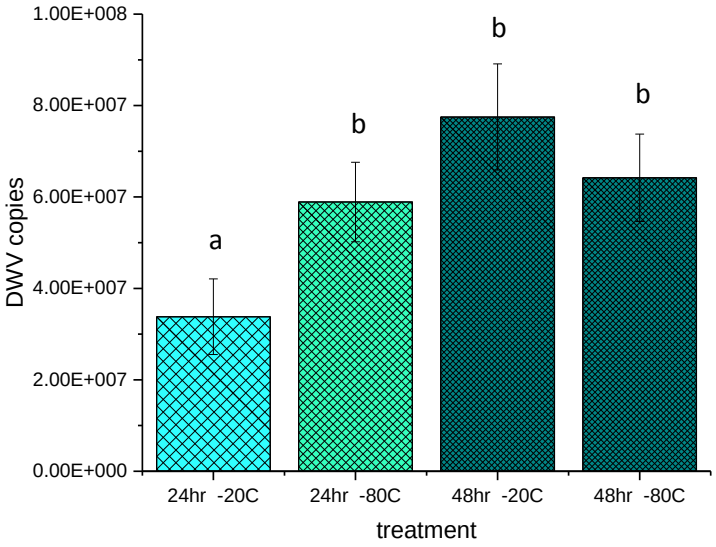

1.00E+008
8.00E+007
6.00E+007
4.00E+007
2.00E+007
0.00E+000
DWV copies
a
b
b
b
24hr -20C
24hr -80C
48hr -20C
48hr -80C
treatment

Fig. 3.5 – A) A bar graph depicting the effect two different storage temperatures (-20°C and -80°C) have on the efficacy of three storage media (nothing, RNAlater™, ethanol) to preserve DWV RNA. B) A bar graph depicting the effect two different times until storage (24 and 48 hours) have on the efficacy of two temperatures (-20°C and -80°C) to preserve DWV RNA. Relative preservation is indicated in both graphs by the average number of DWV copies detected in the RNA extracted from individual honey bees after the PCR reaction. Standard errors are indicated by the bars on each of the columns. Letters above the bars that are the same indicate means that are not statistically significant from each other ($P>0.05$).

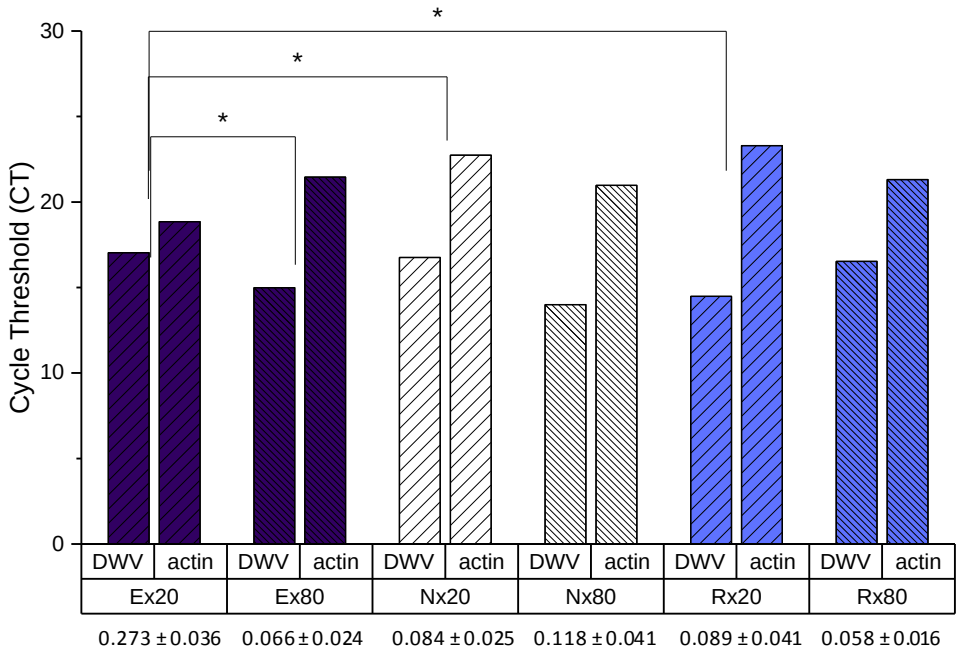


Fig. 3.6 – A bar graph depicting the relative difference between the average cycle threshold (CT) required to detect actin and DWV in RNA extracted from honey bees stored under a variety of treatments (Ex20 = in ethanol at -20°C; Ex80 = in ethanol at -80°C; Nx20 = in nothing at -20°C; Nx80 = in nothing at -80°C; Rx20 = in RNAlater™ at -20°C; Rx80 = in RNAlater™ at -80°C). Sets of columns connected by an asterisk showed significant differences in their average relative CT values when analyzing the data using a one-way ANOVA. This dataset is made up of honey bees stored 24 hours or more after capture and crushed for extraction using the bead homogenization method. . Values below the graph are the average differences adjusted by the 2^-ΔCT method (as well as the standard error) that were used in the statistical comparison.

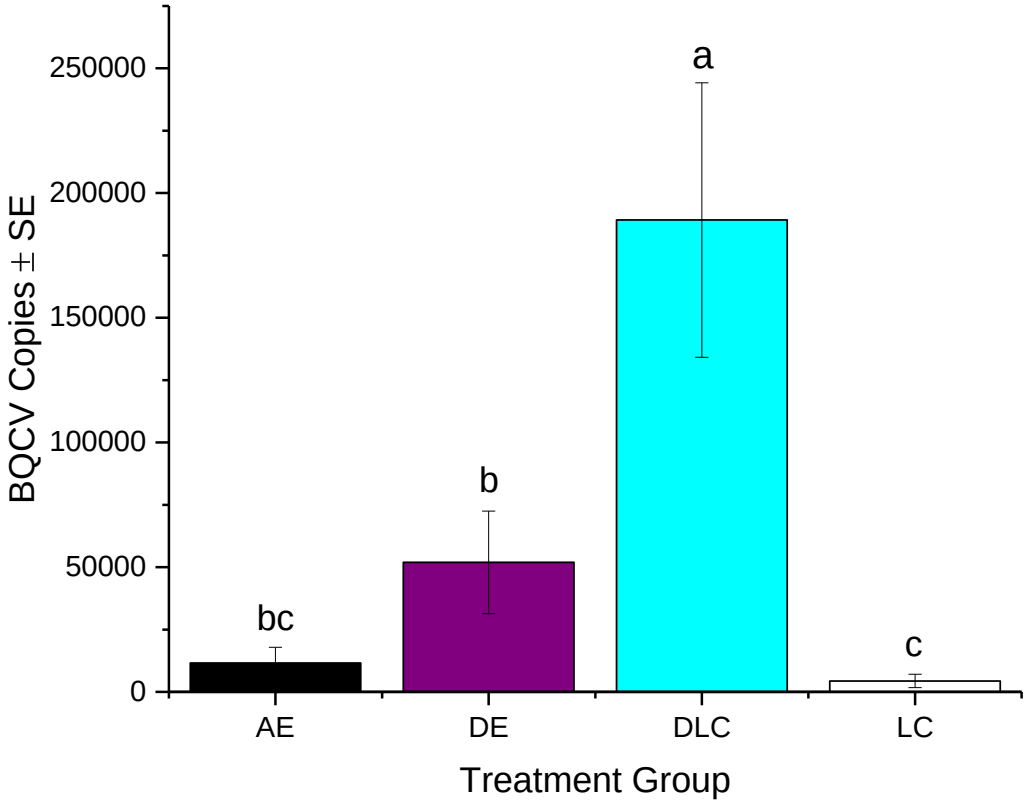


Fig. 3.7 - Results from the first of two bee bowl experiments where alfalfa leafcutting bees and honey bees were placed within bee bowl traps in a temperature and humidity controlled room in various treatments (AE = alive experimental; DE = dead experimental; DLC = dead leafcutter control; LC = leafcutter control [alive]). A bar graph depicting the amount of BQCV copies detected on average in leafcutting bees from various treatment groups after the PCR reaction. Standard errors are indicated by the bars on each of the columns. Letters above the bars that are the same indicate means that are not statistically significant from each other (P>0.05).

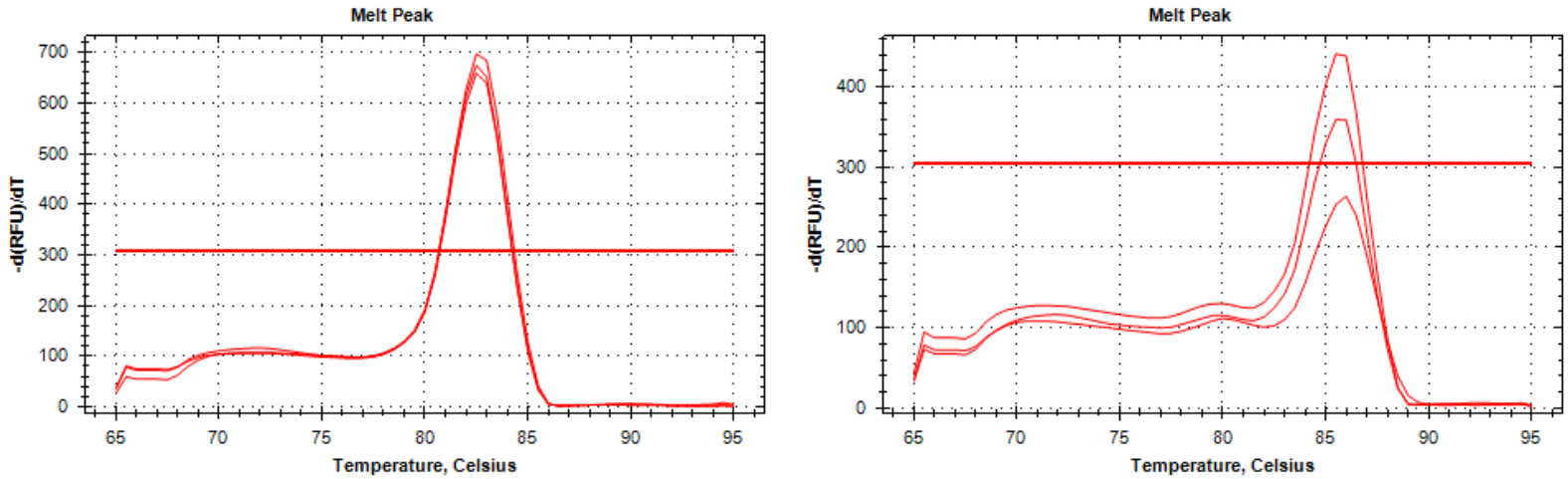


Fig. 3.10 - Melt curves generated by the denaturation step of the PCR reaction looking for copies of BQCV in the bee bowl experiment. The product amplified in honey bees (left) has a distinct melting peak at 82.5°C, whereas that amplified in alfalfa leaf-cutter bees peaks at roughly 85.5°C.

CHAPTER 4. GENERAL DISCUSSION

Across the world, bumble bee species are in decline and a suite of picorna-like RNA viruses originally discovered in honey bees is thought to be contributing to their disappearance. This study examined the possibility that these viral pathogens are spilling over from managed honey bee colonies into wild bumble bees in Canada. Bumble bees from sites where honey bees were present (and associated honey bees) and bumble bees from sites where honey bees were deemed absent were collected and analysed individually in the lab for up to seven different viruses in order to address my main objectives: 1) to compare prevalence and intensity of these viruses in bumble bees captured in proximity to and isolation from honey bees and 2) to compare prevalence and intensity of these viruses among different wild bumble bee species, between bees captured with sweep nets versus bee bowls and between bees captured at various periods over the summer field season to see if they would influence our determination of prevalence and intensity among different populations of bees. With a mind to the lack of information on how best to handle field-caught specimens for virus quantification studies this study also looked at: 1) the effects different combinations of storage mediums, temperatures and durations prior to storage had on the preservation of host and virus RNA molecules and 2) whether or not bee bowl traps used in the passive capture of bees represented a possible contamination source between infected and uninfected bees. This study determined that these viruses are widespread in native bumble bee populations and provided further evidence to contribute to the study of pathogen spillover, as well as provided useful

information on the proper handling and storage of field caught specimens for future work on these viruses.

Pathogen spillover of RNA viruses

Pathogen spillover is well studied for gut parasites from commercial *Bombus* to wild species (Colla et al. 2006, Otterstatter and Thomson 2008, Arbetman et al. 2013, Murray et al. 2013) as well as from managed honey bees into wild bumble bee species (Imhoof and Schmid-Hempel 1998, Graystock et al. 2013a, Graystock et al. 2016). In these cases the introduced pathogen is typically more infective to its new host. Synergistic reactions between pathogens that infect honey bees increase the health risk for the colonies (Genersch and Aubert 2010, Cornman et al. 2012). Therefore, multiple detections of RNA viruses in wild bee populations around the world (Singh et al. 2010, Evison et al. 2012, Zhang et al. 2012, Reynaldi et al. 2013, Fürst et al. 2014, Ravoet et al. 2014, Guzman-Novoa et al. 2015, McMahon et al. 2015, Dolezal et al. 2016, Tehel et al. 2016), as well as in other arthropod species (Levitt et al. 2013, Lucia et al. 2014, Manley et al. 2015), raise concern for the potential health risk to wild populations of pollinators. In this study it was shown that Deformed Wing Virus (DWV), Black Queen Cell Virus (BQCV), Sacbrood Virus (SBV) and Israeli Acute Paralysis Virus are widespread in wild bumble bee populations. If we are expecting that the original host is the honey bee, then prevalence should be lower in bumble bee populations, and as they are a new host with no evolved immune defense against the virus, intensity should be higher in infected bumble bee samples (Woolhouse et al. 2005). This was the first study to extend this theory to bumble bees that are

not associated with honey bee populations, arguing that prevalence in secluded populations should be even lower than that in exposed populations of bumble bees, and measured intensities even higher. Measured prevalence of DWV and SBV fit these assumptions providing evidence of pathogen spillover from managed honey bee colonies into native bumble bees. BQCV was less prevalent in isolated bumble bee populations, but prevalence was at 100% for most bee populations captured from sites where honey bees were present (including in the honey bees themselves), which limits our ability to determine any sort of directionality on the basis of prevalence alone. Additionally, although IAPV infection was still the most prevalent in sampled honey bees, it was more prevalent in isolated bumble bees than in bumble bees that were exposed to sites with infected honey bees. Previous studies on viruses did not venture strong conclusions on the directionality of spillover for these viruses and some speculated that pathogen 'spillback' (Daszak et al. 2000) events may already be occurring between wild and commercial populations of bees (Graystock et al. 2016), or that multiple spillover events may have already occurred (Manley et al. 2015). Ravoet et al. (2014) speculates that solitary bees may act as reservoirs for DWV, BQCV and SBV, and commercial *Bombus impatiens* have been determined to be reservoirs for several pathogens infective to wild bees including RNA viruses (Sachman-Ruiz et al. 2015). This may be the case with what we are seeing with IAPV in this study.

Kashmir Bee Virus (KBV), Acute Bee Paralysis Virus (ABPV) and Chronic Bee Paralysis Virus were detected in very small sample sizes in this study. CBPV is typically very widespread in honey bees (Bailey 1967, Ribière et al. 2010), but other studies have detected it rarely in non-honey bee hosts (Gamboa et al. 2015). ABPV, on the other hand is rarely detected in living

specimens and often in low quantities when it is given that increased loads quickly lead to death of the bee (Highfield et al. 2009, Genersch and Aubert 2010). KBV was detected in honey bee populations with a prevalence of 10-40% in similar areas sampled for a previous study (Desai et al. 2016), but in this experiment was only detected in bumble bees. In fact, all three of these viruses when detected were represented in bumble bees suggesting they too are infective to native bumble bees.

It was expected that virus intensity would be higher in bumble bees if they were a new host for the virus, but in this study this was never the case. Averaged intensities for DWV, BQCV, SBV and IAPV were never higher in bumble bee populations than in associated honey bee populations. Additionally, average BQCV intensities were determined to be lower in isolated bumble bee populations than in exposed bumble bee populations, whereas intensities of the other three viruses were no different between pooled populations of bumble bees. Dolezal et al. (2016) also found intensities to be lower in non-honey bee hosts. This appears to argue against pathogen spillover supported by what was seen with prevalence, and may be indicative of incidental contaminations with the virus that do not replicate in wild bee hosts. When comparing intensities among captured *Bombus* species in this study some differences were found. This could be due to differences in immune responses of unique species groups or could simply be from differences in environmental exposure to sources of virus. For isolated bumble bees the virus sources are not well understood although there is some evidence that viruses can be transmitted through shared flower use (Singh et al. 2010) or other potential insect vectors such as ants (Celle et al. 2008), small hive beetles (*Aethina tumida* Murray [Coleoptera : Nitidulidae]) (Eyer et al. 2009) and wax moth larvae (*Galleria mellonela* Fabricius

[Lepidoptera : Pyralidae]) (Triyasut et al. 2015). One of the weaknesses of this study is that it does not test for the minus, or replicating, strand which could tell us whether or not these viruses are infective to wild bumble bee populations. With this missing piece data on measured intensities may be more informative to the question of pathogen spillover.

Lastly, Fürst et al. (2014) provides some evidence for pathogen spillover in determining that DWV strains are identical between honey bees and associated bumble bees. Sequencing of the viral targets in this study could also provide useful information as to the directionality of the spread of these viruses. IAPV in isolated bumble bees could be of a different variant, which may explain its higher prevalence in isolated populations than in exposed populations. BQCV detected by our primers in the bee bowl study produced a distinct melt curve from that detected in the honey bees (Chapter 3) and so is likely a different strain. However, even with increased evidence provided by detection of the minus strand and sequencing of targets there is still no proof that viral infections have a negative impact on wild bee health. Infections with these viruses do not normally present visible symptoms (even in honey bees) and *Bombus atratus* in South America that are co-infected with DWV, BQCV and SBV are asymptomatic (Reynaldi et al. 2013). Experimental inoculations with mixed doses of viruses that would each be lethal to a honey bee had no effect on treated *Megachile rotundata* and *Colletes inaequalis* (Dolezal et al. 2016). Therefore, there is still much work to be done for a proper assessment for the risk these viral pathogens pose to native pollinators.

Virus quantification from field-caught specimens

Prior to this study there was little information available to recommend how best to capture and store field-caught specimens for virus quantification studies. Chen et al. (2007) concluded that storage at -80^{o}C best preserved viral RNA and that use of 70% ethanol for storage lead to enhanced levels of degradation. They also demonstrated that viruses in specimens stored at -20^{o}C or at 4^{o}C with the use of RNAlater^{TM} could still be detected. In general, viral RNA was perceived to be less prone to degradation in unsuitable conditions than host mRNAs (Dainat et al. 2011). This bodes well for studies looking to detect the presence or absence of RNA viruses in field-caught specimens, but is troublesome for quantification studies where calculated concentrations of virus are relative to the amount of host RNA degradation in the sample.

In this study it was shown that storage at -80^{o}C better preserved host and viral RNA molecules alike, than storage at -20^{o}C usually did. Storage mediums and durations prior to storage up to 48 hours were not significant on their own without considering other factors such as temperature. At higher temperature (ie. -20^{o}C) actin appeared to be relatively more preserved by ethanol and DWV relatively more preserved by RNAlater^{TM}. For virus quantification studies using RT-qPCR a standardized concentration based upon 2µg of RNA is required for cDNA synthesis prior to amplifying PCR products. The relative make-up of this sample is an important consideration as a bias on viral concentration one way or the other will change the estimate of viral load in your sample. For this reason use of storage techniques that preferentially preserve either host or viral RNA would be detrimental to the results. Therefore, no use of storage media would be recommended for study of DWV. BQCV preservation, on the

other hand, did not show significant interactions with storage media and the recommendation would be to use whatever preserves your specimen best.

Bee bowls are a passive pan trapping technique utilizing blue, yellow and white plastic bowls 2/3 filled with water mixed with DAWN™ original scent dish soap (NSERC-CANPOLIN 2009) and commonly used for the capture of bees in the field. Bees captured in these bowls may remain there in the heat of the day for hours before collection. Thus, there is some concern that an uninfected bee sharing that bowl with an infected one could be contaminated and later test positive for a virus it was not infected with. This would lead to misleading results in studies of both virus prevalence and quantification. This study showed that uninfected alfalfa leafcutting bees did not acquire DWV or BQCV particles from infected honey bees, meaning the bee bowl method is still viable for virus research.

Summary

In conclusion, this study has shown that honey bees have some effect on the suite of viruses infecting native bumble bee populations within the vicinity of their colonies in Canada, though conclusions about the directionality of pathogen spillover can still not be made. Viruses, such as DWV, BQCV, SBV and IAPV were found to be widespread and existed in populations well isolated from direct contact with the suspected original host (the honey bee, *Apis mellifera*). Average intensities were never higher in infected bumble bee populations than they were in managed honey bee populations and the impact these lower intensities have on bumble bee health is yet to be determined. Future researchers studying RNA viruses, should

store field caught specimens at -80^{o}C without the use of storage media and continued use of bee bowl trapping is viable.

THE NEED FOR FUTURE RESEARCH

Arising from my thesis are several topics and considerations for future research on RNA viruses in wild bees.

1. Sequencing of amplified targets to determine differences in viral strains will give a clearer picture of directionality of the transmission of these viruses.
2. For viruses like KBV, ABPV and CBPV that did not show up in large sample sizes in this study it is suggested that associated honey bee colonies be pre-screened for the viruses in question.
3. All future studies on viral dynamics among bee populations should test for the minus, or replicating, strand of RNA for conclusive evidence that it is infective to the host in which it is detected and not just an incidental accumulation of virus particles.
4. Locating and sampling directly from distinct wild bumble bee colonies will help us get a better handle on species differences for prevalence and intensity of virus infection.
5. Future studies should shift focus to the impact viruses reported in wild bees have on their fitness, including the increased risk with co-infections we see in honey bees.

Appendix 1. Average gene copies of each of seven viruses (± SE) detected in 1 µg of extracted RNA from groupings of bee samples used for different analyses: honey bees (*Apis mellifera*), bumble bees captured at sites where honey bees were present (HB-exposed) further broken down by species, bumble bees captured at sites where honey bees were absent (HB-absent) further broken down by species and bumble bees captured using bee bowls or sweep nets. Commonly detected viruses (Deformed Wing Virus [DWV], Black Queen Cell Virus [BQCV], Sacbrood Virus [SBV] and Israeli Acute Paralysis Virus [IAPV]) as well as rarely detected viruses (Kashmir Bee Virus [KBV], Acute Bee Paralysis Virus [ABPV] and Chronic Bee Paralysis Virus [CBPV]) are included.

	DWV	**BQCV**	**SBV**	**IAPV**	**KBV**	**ABPV**	**CBPV**
Apis mellifera	$3.286 \times 10^7 \pm 1.648 \times 10^7$	$2.028 \times 10^9 \pm 1.349 \times 10^9$	$3.995 \times 10^6 \pm 3.038 \times 10^6$	$3.962 \times 10^8 \pm 2.285 \times 10^8$	---	---	593334512 ± 593332844
HB-exposed	15571 ± 3684	$2.17 \times 10^7 \pm 1.294 \times 10^7$	15390 ± 6983	12473 ± 7930	575 ± 162	753 ± 193	4518 ± 2759
B. terricola	---	---	3870	---	---	---	---
B. borealis	9299 ± 8963	$1.126 \times 10^6 \pm 1.019 \times 10^6$	616 ± 209	2131	---	---	---
B. vagans	16439 ± 5887	$7.575 \times 10^7 \pm 6.366 \times 10^7$	8958 ± 3572	1307 ± 672	---	---	---
B. ternarius	8975 ± 5646	$8.855 \times 10^5 \pm 3.018 \times 10^5$	6644 ± 3254	2085 ± 729	1474	---	507
B. bimaculatus	44281 ± 28193	$1.16 \times 10^7 \pm 5.546 \times 10^6$	21683 ± 19441	52206 ± 40781	---	---	8800 ± 3000
B. sandersoni	11009 ± 5712	$1.746 \times 10^6 \pm 6.79 \times 10^5$	6102 ± 3162	---	---	---	438
B. rufocinctus	13591 ± 5866	$1.561 \times 10^7 \pm 1.119 \times 10^7$	24984 ± 15677	7449 ± 6361	827	753 ± 193	---
B. perplexus	19110	---	11500	---	---	---	---
B. griseocollis	2.44×10^5	$4.316 \times 10^6 \pm 1.857 \times 10^6$	2018 ± 1203	---	---	---	---

HB-absent	33840 ± 13092	$4.512x10^5$ ± $2.294x10^5$	319 ± 64	8727 ± 2636	1710 ± 36	---	803
B. borealis	13962 ± 13717	45222 ± 36638	900 ± 557	976 ± 541	3348	---	---
B. vagans	40729 ± 17782	$3.6x10^5$ ± $1.954x10^5$	157 ± 81	13791 ± 4937	---	---	---
B. ternarius	18313 ± 15516	$2.823x10^5$ ± $2.512x10^5$	208 ± 28	1603 ± 650	---	---	---
B. sandersoni	51845 ± 37534	$3.451x10^5$ ± $2.288x10^5$	320 ± 49	18284 ± 9806	3490	---	803
B. rufocinctus	2992	14198 ± 7055	116 ± 20	5719	---	---	---
B. perplexus	4969	26120	---	---	---	---	---
B. griseocollis	8015 ± 3075	29505 ± 18899	449 ± 237	2573 ± 1715	---	---	---
B. fervidus	---	$8.345x10^6$ ± $7.553x10^6$	242	3082	---	---	---
B. fernaldae	---	---	252	---	---	---	---
Bee bowl	6333 ± 1840	$5.315x10^6$ ± $2.478x10^6$	3403 ± 951	4869 ± 1801	3348	---	
Sweep net	30727 ± 8629	$1.884x10^7$ ± $1.245x10^7$	9688 ± 5185	11918 ± 4189	965 ± 401	753 ± 193	3695 ± 2290

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