

The Pennsylvania State University

The Graduate School

Department of Entomology

**ECOLOGY AND EPIDEMIOLOGY OF RNA VIRUSES IN HYMENOPTERAN  
POLLINATORS**

A Dissertation in

Entomology

by

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Submitted in Partial Fulfillment  
of the Requirements  
for the Degree of

Doctor of Philosophy

December 2011

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## ABSTRACT

Pollinators are critical to agricultural production and maintenance of natural plant communities. However, widespread declines in pollinator populations have become a cause of concern. RNA viruses are suspected as one of the major contributors to honey bee (*Apis mellifera*) losses including the recent malady, colony collapse disorder (CCD). But, our knowledge of viral diseases in other managed and wild bee species is very limited. This dissertation investigates the ecology and epidemiology of RNA viruses in different hymenopteran pollinators. Detection of RNA viruses in thirteen non-*Apis* hymenopteran species, using reverse transcriptase-PCR, indicated widespread occurrence of these viruses. This study provides the first evidence of pollen as a route of inter-taxa transmission of RNA viruses in hymenopteran pollinators. Molecular detection of viruses in pollen pellets from foragers and the subsequent phylogenetic analyses support that these viruses are disseminating freely among the pollinators via the pollen itself. The viruses in the pollen and honey stored in the hive were demonstrated to remain infectious for at least six months of storage. RNA viruses were also widespread in commercial colonies of the bumble bee, *Bombus impatiens*, the major pollinator of greenhouse crops, but were less prevalent in the alfalfa leafcutting bees (ALCB) (*Megachile rotundata*) and the alkali bees (*Nomia melanderi*), the two solitary bees essential for the alfalfa seed crop. Inter-taxa transmission of one of these viruses was further demonstrated in containment greenhouse experiments, where the recently described Israeli acute paralysis virus (IAPV) moved between honey bees and bumble bees within ten days. IAPV disseminated throughout the bee body including the ovaries and eggs when fed to virus-free bumble bee queens, but remained restricted in the gut of the ALCB larvae. IAPV infection negatively impacted the health of bumble bee and honey bee colonies under greenhouse conditions, and adult survivorship and larval diapause in ALCB laboratory bioassays. However, further studies are needed to understand the role of IAPV

and related viruses in the recent declines in field populations of different pollinators. This study increases our understanding of RNA virus ecology and epidemiology and may help explain bee disease patterns and pollinator declines in general.

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## ACKNOWLEDGEMENTS

There are many people whose help and support made this work possible. First and foremost, my thanks go to the many people in the Cox-Foster lab who helped me along the way, especially technicians Amanda Mahoney, Monica Kalkstein, Jeremy Fitzgerald, Owen Thompson and, fellow graduate students Abby Levitt, Michael Freiberg, Michael Williams, Kim Nguyen (from Gembloux University, Belgium), and undergraduates Mei Kishiki and Xavier Flores (from Puerto Rico under Summer Research Opportunity Program). All of these people, and many more, contributed in incalculable ways, be it with helping design and run experiments or with helpful comments and discussions of my work and ideas. I greatly appreciate Rosalind James's lab crew from USDA bee lab, Logan, UT, especially Ellen Klinger and Craig Huntzinger for their help in collecting field bee samples and in setting-up bioassays.

I would also like to thank my advisors, Diana Cox-Foster and Edwin Rajotte, who mentored my work and professional development throughout my time as a student. Not only did they support my research and course work, they also provided opportunities to develop the other aspects of my professional career. My thanks also go to the other members of my committee, Rosalind James, Edward Holmes and Fred Gildow, for their helpful comments and support. Also, thanks to the Integrated Pest Management Collaborative Research Support Program (IPM CRSP) for funding me throughout most of this work.

I also thank USDA-ARS Subtropical Agricultural Research Center at Weslaco, TX, for irradiation services and the Penn State Genomic Core Facility at University Park campus for their sequencing services, required through the course of this work.

I thank my parents, my in-laws and my sisters for giving me their love and support through all the ups and downs of being a graduate student. Finally, I thank my wife Bindu, son Aviraj and my cute little 3-months old daughter Jocelyn for making this work a lighter load to bear.

## Chapter 1

### Introduction

Pollinators are integral to agricultural production and provide a crucial ecosystem service by helping the plants reproduce [1]. The worldwide economic value of animal-mediated pollination has been estimated around 225 billion US dollars [2]. The animal pollinated crops contain the majority of the dietary lipids and several micronutrients including vitamins (A, C and E) and minerals (calcium, fluoride and iron), and any decline in pollinators could drastically impact human nutrition worldwide [3]. The demand for such nutrient rich crops is rapidly increasing along with the fast growing human population and the heightened consumer consciousness about the importance of food diversity and balanced diet. However, the widespread declines in pollinators, particularly the bees (Hymenoptera: Apiformes), have raised serious concerns over the stability of global food supply and natural plant communities [4-6].

A variety of factors, including a parasitic mite (*Varroa destructor*), RNA viruses, pesticides and the microsporidians, *Nosema ceranae* and *N. bombi* have contributed to honey bee population losses across the United States and several other countries in Europe and the Middle East [7-9]. The large-scale honey bee losses, particularly in the U.S. over the last five years, have been partially attributed to a syndrome named the colony collapse disorder (CCD) [9,10]. CCD is characterized by the rapid disappearance of adult workers, leaving behind a queen along with few young workers and lots of brood and food stores. Eventually the colony dies and oddly, the food stores remain untouched by robbers and hive pests for unusually long periods of time.

Epizootiological and metagenomic studies have revealed higher pathogen loads and co-infections in bees from CCD colonies, suggesting either an increased exposure to pathogens or a compromised ability of bees to fight diseases as a result of other stress factors such as pesticides, poor nutrition, etc. [11,12]. Although unusually high and apparently unexplained colony losses

have been reported throughout the documented history of beekeeping [13], this CCD phenomenon has generated scientific, public and political interest in pollinator health, and has provided an incentive to study bee pathology.

For most of our crop monocultures, the honey bee (*Apis mellifera*) is the preferred pollinator, mainly because an elaborate management system has evolved that allow pollinators to be moved in and out of crops as needed. However, there are other non-*Apis* bee species, which are far more efficient pollinators of specific crops and/or specific crop production systems, and are therefore commercially domesticated [14-17]. In addition, resident ‘wild’ solitary and social species may be reliable pollinators if the local ecosystem is managed appropriately. More than 4000 species of bees are native to North America and these non-*Apis* hymenopteran pollinators alone may be responsible for more than \$3 billion of fruits and vegetables produced in the US [18]. Moreover, diverse pollinator communities can improve reproduction in individual crops and wild plants, while providing stability and assurance of the pollination services [19,20].

The honey bee, *A. mellifera* L. (Hymenoptera: Apidae), is a eusocial insect that lives in highly organized, perennial colonies consisting of a single reproductive queen and up to 60,000 workers, with well defined division of labor [21]. After honey bees, the bumble bee, *Bombus impatiens* (Apidae) and the alfalfa leafcutting bee, *Megachile rotundata* F. (Megachilidae), are the other two most intensively managed bees in the United States.

Bumble bees (genus *Bombus*) are primitively eusocial insects with an annual life cycle. A single queen starts the colony in spring, which then expands in worker numbers throughout the summer. Males and future queens (larger workers) are produced late in the season and only the mated young gynes hibernate during winter, starting their own colonies the following spring [22]. Each year over a million bumble bee colonies with estimated value of around \$80 million are reared commercially around the world [23]. Bumble bee colonies are used extensively for pollination of high-value crops such as greenhouse tomatoes (*Solanum lycopersicum* L.) and

peppers (*Capsicum annuum* L.) worth more than \$17 billion.

On the other end of the sociality spectrum, the solitary alfalfa leafcutting bee (ALCB) has revolutionized the alfalfa seed production industry in the northwestern United States and the central Canada. In the process, the ALCB has become the most intensively managed solitary bee in the world [24]. ALCB females actively forage only for 6-8 weeks each year, and use existing above ground cavities (eg. plant stems, abandoned beetle bore-holes, and pre-drilled, man-made ‘nest blocks’) to individually build linear nests with multiple cells made from leaf cuttings.

Since 1963 more than 18 viruses are known to infect honey bees, with the majority being non-enveloped, positive-sense single-stranded RNA viruses [25-27]. Their RNA serves both as a genome and as mRNA that is translated into polyproteins, which are cleaved into functional proteins at conserved proteolytic cleavage sites. Among these, the following four viruses are most common in the United States: deformed wing virus (DWV), black queen cell virus (BQCV), sacbrood virus (SBV), and Kashmir bee virus (KBV) [28,29]. In contrast, the chronic bee paralysis virus (CBPV) and the acute bee paralysis virus (ABPV) are more prevalent in Europe [30,31]. In addition, a recently described virus, Israeli acute paralysis virus (IAPV) was found to be associated with CCD-affected beekeeping operations throughout the US [11]. All of these except CBPV have monopartite RNA genome and icosahedral particle symmetry and have been assigned to the families *Dicistroviridae* and *Iflaviridae*, order Picornavirales [32,33]. CBPV has a multipartite genome organization and different particle morphology and has not yet been assigned to any genus or family [34].

These obligate intracellular parasites usually persist or co-exist with honey bees as asymptomatic, covert infections. However, they are capable of rapid multiplication often resulting in disease manifestation [35-37]. Overt viral infections in honey bees provoke a wide range of symptoms including wing deformities, discoloration, hair loss, bloated abdomens, trembling, paralysis, and brood and adult mortality, with serious consequences in terms of colony survival

[28,38]. The spread of a parasitic mite (*V. destructor*) in the last couple of decades, and its dual role as a virus vector and bee immune system suppressor, has led to the emergence of viral infections in honey bees with apparent symptoms and significant health impacts at both the individual bee level and the colony level [39]. In addition, other epidemiological factors such as high host contact rates as a result of modernization of agriculture and commercial beekeeping may also be responsible for the enhanced virulence of these viruses [20,40]. Long distance hauling and unnaturally high-density congregations of bees such as for California almonds, leads to high horizontal transmission favoring the spread and evolution of more virulent pathogen strains [41,42].

DWV and CBPV are among the few viral diseases of bees with well-defined symptoms, and as a result have attracted the most scientific attention over the last 3-4 decades. CBPV follows a classical dose-mortality model in the laboratory bioassays, while field studies have shown direct association between CBPV outbreaks and increased host density in Europe [43,44]. DWV and SBV belongs to the family *Iflaviridae*, members of which have a genome organized as a single open reading frame (ORF) flanked by a long 5'-untranslated region (5'-UTR) and a short, highly conserved 3'-UTR terminated with 3' poly-A tail [39].

KBV, ABPV and IAPV forms a closely related complex of viruses with a RNA genome organized into two ORFs, separated by an intergenic region and flanked by non-translated regions [45]. The viruses are rapidly lethal to honey bee adults in the laboratory when injected into pupae or adults at low doses or when fed to newly emerged adults at larger doses [46,47]. Sharp declines in adult honey bee populations have been associated in the past with severe ABPV and KBV infections, resulting in the appearance of dead/diseased brood due to the lack of enough nurse bees [46,48].

Although most viruses have been known from honey bees for about three to four decades, IAPV was isolated and characterized only in 2002 in Israel [47]. Later on, IAPV was also



detected sporadically in honey bees from other parts of the world [11,49-51]. Due to its rather recent discovery, very little is known about the host range, geographical distribution, pathology and epidemiology of IAPV. One study reported up to 80% mortality in adult honey bees infected orally with IAPV, within a week of infection, with little difference across a 1000-fold range of inoculum concentrations [52]. ABPV and IAPV overt infections are characterized by trembling, paralysis, gradual darkening and loss of hair, followed by death; however, KBV does not provoke typical symptoms of paralysis [46,47]. This rapid adult mortality associated with IAPV is especially interesting in the wake of CCD, wherein the sudden disappearance of adult workers from the colony is the distinctive characteristic [9]. Moreover, elevated levels of multiple viruses including IAPV have been detected in bees from colonies with CCD symptoms [12,53].

Honey bee virology has advanced rapidly over the last decade, however most of the research has been focused on the virus characterization, diagnosis, incidence and transmission within honey bee colonies. Complex horizontal as well as vertical transmission pathways have been documented for the spread of viruses within honey bees (Figure 1) [54], but very few studies have looked at virus epidemiology and virulence from the colony level perspective [55]. Further, the basic physiological, immunological and epidemiological mechanisms underlying virus pathology and virulence are poorly understood. Such detailed experimental studies are very demanding and complicated due to: (i) the lack of any established bee cell lines, (ii) the difficulty in obtaining virus-free bee colonies that can be used as a control, (iii) the difficulty of obtaining single-virus infected bees to prepare pure virus inoculum and infectious clones, and (iv) the complex interactions among multiple factors in the bee colony that can affect pathogen transmission and bee susceptibility. The chronic effects of disease, such as shortening of the adult bee life span (which is usually masked by heavy brood rearing in the spring and summer), reduced tolerance to cold and other stresses and adverse behavioral changes, are much harder to measure experimentally. IAPV, being a relatively new virus, provides an opportunity to conduct

experimental studies, since IAPV-free honey bees and other non-*Apis* managed bees can be obtained.

The host range of RNA viruses and their prevalence and health impacts in other managed and wild bee species are not known. Bees can acquire infectious propagules from the environment. Recent studies in bumble bees have demonstrated pathogen spillover, involving the spread of intestinal protozoans, *Crithidia bombi* and *Nosema bombi*, from commercial greenhouse bumble bees to wild native bumble bee species via shared use of flowers [56,57]. “Pathogen spillover” occurs when a pathogen spreads from a heavily infected ‘reservoir’ host population to a sympatric ‘non-reservoir’ host population [58]. Since honey bees share floral resources with a variety of pollinators, viruses can also potentially spread between different pollinator species. More research on the ecology and epidemiology of viruses in the pollinator community is thus vital for understanding the dynamics underlying disease outbreaks and to shed light on the current pollinator crises.

### **Thesis objectives:**

- Elucidate the spread and distribution of bee-pathogenic RNA viruses in the hymenopteran floral communities in the northeastern United States
- Characterize the inter-taxa transmission dynamics of RNA viruses in hymenopteran pollinators
- Determine the prevalence of RNA viruses that are common in honey bees, in non-*Apis* domesticated bees
- Determine the health impacts of Israeli acute paralysis virus (IAPV) on domesticated bees
- Examine the pathology, tissue specificity and epidemiology of Israeli acute paralysis virus (IAPV)

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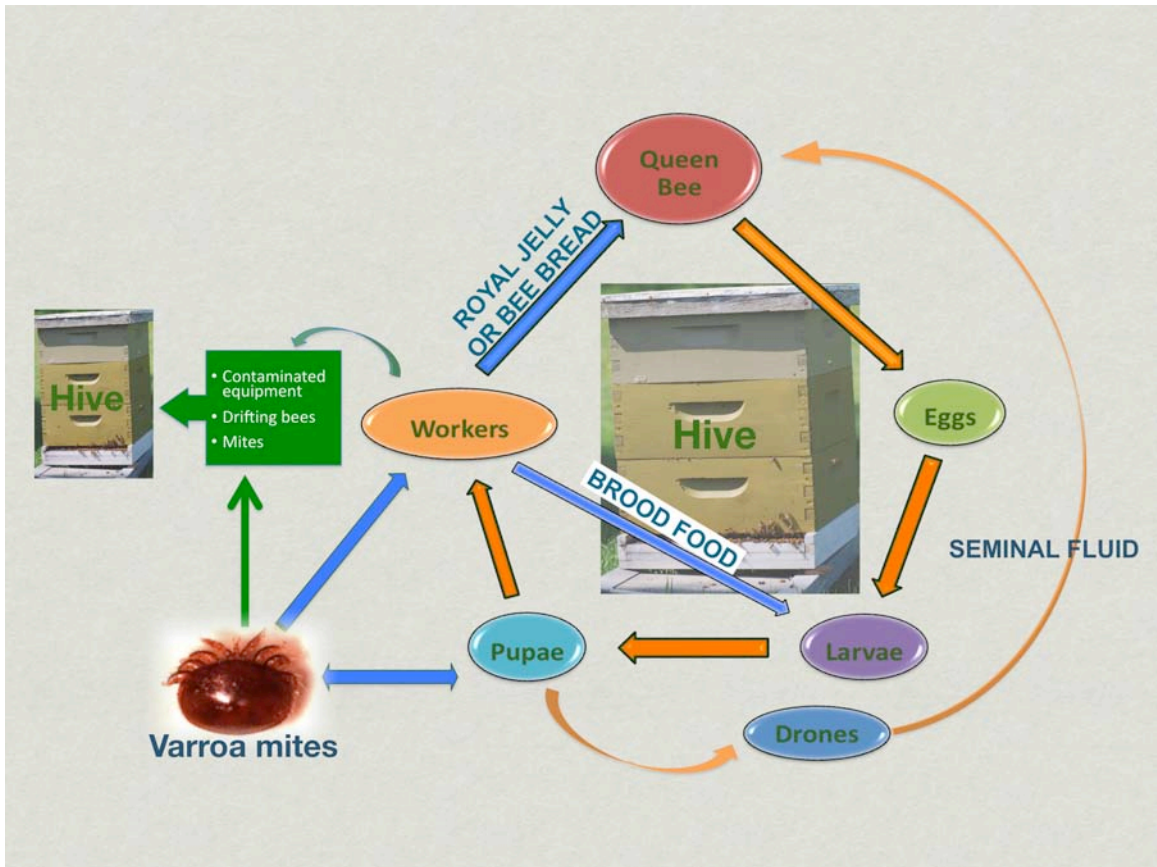
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**Figure 1: Known routes for horizontal and vertical transmission of RNA viruses in the honey bee, *Apis mellifera*.** Orange arrows represent intra-colony vertical transmission routes; blue arrows represent intra-colony horizontal transmission routes; green arrows represent inter-colony transmission routes.

## Chapter 2

# **RNA Viruses in Hymenopteran Pollinators: Evidence of Inter-taxa Virus Transmission via Pollen and Potential Impact on Non-*Apis* Hymenopteran Species**

### **Author contribution**

Rajwinder Singh collected and analyzed the pollinator and pollen samples for RNA viruses, and performed most of the experiments in this chapter, which was published in PLoS ONE in 2010. Rajwinder Singh also obtained viral sequences, while the phylogenetic analysis was performed mainly by Abby Levitt with the help of Edward Holmes from the department of biology. Field experiment with honey bee colonies to test the pathogenicity of stored pollen and honey was performed by Diana Cox-Foster and Nancy Ostiguy's lab technicians. Nancy Ostiguy also helped with statistics to analyze the data in this chapter. Rick Donvall of the Pennsylvania Department of Agriculture identified pollinator species.

### **Abstract**

Although overall pollinator populations have declined over the last couple of decades, the honey bee (*Apis mellifera*) malady, colony collapse disorder (CCD), has caused major concern in the agricultural community. Among honey bee pathogens, RNA viruses are emerging as a serious threat and are suspected as major contributors to CCD. Recent detection of these viral species in bumble bees suggests a possible wider environmental spread of these viruses with potential broader impact. It is therefore vital to study the ecology and epidemiology of these viruses in the hymenopteran pollinator community as a whole. We studied the viral distribution in honey bees, in their pollen loads, and in other non-*Apis* hymenopteran pollinators collected from flowering plants in Pennsylvania, New York, and Illinois in the United States. Viruses in the samples were detected using reverse transcriptase-PCR and confirmed by sequencing. For the first time, we

report the molecular detection of picorna-like RNA viruses (deformed wing virus, sacbrood virus and black queen cell virus) in pollen pellets collected directly from forager bees. Pollen pellets from several uninfected forager bees were detected with virus, indicating that pollen itself may harbor viruses. The viruses in the pollen and honey stored in the hive were demonstrated to be infective, with the queen becoming infected and laying infected eggs after these virus-contaminated foods were given to virus-free colonies. These viruses were detected in eleven other non-*Apis* hymenopteran species, ranging from many solitary bees to bumble bees and wasps. This finding further expands the viral host range and implies a possible deeper impact on the health of our ecosystem. Phylogenetic analyses support that these viruses are disseminating freely among the pollinators via the flower pollen itself. Notably, in cases where honey bee apiaries affected by CCD harbored honey bees with Israeli Acute Paralysis virus (IAPV), nearby non-*Apis* hymenopteran pollinators also had IAPV, while those near apiaries without IAPV did not. This study adds to our present understanding of virus epidemiology and may help explain bee disease patterns and pollinator population decline in general.

## **Introduction**

Pollinators of all types are vital to agriculture and are responsible for reproduction of crops worth >225 billion US dollars worldwide [1]. Honey bees (*Apis mellifera* L.) alone in the United States, account for an added market crop value exceeding 15 billion dollars [2]. However, pollinator populations in general have been declining over the last couple of decades [3-5]. The recent dramatic losses of thousands of honey bee colonies due to colony collapse disorder (CCD) and other causes [6,7] has not only created great concerns in the scientific and agricultural community but has also highlighted the ever increasing risk of future crises in the global food supply due to our sole dependence on single pollinator species [8]. Apart from a pollination industry relying on only a few managed pollinators, more than 4000 other species of bees are

native to North America. These non-*Apis* hymenopteran pollinators alone may be responsible for more than \$3 billion of fruits and vegetables produced in US [9].

Being social insects, honey bees live in compact, highly organized and productive colonies consisting of up to 60,000 individuals. However, this social organization and the close interactions among colony members makes them highly susceptible to a variety of infectious diseases, among which viral pathogens are emerging as a serious threat to their health and survival [10,11]. More than 18 viruses have been identified from different stages and castes of honey bees including eggs, larvae, pupae, adult workers, drones and queens from different parts of the globe [12-14]. Among these, four positive-sense, single-stranded RNA viruses are most common in the United States: Deformed wing virus (DWV), Black queen cell virus (BQCV), Sacbrood virus (SBV), and Kashmir bee virus (KBV) [10,15]. Less commonly found are Acute bee paralysis virus (ABPV) and Chronic bee paralysis virus (CBPV). In addition, a recently described virus, Israeli acute paralysis virus (IAPV) was found to be highly associated with CCD-affected beekeeping operations throughout the US [16] and now appears to be more widely distributed nationwide [6]. IAPV sequence analysis across three genomic domains suggested the existence of three different groups: group 1 (the western strain) includes samples from operations in the western United States, as well as from bee packages imported from Australia; group 2 includes sequences from Israel; group 3 (the eastern strain) includes sequences from three operations in the eastern United States and one operation in Canada [17]. Elevated titers of multiple viruses were detected in bees from colonies with CCD symptoms [18]. All of these except CBPV have symmetric particles and a monopartite RNA genome. BQCV, ABPV, KBV and IAPV belong to a family Dicistroviridae, while DWV and SBV have been assigned to the genus *Iflavirus* [19,20]. In contrast, CBPV has a multipartite genome organization and different particle morphology and has not yet been assigned to any genus or family [21].

Like most of the insect-infecting RNA viruses, so-called honey bee viruses usually persist as inapparent, asymptomatic infections, capable of replicating rapidly under certain conditions, resulting in observable symptoms often leading to colony losses [22,23]. Symptoms of infections of the different viruses in honey bees range from deformed wings, discoloration, hair loss, bloated abdomens to trembling, paralysis, and brood and adult mortality, with serious consequences in terms of colony survival [10,24]; the full impact on bee behavior and health by these different viral infections is not completely understood. A better understanding of the epidemiology of viruses is vital to understanding the dynamics underlying virus outbreaks and to shed light on the current honey bee and pollinator crises. Complex routes of virus transmission involving both horizontal as well as vertical transmission pathways have been documented in honey bees [10]. Transmission pathways include vector-borne transmission via *Varroa* mites (DWV [25], KBV [26], and recently IAPV [27]) and vertical transmission from infected queens and drones to their offspring [28-30]. In addition, detection of some of these viruses in glandular secretions of worker bees [31], in colony foods including pollen, honey and royal jelly [28,29] as well as in bee feces [32,33], suggests potential food-borne and fecal-oral routes of horizontal virus transmission inside the colony. Like other picornaviruses such as poliovirus [34], these viruses may infect a variety of tissues, with dissemination from the gut or site of infection affected by host conditions. The dissemination to other tissues and the impacts on bee health has not been extensively studied for these viruses [19].

Despite their designation as honey bee viruses, their host range is not restricted to *A. mellifera*, as there are some previous reports of these viruses from other pollinator species. Bailey and Gibbs [35] described ABPV as inapparent infection in bumble bee species. Later, KBV was detected in yellow jacket wasps (*Vespula germanica*) in Australia [36]. Recently, Genersch et al. [37] reported the occurrence of wing deformities in two bumble bee species (*Bombus terrestris* and *B. pascuorum*) in Europe, resembling those seen in DWV-infected honey bees. With

molecular methods, they demonstrated that those bumble bees were indeed infected with DWV. A method has been recently published to detect ABPV, KBV, and DWV in bumble bees [38]. These reports suggest the possibility of wider environmental spread of these viruses with potential broader impact on the overall hymenopteran pollinator community.

Although, our understanding of viral epidemiology in honey bees has rapidly advanced over the last decade, most of the work has been focused on elucidating the routes of virus transmission within honey bee colonies. The intricate dynamics of interspecies virus transmission in the pollinator community has not been studied to date. Honey bees do not live in isolation in the environment, but mingle with other species on flowering plants. Other species include bumble bees, solitary bees, wasps, flies, ants, butterflies, mites and spiders, with which honey bees interact quite freely and frequently [39-42]. These interactions can lead to pathogen transmission; recent studies in bumble bees in Ontario, Canada have demonstrated pathogen spillover involving the spread of intestinal protozoa *Crithidia bombi* and *Nosema bombi* from commercial greenhouse bumble bees to wild native bumble bee species [43,44]. The status of pollinators both in North America and Europe appears to be declining [4,5]; how the decline of these essential members of our ecosystems relates to diseases is not known.

The focus of this study was to determine if pollen and/or pollinator species are involved in inter-taxa virus transmission in the hymenopteran pollinator community and to characterize the host range of RNA viruses. We have focused on viruses commonly associated with honey bees in the United States; DWV, BQCV, SBV and KBV, and the relatively newly-detected virus, IAPV. Viruses were detected using reverse transcriptase-PCR and confirmed by sequencing. In particular, we used phylogenetic analysis to study the distribution and sequence comparison of viruses in honey bee populations, the pollen loads collected by them from endemic, wild, flowering plants as well as agricultural crops, and other non-*Apis* pollinator species. We addressed several key questions: (i) Is the source of the RNA viruses in the stored pollen or bee

bread potentially from the pollen forager or the pollen itself? (ii) How does the prevalence of viruses detected in pollen pellets compare to those found in foragers carrying those pellets? (iii) What is the association of the viruses with the pollen? (iv) Is the virus found in stored pollen infectious? (v) Are these viruses specific to honey bees or are they widespread in hymenopteran pollinators? (vi) Does phylogenetic analysis of viral sequences indicate interspecies viral transmission in the hymenopteran pollinator community?

## **Materials and methods**

### **Sample collection to determine the source of the RNA viruses in the stored pollen or bee bread:**

In a preliminary study to determine if pollen foragers themselves were responsible for the virus being found in their pollen loads, 12 pollen foragers were collected as they were entering a colony at the Hill Top apiary at Penn State in 2005. The pollen pellets or loads (mass of pollen grains collected by honey bees, bumble bees and many other bee species, in their pollen baskets or corbiculae on hind legs) were removed from the honey bees with one pollen pellet extracted for RNA and the other kept for pollen identification. Some of the foragers were directly assayed for viruses and eight were kept for 24 hrs at 34 °C, 50% RH (Relative Humidity) with sugar syrup and water. The foragers were dissected into two body regions, head plus first thoracic segment (containing salivary glands) (H/T1) and the remainder of the body consisting of second and third thoracic segments and abdomen (T2,3/A).

In a more expanded study, 65 incoming honey bee pollen foragers were randomly collected from the landing board at the entrance of the five hives in two different locations (24 km apart) in Centre County, Pennsylvania during the summer of 2007. Bees were put individually into plastic tubes with pollen pellets still intact on their legs. Both apiaries were free from any CCD symptoms and all hives appeared to be normal and highly productive, with many

individuals and with most cells in the brood nest filled with either larvae or pupae (indicative of a healthy colony and queen). During the same time period other non-*Apis* hymenopteran species were collected using sweep nets from flowering plants near these apiaries. Non-*Apis* hymenopterans were also collected near apiaries harboring IAPV-infected honey bees and with a known history of CCD in Pennsylvania and New York. Some *Polistes* wasps were collected from Illinois near Urbana, Illinois from the field as well as established colonies. All the specimens were photographed for identification and whenever possible one representative of each type was pinned for proper identification and kept as a voucher specimen. All samples were immediately put on dry ice and stored at -80°C in the laboratory until analysis. Sixty-five honey bee foragers, 68 pollen pellets (including 65 from honey bees and 3 from non-*Apis* hymenopterans) and 55 other non-*Apis* hymenopteran specimens were analyzed for RNA viruses. Pollen pellets were carefully removed from frozen specimens and stored in separate 1.5ml centrifuge tubes. After removing pollen, hind legs of bees were discarded and the remaining body was washed thoroughly with distilled water to remove any pollen. Pollen pellet color was recorded and a subsample of pollen was mounted on a slide for identification as described below.

#### **Pollen identification:**

Pollen pellets were separated according to the color and microscopic slides were prepared by mounting unacetolyzed pollen in high viscosity silicone oil [Poly (dimethylsiloxane), 200<sup>®</sup> fluid, viscosity 30,000 cSt]. Slides were then observed and photographed under a ZEISS Axioskop compound microscope for morphological characters including overall shape and size of pollen grains, the number, shape and arrangement of wall apertures, and the structure and orientation of the exine surface [45,46]. Source plants were identified by comparing with the reference collection of microscopic slides prepared directly from identified plants collected from the same region.



**Association of the viruses with pollen:**

To obtain information about the association of the virus and pollen, a pollen washing experiment was conducted following the procedure used by Aparicio et al. [47], with slight modifications. About 10g of pollen pellets collected from honey bee hives with pollen traps, were mixed together. A 100 mg subsample was then suspended in 1ml Trizol, vortexed for 30 sec. and centrifuged at 5000 rpm for 5 min. Supernatant was then removed and this whole procedure was repeated four times. The washed pollen was then homogenized with Geno / Grinder 2000 (SPEX SamplePrep LLC) at 1300 strokes/min. for 3 min in 1ml Trizol. This whole washing process was also performed using 1ml of phosphate saline-Tween polyvinylpyrrolodone buffer [1M Phosphate buffer saline (PBS), 0.05% Tween-20, 2% Polyvinylpyrrolodone] at pH 7.4, except that the fourth washing in this case was done with 1% SDS (Sodium dodecyl sulfate) to remove virus particles tightly bound to pollen grains. Viral RNA was extracted from all these samples using the extraction process discussed below. These samples were then analyzed for DWV, and BQCV using RT-PCR.

**Test of infectivity of virus in stored pollen and honey:**

In Fall 2005, frames of bee bread (stored pollen) and honey were collected from colonies previously determined to have DWV (both from symptoms and with RT-PCR). Multiple cells of bee bread or honey were sampled at random from both sides of each frame and RNA was extracted from groups of 2-3 cells. RT-PCR was performed for DWV, SBV, and KBV. Only DWV was detected in the majority of the cells both for frames of honey or bee bread. These frames were stored at ambient temperature over the winter (fluctuating from below -6 °C to 32°C), with protection from pests. Additional frames were power-washed to remove all deposits, leaving some wax; these were designated as “clean” frames. The wax did not have DWV as tested by RT-PCR.

Six months later in Spring 2006, new packages were placed into new hive equipment in an isolated apiary (Rock Springs Apiary) that had no known feral or managed colonies of honey bees located within 8 km. The surrounding area was forest, meadow and farmland. After one week when the colonies had established and the marked queens had begun to lay eggs, egg samples (N=4 samples of 5 eggs each, or 20 eggs per colony) and worker attendants (N=15) were collected for each colony and analyzed for DWV, BQCV and SBV. A total of twelve packages or colonies were found to have workers free of DWV, KBV, and SBV; and the queens were laying virus-free eggs. These packages were randomly divided into three treatments with four colonies each: Controls (fed artificial bee pollen and sugar syrup, given “clean” frames), DWV-Honey (fed a frame of honey contaminated with DWV and artificial bee pollen), or DWV- Bee Bread (fed a frame of bee-bread contaminated with DWV and sugar syrup). Egg samples from each colony (N=4 samples of 5 eggs each, or 20 eggs per colony) were collected every week for five weeks following introduction of the frames of food; and DWV and SBV infections and actin were determined by RT-PCR. Each marked queen was observed in its colony during the experiment, ensuring that the same individual queens were being monitored for viral infection.

**RNA extraction:**

Total RNA was extracted from individual samples using TRIzol<sup>®</sup> reagent (Invitrogen) and was resuspended in 20µl DEPC-treated water. Concentration of total RNA was determined spectrometrically (Spectra Max 250, Molecular Devices).

**Reverse transcriptase-polymerase chain reaction (RT-PCR) for diagnostic and phylogenetic analysis:**

For diagnosis of the viruses, cDNA was synthesized using random hexamers and M-MLV reverse transcriptase (Promega), using the protocol of Cox-Foster et al. [16]. Primers were

designed using Primer3 [48] except for BQCV primers that were obtained from Benjeddou et al. [49]. Different primer sets and their gene regions are listed in (Appendix B). RT-PCR was carried out for DWV, IAPV, KBV and SBV using a program of initial denaturing for 8 min at 94°C and 35 cycles of 94°C for 55 s, 51.5°C for 55 s, and 72°C for 1 min 25 s, with a final extension step for 10 min at 72°C. For BQCV and ABPV, PCR was carried out using a program of initial denaturing for 8 min at 94°C and 38 cycles of 94°C for 1 min, 55° for 1min and 72°C for 1 min 15 s, with a final extension step for 10 min at 72°C. Five micro liters of the RT-PCR products were electrophoresed in a 1.5% agarose gel, stained with SYBR<sup>®</sup> Safe DNA gel stain (Invitrogen), and imaged using a Gel Doc XR (BIO-RAD). Primers (actin-F, ATGAAGATCCTTACAGAAAG; actin-R, TCTTGTTTAGAGATCCACAT) were used to amplify 514 bp of the honey bee actin gene (GenBank accession no. BI504901), serving as an internal control for the quality of RNA extraction. Detection of actin in the honey bees was used as a positive indicator of intact mRNA being assayed and also serves as an internal loading standard. A negative control lacking template DNA and a positive cDNA control were performed for each PCR reaction. Positive identification was confirmed by sequencing the PCR products. Primer sequences for DWV would have also amplified *Varroa destructor* virus-1 and Kakugo virus; sequence data did not find these viruses in our samples.

### **Sequence analysis:**

PCR products were treated with ExoSAP-IT (USB) and sequenced on both strands. PCR products were also cloned using TOPO TA Cloning Kits with One Shot<sup>®</sup> Chemical Competent Cells (Invitrogen) and several individual clones were sequenced for each amplification. Sequence data were aligned and analyzed using the MEGA package. Nucleotide sequences determined in this study are being deposited under GenBank accession numbers. (Numbers will be added to manuscript upon acceptance).

**Phylogenetic analysis:**

Maximum likelihood (ML) phylogenetic trees were inferred for DWV, BQCV and IAPV. In all cases phylogenetic trees were estimated using the ML method implemented by the PAUP\* 4.0 package [50] and utilizing the best-fit model of nucleotide substitution as determined by MODELTEST [51], which in each case was the most general time-reversible GTR+ $\Gamma_4$ +I model (full parameter values available from the authors on request). A bootstrap resampling analysis of 1000 replications was performed to assess the support for specific nodes.

To statistically test if the evolutionary structure of BQCV, DWV and IAPV sequences is distinct among species, we computed the association index statistic (AI) [52] and parsimony score (PS) [53] statistic of clustering strength, using the BaTS (Bayesian tip-association significance testing) program developed by Parker et al. [54]. This analysis was based on a posterior distribution of phylogenetic trees inferred using the Bayesian Markov Chain Monte Carlo (MCMC) available in the BEAST package [55]. The BEAST analysis utilized the GTR+ $\Gamma_4$ +I model of nucleotide substitution, a Bayesian skyline coalescent prior, and an uncorrelated lognormal relaxed molecular clock.

**Statistical Tests:**

SAS 9.1 statistical package (SAS Institute Inc.) was used to analyze the data by two-way ANOVA, Cochran-Mantel Haenszel statistical analysis, Fisher Exact test and Tukey-Kramer post hoc analysis. The BaTS program [54] was used to compute the association index statistic and parsimony score.

## **Results**

### **Is the source of the RNA viruses in the stored pollen or bee bread potentially from the pollen forager or the pollen itself?**

Of the 12 initial honey bee pollen foragers analyzed for SBV and DWV and either kept for 24 hrs after removing their pollen pellets or directly frozen, there were no detectable differences in the prevalence of the viruses in these two groups. This suggests that the supply of virus associated with either the salivary glands or digestive tract was not reduced in those assayed immediately after collection as compared to those kept for 24 hrs. All foragers were dissected into two regions prior to virus analysis, the head and first thoracic segment containing the salivary glands and the remainder of the body. Three of the foragers were found to have detectable DWV in their pollen loads, without any detectable viral infections in the head/thoracic segments and two of these did not have any detectable infections anywhere (Figure 1) indicating that the salivary secretions of the forager were not a likely source of viral contamination on the pollen. One forager had a high level of DWV in her head/thoracic segment but there was no detectable DWV in her pollen load. SBV was detected in most of the pollen pellets as well as in the abdomen/thorax of the foragers, but few of the foragers had detectable virus in their heads/thoracic segments. These data therefore suggest that there is an alternative source of bee bread contamination.

### **How does the prevalence of viruses detected in pollen pellets compare to those found in foragers carrying those pellets?**

In an expanded collection of 65 honey bee pollen foragers in 2007, all foragers were infected with at least one virus with most having multiple infections. A large number of pollen pellets also were positive for one or more virus species. BQCV was the most prevalent species detected in the honey bee samples (98.5%). In comparison, only 30.8% pollen pellets were

positive for this virus (Figure 2, Table 1). SBV was less common with only 24.6% bees and 3.1% pollen pellets detected positive. The incidence of DWV was relatively high, and this virus was almost equally detected among foragers (61.5%) and their pollen loads (58.5%). All forager honey bees and their pollen loads tested negative for IAPV and KBV. DWV was the most commonly detected virus in pollen loads of honey bees. More importantly, there were forager/pollen pellet pairs where the uninfected forager was carrying pollen loads positive either for DWV (13.9%) or SBV (1.5%) (Table 1, Figure 2).

Overall, there was a significant, positive association between the prevalence of virus species found in the forager and in the pollen pellet of that forager (Cochran-Mantel Haenszel Statistic = 9.46; df = 1; p = 0.002). However, this relationship seemed to be driven mainly by DWV frequencies. Analyzing frequencies of the three viruses separately revealed a significant correlation between the prevalence of DWV positive foragers and the DWV positive pollen pellets (Fisher Exact test; p = 0.005). This relationship between forager and pollen also extended to the other pollinator species collected at the same time; among three pollen pellets taken from non-*Apis* hymenopteran pollinators, one was positive for DWV (data not shown) and the corresponding forager bumble bee was also positive for DWV. However, there was no significant, positive correlation between the frequencies of foragers and pollen pellets with SBV (Fisher Exact test; p = 0.435) or BQCV (Fisher Exact test; p > 0.999). There were significantly fewer samples where both forager and corresponding pollen pellet were positive for SBV or BQCV in comparison to the pairs where only the forager was positive while her corresponding pollen pellet was negative (Table 1).

The frequency of the number of co-existing virus species was significantly different between the honey bee foragers and the pollen pellets (Mantel-Haenszel  $\chi^2 = 36.36$ ; p < 0.001). A higher percentage of bees had two or three viruses co-existing versus only one virus; however, the trend was opposite in pollen (Figure 2). All the honey bees were infected with at least one virus;

in comparison, 30.8 % pollen pellets were free from any virus tested in this study. Co-infections of three viruses (DWV, SBV and BQCV) were detected in 16.9 % of honey bee foragers; while, only 1.5 % pollen pellets tested positive for all three viruses.

### **What is the association of the viruses with the pollen?**

Based upon color of pollen pellets (Appendix A) and pollen morphology, the virus-positive pollen belonged to many plant species including goldenrods (*Solidago* spp.), thistles (*Cirsium* spp.), clovers (*Trifolium & Melilotus* spp.) and common burdock (*Arctium minus*).

The pollen-virus association was further examined to determine if there was potential for the viruses to be inside the pollen grain as opposed to just being on the outside of the pollen exine. Following extensive rinses with Trizol, both DWV and BQCV were present in the supernatants from first two washings. However, no virus was detected in the third and fourth washings but was again detected in the homogenized pollen, although the viral RT-PCR bands on the gel were less intense as compared to the bands in the first two washings (Figure 3). Similar results were obtained with phosphate saline-Tween polyvinylpyrrolodone buffer (PBS+) and sodium dodecyl sulfate (SDS) washings. This suggests a possibility for a more intimate association between these RNA viruses and plant-pollen.

### **Is the virus found in stored pollen infectious?**

A field experiment demonstrated that viruses detected in bee bread (stored pollen) and honey were infectious. In an apiary isolated from other known honey bee colonies, DWV-free colonies were given either bee bread with DWV, honey with DWV, or virus-free artificial foods (control). To avoid false positives originating from the detection of virus in the gut of the honey bees that had consumed the virus-contaminated bee bread (stored pollen), virus infection was monitored in eggs laid by the queen. For the queen to become infected and to lay infected eggs,

workers that attend to the queen and feed her royal jelly from their salivary glands would need to become infected and actively secrete virus in the royal jelly. Any virus infection in the colony workers would have to be via the consumption of the virus-contaminated foods. By the end of week 2, the bee bread was entirely consumed by the workers in all four colonies in the treatment; the frames of honey were consumed by the end of week 3 (Figure 4).

DWV was not detected in egg samples from all 12 colonies for the first week following introduction of the frames of virus-contaminated bee-bread, honey, or “clean” frames (Figure 4). At week two, three out of four colonies fed virus-contaminated bee-bread were found to have queens laying eggs with detectable DWV; in subsequent weeks, the percentage of the egg samples infected with the virus increased in these three colonies. With a delay of one week, a similar pattern was observed in colonies fed with DWV-contaminated honey, with three out of four colonies having eggs positive for DWV. Only one control colony had a few DWV infected eggs by weeks four and five. The percentage of colonies infected with DWV over time was significantly higher in treatments where either contaminated bee bread or honey was fed as compared with controls (two-way ANOVA, treatment  $p < 0.0001$ , time  $p < 0.001$ ; Tukey-Kramer post hoc analysis  $p < 0.05$  for treatments). This indicated that DWV virus in the stored pollen or honey was infectious, even after storage in a pest-free building at ambient outdoor temperature (fluctuating from approximately  $-6^{\circ}\text{C}$  to  $32^{\circ}\text{C}$ ) for six months.

In this experiment, SBV was also monitored in the same samples and from the same cDNAs tested for DWV, as described above. Although initially all colonies and the foods fed to the bees were SBV-free, all 12 colonies had eggs positive for SBV beginning in week 1 (Figure 4), with no significant differences in SBV prevalence among the treatments (ANOVA,  $p = 0.84$ ). This suggested that SBV came into the colonies from an outside source. However, by the end of week 5, SBV was no longer detectable in any of the egg samples from the 12 colonies. Interestingly, these samples with declining prevalence of SBV were eggs in which the DWV



prevalence was increasing (Figure 4). The disappearance of SBV but continued infection by DWV suggested that the queen could selectively clear infection by one virus species while continuing to be infected by another related virus.

### **Are these viruses specific to honey bees or are they widespread in hymenopteran pollinators?**

Eleven non-*Apis* hymenopteran species, collected from flowering plants near the honey bee apiaries, were positive for one or more virus species (DWV, BQCV, SBV, KBV, IAPV) (Table 2). These included three common bumble bee species (*Bombus impatiens*, *B. vagans*, *B. ternarius*), the eastern carpenter bee (*Xylocopa virginica*), the small carpenter bee (*Ceratina dupla*), a sweat bee (*Augochlora pura*), mining bees (*Andrena* sp.), a yellow jacket (*Vespula vulgaris*), paper wasps (*Polistes metricus*, *P. fuscatus*) and sand wasp (*Bembix* sp.). Moreover, IAPV was detected only in non-*Apis* hymenopteran pollinators collected near the apiaries harboring honey bees with IAPV, from Pennsylvania and New York.

### **Does phylogenetic analysis of viral sequences indicate interspecies viral transmission in the hymenopteran pollinator community?**

A comparison of viral sequences from the honey bee foragers, their pollen pellets and from the non-*Apis* hymenopteran pollinators revealed the relationship among viral strains and pollinator species as well as the relationship between each forager and pollen pellet combination. The analysis of samples taken near IAPV-free apiaries was restricted to BQCV and DWV, given the lack of significant number of samples positive for SBV. The IAPV sequences from the non-*Apis* hymenopteran pollinators were also compared to the sequences obtained from honey bees taken from apiaries with IAPV and a known history of CCD symptoms.

Maximum likelihood (ML) phylogenetic trees were inferred for DWV (Figure 5) and BQCV (Figure 6) using a region of genes (see Appendix B for exact location) encoding the structural capsid proteins in the polyprotein for each of these viruses detected in the *Apis mellifera* foragers, their pollen pellets, and other non-*Apis* pollinators. The strength of species-specific association of the viruses was then assessed statistically using the BaTS (Bayesian tip-association significance testing) program. For both DWV and BQCV, there was no obvious clustering of viral sequences by the type of pollinator from which it was taken (Figures 5 & 6); instead, the sequences were intermixed across the trees. Indeed, there was no significant signal for clustering of the virus by host species in the BQCV phylogenetic tree [Association Index (AI),  $P = 0.167$ ; Parsimony Score (PS),  $P = 0.119$ ], such that BQCV strains are no more associated with specific pollinator species than random. DWV, however, had a weakly significant phylogenetic separation by species (AI,  $P = 0.023$ ; PS,  $P = 0.02$ ). This significance in DWV clustering, however, is strongly confounded by collection dates (AI,  $P = 0$ ; PS,  $P = 0$ ), as opposed to an actual species-specific difference. This in part reflects the difference in collection dates for the forager honey bees and the other pollinators, and further suggests that any species-specific clustering in DWV is very weak.

In addition, there was no obvious clustering of sequences of the virus found in the pollen pellets separately from the viral sequences from the pollinators (Figures 5 & 6). This suggests that the virus found in pollen was most likely previously deposited on the flowers by pollinators infected with the virus.

Similarly, for IAPV, an ML phylogenetic tree was inferred using a part of the structural polyprotein for the virus from the *Apis mellifera* specimens taken from apiaries diagnosed with CCD [16,17] and the non-*Apis* pollinators collected from near two of the IAPV-infected apiaries (associated with Operation 3 [16]). The IAPV detected in the non-*Apis* pollinators did not cluster separately from the IAPV in the honey bees in nearby apiaries (Figure 7) (AI,  $P = 0.083$ ; PS,  $P =$

1). Interestingly, the viral sequences from non-*Apis* hymenopteran pollinators collected from the two apiaries segregated by sampling location, with the non-*Apis* hymenopteran pollinators from each site possessing a clearly phylogenetically distinct lineage of IAPV.

## **Discussion**

### **Is the forager transferring the virus into the pollen pellet that she carries?**

We report in this paper the first molecular detection of DWV, BQCV and SBV in the pollen loads directly taken from the pollen baskets of forager honey bees. A high percentage of tested pollen loads were positive for these viruses, especially for DWV. Similarly, one of three analyzed pollen pellets from non-*Apis* hymenopteran pollinators had DWV. These data suggest that pollen can be frequently associated with RNA viruses that infect bees and that the virus detected in the bee bread or pollen stores in the hive is in part due to virus associated with the pollen itself.

Overall, there was significant variation in the distribution of different virus species in foragers and their corresponding pollen loads. While BQCV was the most prevalent virus in the honey bee foragers, comparatively fewer pollen loads carried the virus. Conversely, the incidence of DWV was equally prevalent in foragers and their pollen loads. These differences in prevalence rates suggested that the different viruses differ in their viral ecology in this environment, either in different infection rates among the pollinators or in their transfer to the pollen. Given that one third of all pollen pellets from honey bee pollen foragers were free of detectable virus in spite of all foragers being infected, these viruses may not be frequently transferred to the pollen loads by pollen foragers. Instead, a different group of pollinators may be responsible for contamination of pollen. DWV was in fact the most prevalent virus in the wild pollinators, which may help explain the increased detection of DWV in pollen pellets of honey bee foragers. The viral ecology and infection dynamics in pollinators demand further study.

The high prevalence of BQCV and DWV in honey bees in our study was consistent with results from other virus surveys in the US [10,15]. Notably, multiple virus species were found co-infecting non-*Apis* hymenopteran pollinators as well as honey bees in this study, which corroborate many reports of multiple viral co-infections in honey bees throughout the world [15,18,56,57]. This is especially important since multiple viruses have been found associated with CCD, without one viral agent or other pathogen being linked to CCD by itself [57-59]. In addition, multiple viruses were detected in the pollen loads, although the percentage of pollen loads with multiple virus species was significantly less compared to the foragers.

Perhaps the most important observation was the detection of DWV and SBV in pollen loads of uninfected foragers suggesting that some foragers were bringing in virus from outside and thereby directly implicating pollen as a source of virus infection for healthy colonies. We did not find any uninfected foragers bringing in BQCV in pollen loads, since the incidence of this virus was very high in foragers (almost 100%). Previous research on plant viruses demonstrated that, by moving pollen from plant to plant, honey bees play an important role in the transmission of some pollen-borne plant viruses [60,61], which complements our findings. The implication of our finding is that pollinators may become infected with a new virus introduced to the environment by another species, mediated by plant pollen.

How the virus becomes associated with the pollen is not known. Historically, it was assumed that the forager collects the pollen and, through the addition of salivary secretions to moisten the pollen, she molds the pollen into a pellet to pack it into her pollen basket [62]. Previous studies have reported the detection of some of these RNA virus species in the thoracic and hypopharyngeal salivary glands of honey bees [24,31]. This is further supported by detection of several of these viruses in the colony foods including honey, pollen and royal jelly [28,29]. Our data indicate it is unlikely that the salivary secretions of the foragers transfer the virus to the pollen given three findings: 1) pollen pellets can be found to have virus without the forager

herself having detectable virus, 2) not all the pollen pellets from infected foragers had virus, and 3) only a small fraction of the viral sequences matched between a forager and her pollen pellet, in the pairs with detectable virus.

Alternatively, pollen may be contaminated with virus via other means. One of the potential routes is via random deposition of feces from infected insects on flowers. This alternative is supported by reports of detection of several viruses in the honey bee feces- CBPV [63], KBV [33], DWV & BQCV [32] and most recently IAPV (Singh et al, unpublished). Honey bees and bumble bees are known to defecate in the field while foraging. The role of the digestive tract in virus transmission is supported by detection of significantly higher virus titers in the digestive tracts of honey bees as compared to other tissues [32]. Also the infectivity of virus from bee feces has been proven, both by injecting healthy bees with virus particles obtained from feces of infected bees and more importantly, by keeping naive bees in the feces-soiled environment [64]. Most studies have implicated virus in the fecal matter as one of the routes of horizontal transmission in the honey bee hives, but this could also be the mechanism underlying the inter- and intra-species virus transmission via pollen.

It is unknown how long these ssRNA viruses can survive on flowers under harsh environmental conditions including UV radiation, high temperature and desiccation. On the other hand, for inter-taxa virus transmission to take place, viruses may not need to survive for long periods, as the interactions between pollinators on flowers can be quite intense and frequent, especially during full bloom.

### **What is the nature of the association of viruses with the pollen itself?**

The pollen that tested positive for the virus was not associated with any particular plant species. Rather, the virus-positive pollen pellets belonged to many plant species including goldenrods, thistles, clovers and common burdock. These pollen sources represent a diverse

group, suggesting no restriction by the plant taxonomy on the viral association with the plant. Determining the diversity of plants and the timing of the association with the pollen is important in understanding if the plant plays any role in this viral transmission route in the pollinator community.

Potentially some of the plants may be serving as the reservoirs of these viruses. Detection of viral RNA in the supernatant from the first two pollen washings (washings with Trizol or PBS and SDS solutions) and then pollen homogenate after a fourth washing suggests that either the virus particles were present both on the surface of pollen grains as well as inside the pollen grains or that they were tightly bound to the pollen exine. For pollen-vectored plant viruses, the virions can be located both inside and outside the pollen grains [65,66]. The order Picornavirales, to which these picorna-like viruses belong, is known to contain viruses infecting plants and animals, including humans [67,68]. Recently these viruses were found to have different patterns of dinucleotide bias dependent upon their host (insect, plant, or mammal) [69]; although, some of the insect and plant viruses did not cleanly differ in their dinucleotide bias, suggesting that there is potential for some viruses to infect both insects and plants. A dicistrovirus that infects aphids can also become associated with and persist in plant phloem cells [70].

### **Is the virus associated with pollen or honey infective?**

The viruses detected in the food stores of the honey bees were found to be infective even after being kept at ambient temperature for several months. In the bee bread, the pollen pellets are packed into separate layers, with different pollen sources being found in differently colored layers. Different viruses can be detected in different layers (data not shown), indicating that viruses associated with each layer were present in the forager-collected pollen pellets.

Despite the environmental exposure of the virus to sun and desiccation, these viruses remained infective when fed to honey bee colonies. Environmental stability after exposure to

sunlight, desiccation, temperature fluctuation, and microbial degradation has been reported for other picornaviruses, such as poliovirus in polluted water and for foot and mouth virus transmitted by wind currents [71-73]. Pollen-borne plant viruses vectored by honey bees during pollination are known to remain infective for several weeks after being stored in the bee bread in the hive [74]. It is not known what the infective period is for the picornaviruses detected in pollen and honey, especially under different conditions. This would be valuable information for beekeepers and aide in understanding viral disease dynamics.

**Are these viruses specific to honey bees or are they widespread in non-*Apis* hymenopteran pollinators?**

Knowledge of the degree of host specificity is important to the understanding of pathogen transmission dynamics. Detection of one or more RNA viruses from as many as 11 non-*Apis* hymenopteran species demonstrated that these picornaviruses are widely distributed in the pollinators and are not specific to honey bees or their close relatives, given that each of these viral-species effectively represents a single genetic population. Understanding disease dynamics and tracking outbreaks requires broadening consideration to the community level instead of solely focusing on individual host-pathogen interactions.

For DWV and BQCV, our data indicate that there are not distinct segregations of the viral populations among the pollinators in the temporal and spatial confines of the study. Even when the DWV and BQCV sequences from *Polistes* wasps in Illinois are compared to the viral sequences from pollinators and pollen in Pennsylvania, there is no significant segregation. This suggests that the same viral strains are circulating amongst these diverse species. For other viral diseases in animals, this lack of segregation in phylogenetic analysis of viral sequences taken from different host species has indicated that the cellular mechanisms regulating infection are not highly constrained among these hosts [75]. The honey bees and non-*Apis* hymenopteran

pollinators may have similar viral receptors, permitting each to become infected with these picorna-like viruses. This is not common among all insects, since restrictive host range for other dicistroviruses has been observed in widely separate insect taxa [76].

Importantly, IAPV was detected only in non-*Apis* hymenopteran pollinators collected near IAPV-infected apiaries in New York and Pennsylvania. None of the non-*Apis* hymenopteran pollinators collected from flowering plants around State College, Pennsylvania, where IAPV was not detected in 2007 in honey bees, tested positive for this virus. IAPV may be spreading into non-*Apis* hymenopteran pollinators from honey bees. Alternatively, this virus may have spilled over from some wild species, which may be serving as the reservoir host for this virus, into honey bees. Moreover, results from our greenhouse experiment on interspecies transmission also indicate that this virus does not have any directionality at least between honey bees and bumble bees in a controlled environment.

### **Does pollen play any role in inter-taxa transmission?**

The role of pollen in interspecies virus transmission is supported by phylogenetic analysis. Since there was no clustering of any virus according to whether it was isolated from honey bees, wild pollinators or from pollen, it is highly likely that the same viral strains are freely circulating in different hymenopteran pollinators and that pollen serves as a mediator of viral transmission. The predominant mismatch in sequences of viruses in the forager/pollen pairs also strongly suggests that most foragers were carrying pollen loads with virus originating from other individuals.

Importantly, pollen can have a role in virus transmission among pollinators without the reservoir-host species being in the same locale. More than 200 tons of honeybee-collected and preferably freshly-frozen pollen is used annually for bumble bee rearing worldwide [77]. This same concern may also extend over to honey bees, since many beekeepers purchase pollen to feed



their bees. Pollen has been successfully gamma irradiated without destroying nutritional and physical properties [78], and this practice should be encouraged to prevent introduction of new strains of viruses and other pathogens, irrespective of the source of the pollen.

In conclusion, we propose that pollen serves as one of the major routes of inter-taxa virus transmission in the pollinator community. This is supported by Bailey's report [62] of the presence of SBV and CBPV in the pollen loads of honey bees and presence of ABPV in pollen loads of both honey bees and bumble bees. The dynamics of this viral transmission route via pollen need to be further defined to understand how the multiple viruses move from one species to another, and to determine if pollen and its plant have a greater role than just as a physical carrier of these viruses.

Our finding that RNA viruses have a broad host range and are freely circulating in hymenopteran pollinators has important implications on export/import and movement of managed pollinators that may bring in new or more virulent strains of existing pathogens into the environment, with the potential for deeper impact on our agro-ecosystems and natural ecosystems. Further research is needed to study the impacts of these viruses on specific species of non-*Apis* hymenopteran pollinators and to determine if IAPV or other viruses are linked to additional pollinator decline. The present study, along with the recent lessons learned from dramatic honey bee losses, emphasizes the immediate need to promote honey bee health, encourage use of native pollinators, and focus on the disease dynamics of pollinator community as a whole. The role of diseases in overall pollinator decline demands additional attention.

### **Acknowledgements**

The help of Amanda Mahoney in processing samples is greatly appreciated. Rob Anderson and Jeremy Fitzgerald both helped in collection of samples. We appreciate the help of the Penn State Genomic Core Facility at University Park campus in obtaining sequences. We

appreciate the help of Rick Donvall of the Pennsylvania Department of Agriculture in making species identifications. The Bio414 Plant Taxonomy class at Penn State was helpful in plant identification and in making a pollen reference collection.

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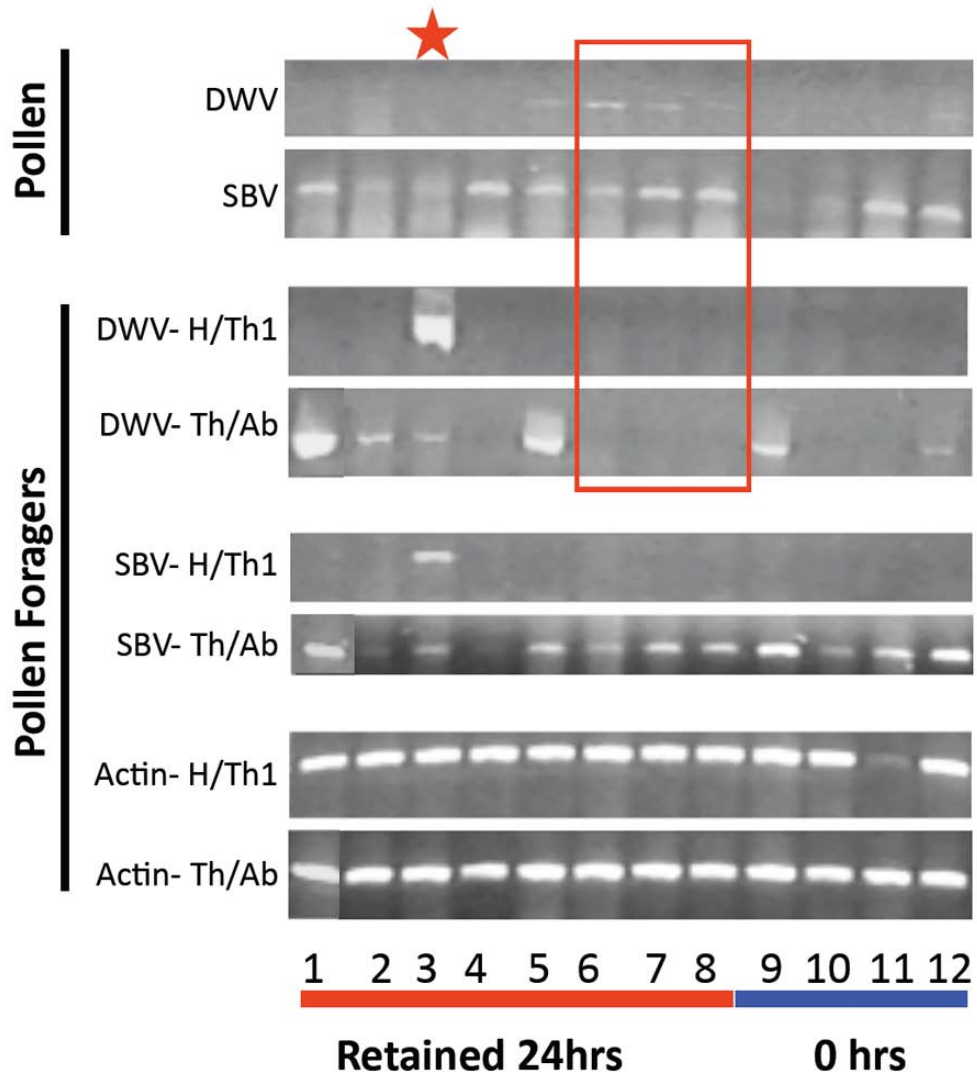
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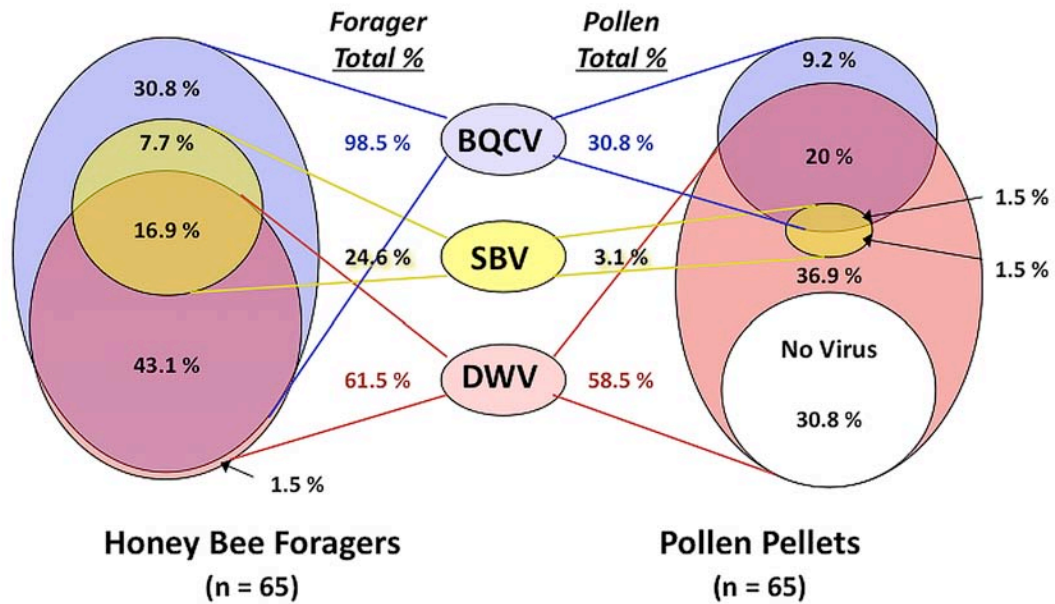
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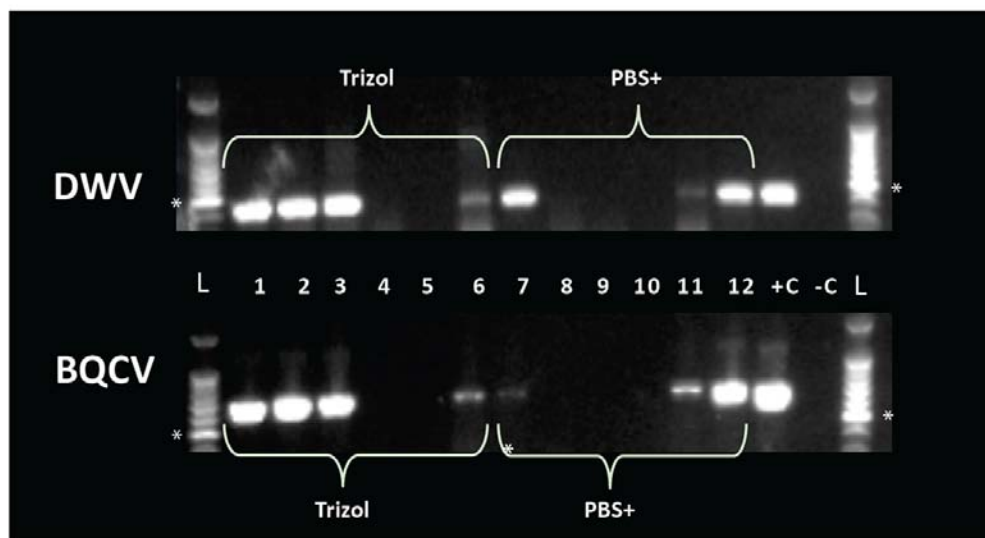


**Figure 1: Comparison of viral presence in pollen pellets and their corresponding forager with her body dissected into two regions with or without salivary glands, to determine if salivary secretions of the forager are associated with virus in pollen pellets.** Incoming foragers with pollen pellets were collected in 2005; pollen pellets removed, tagged with identifier, and frozen at -80 °C. Some foragers (1-8) were kept for 24 hrs at 34°C, 50% relative humidity and fed sugar water; others (9-12) were frozen immediately upon collection. After freezing, all foragers were divided into two regions, head plus prothorax that have salivary glands (**H/T1**) and

the remainder of body lacking salivary glands (**T2,3/A**). Pollen pellets and forager body regions were extracted for detection of deformed wing virus (DWV), sacbrood virus (SBV), and actin mRNA (forager only). Actin mRNA was used as an internal control for methods and loading. Red box indicates three foragers that lack detections of DWV but had pollen pellets with detectable DWV. The red star (lane 3) indicates a forager with heavy DWV infection in Head/Prothorax but no detectable DWV in her pollen pellets. Size of DWV reaction = 424 bp, SBV reaction = 693 bp and Actin reaction = 514 bp.

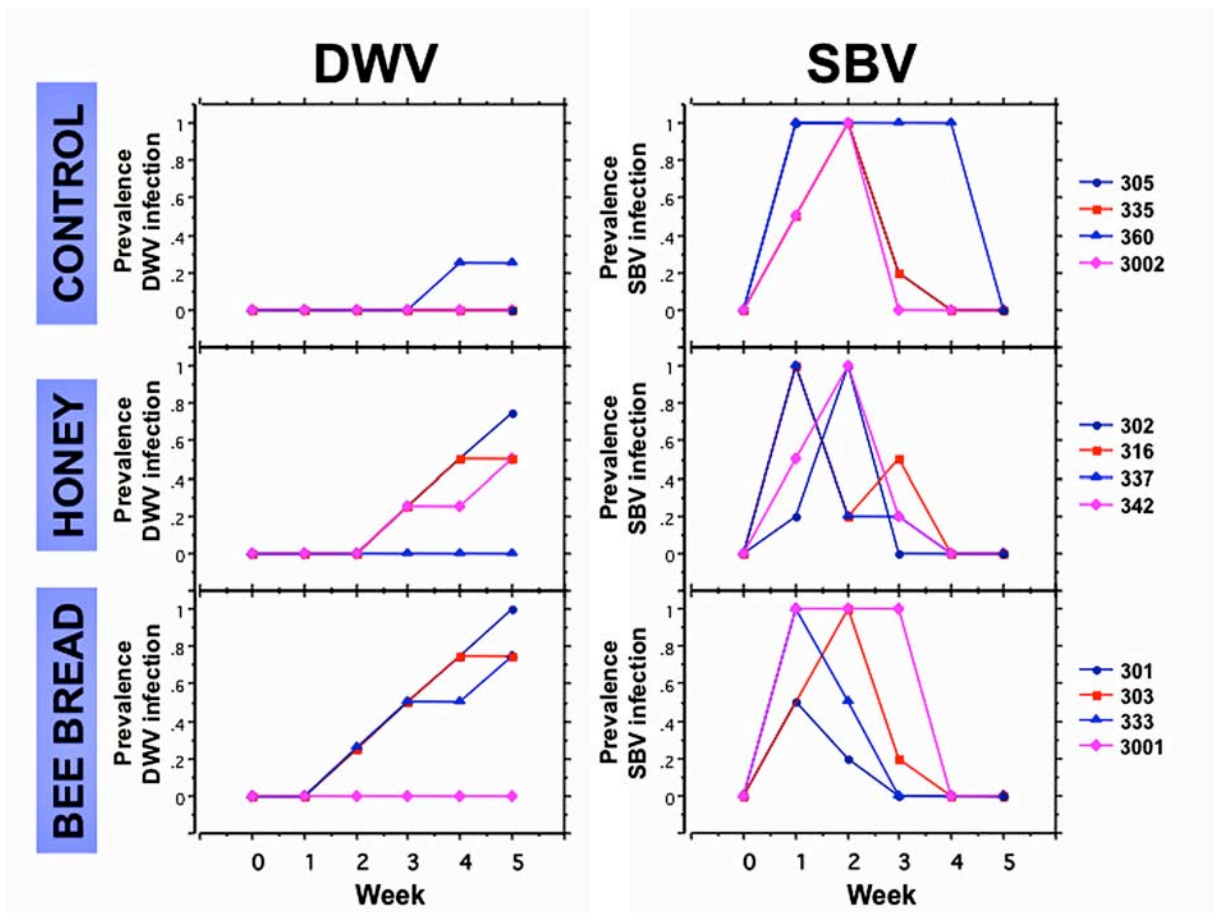


**Figure 2: Proportion of virus species detected in 65 honey bee pollen foragers versus their pollen pellets.** For both the foragers and their pollen pellets, Venn diagrams depict the percentage of DWV (Deformed wing virus in red), SBV (Sacbrood virus in yellow), BQCV (Black queen cell virus in blue), or virus-free samples (white). Overlapping colored circles indicate samples wherein more than one virus was detected. Total percentages of these viruses in either foragers or pollen pellets are given in the middle of the figure. N=sample size.

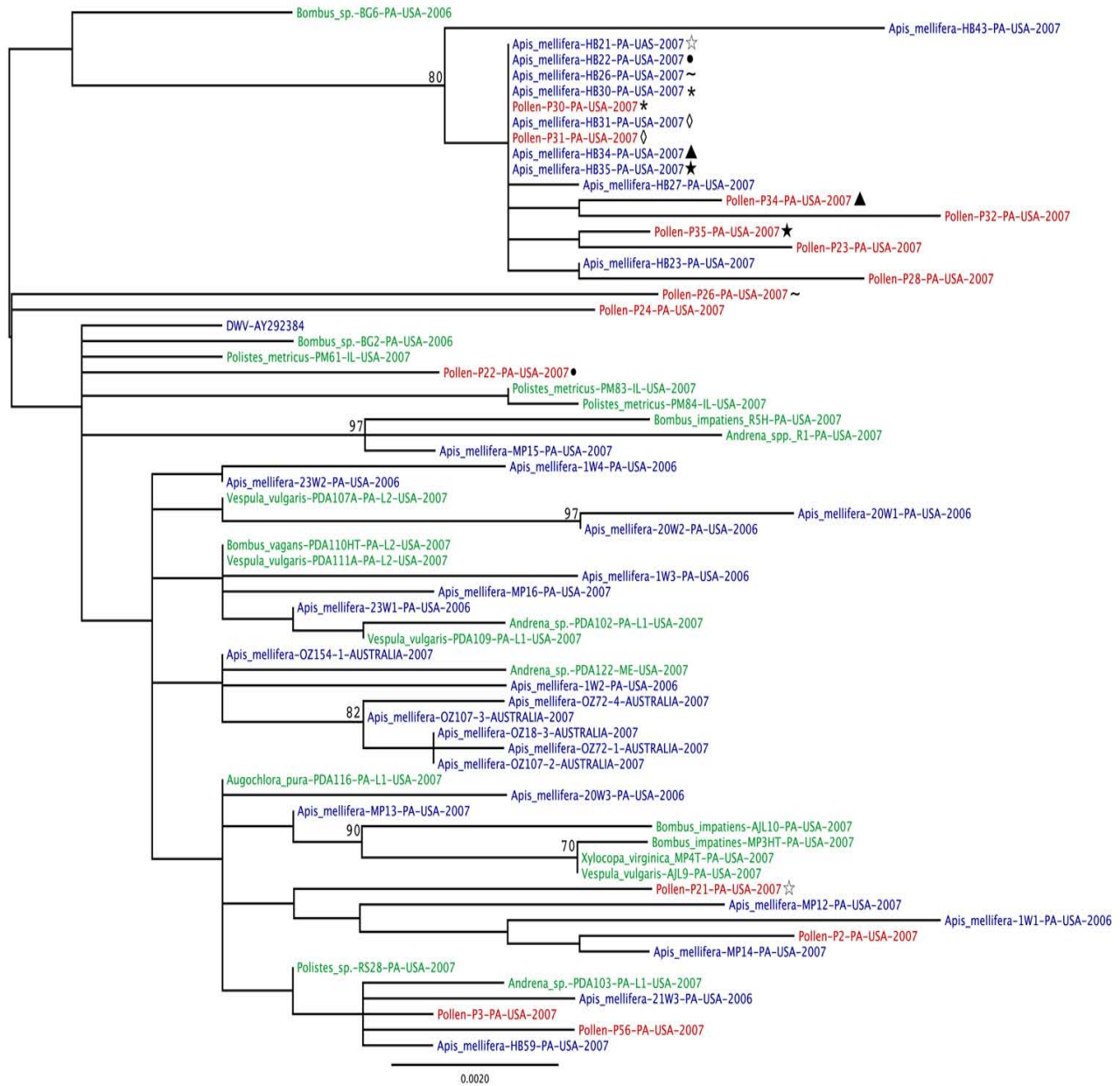


lane #	Treatments	lane #	Treatments
1	Direct homogenization in Trizol	7	PBS+ washing 1
2	Trizol washing 1	8	PBS + washing 2
3	Trizol washing 2	9	PBS + washing 3
4	Trizol washing 3	10	SDS washing
5	Trizol washing 4	11	Homogenization of pellet
6	Homogenization of pellet in Trizol	12	Direct homogenization in PBS +
-C	Negative control	+C	Positive control

**Figure 3: Determination of how DWV and BQCV are associated with pollen.** Virus was detected in extracts of supernatants and homogenates after various washings of an aliquot of combined pollen pellets, in either Trizol or PBS+ (1M PBS, 0.05% Tween, 2% Polyvinylpyrrolodone) followed by SDS (Sodium Dodecyl Sulfate). Standard size ladders (L) are shown at beginning and end of gel images. Size marker of 500 bp is indicated by \* on the ladder. Lane loadings are indicated below the gel image. Size of DWV reaction = 424 bp and BQCV reaction = 700 bp.



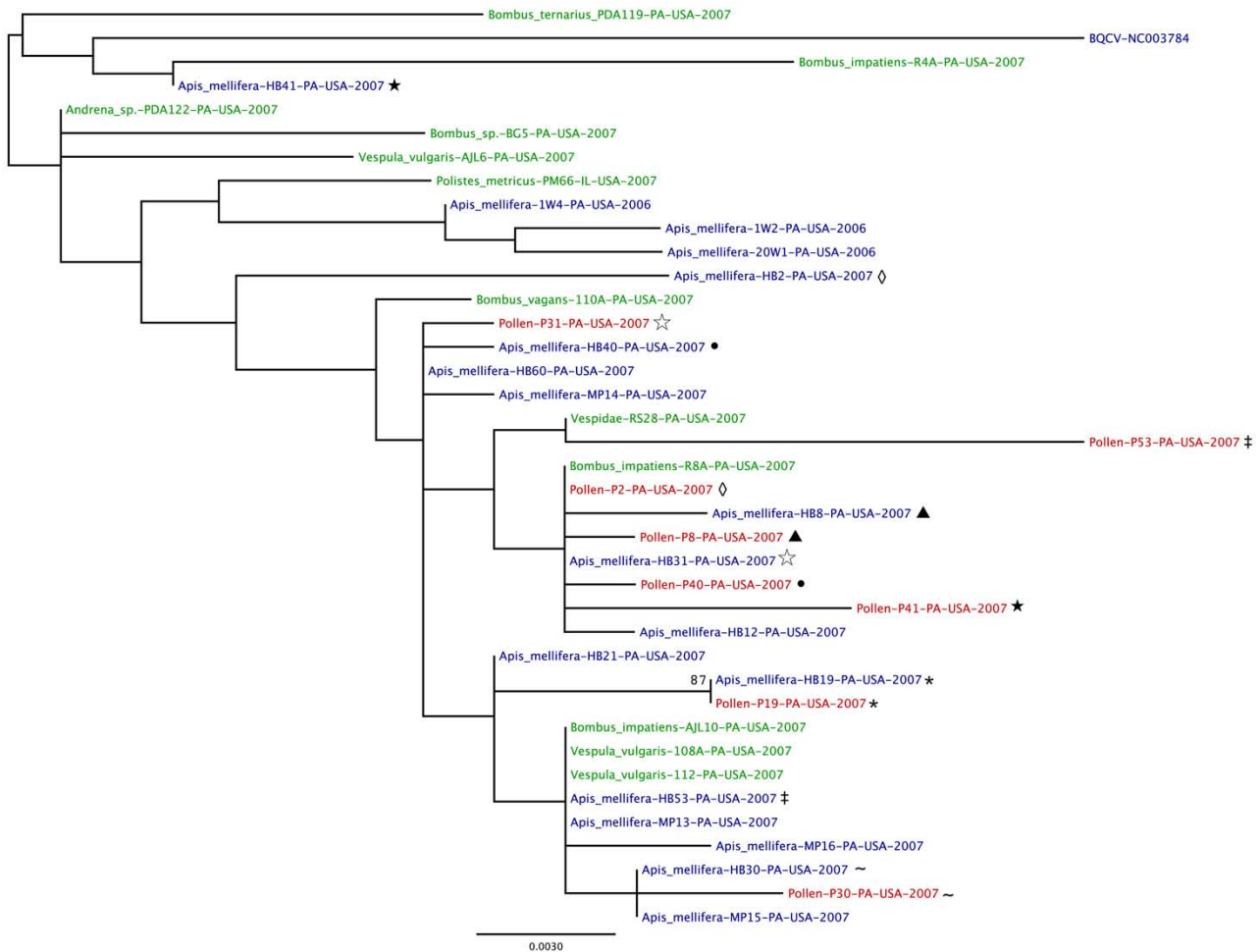
**Figure 4: Test of DWV infectivity in stored pollen and honey and detection of SBV movement from outside source through transmission of DWV and SBV to eggs by queens.** DWV- and SBV-free colonies were installed into new equipment in an isolated apiary near State College, Pennsylvania in the spring of 2005. Colonies in the **Control** treatment were each fed sugar water and artificial pollen, plus given a washed frame. In the **Bee Bread** or stored pollen treatment, colonies were each given a frame of bee bread with detectable DWV and sugar water. In the **Honey** treatment, colonies were each given a frame of capped honey with detectable DWV and artificial pollen diet. No SBV was detected in the workers or eggs from the queens in the colonies or the frames of honey or stored pollen prior to experiment. Egg samples (4 samples of 5 eggs each; 20 eggs total per colony) were collected weekly from each of four colonies (colony numbers listed on right of figure) in three treatment groups, at time of feeding and for five additional weeks. Eggs were extracted and used for detection of DWV, SBV and actin mRNA (present in 100% samples, not shown).



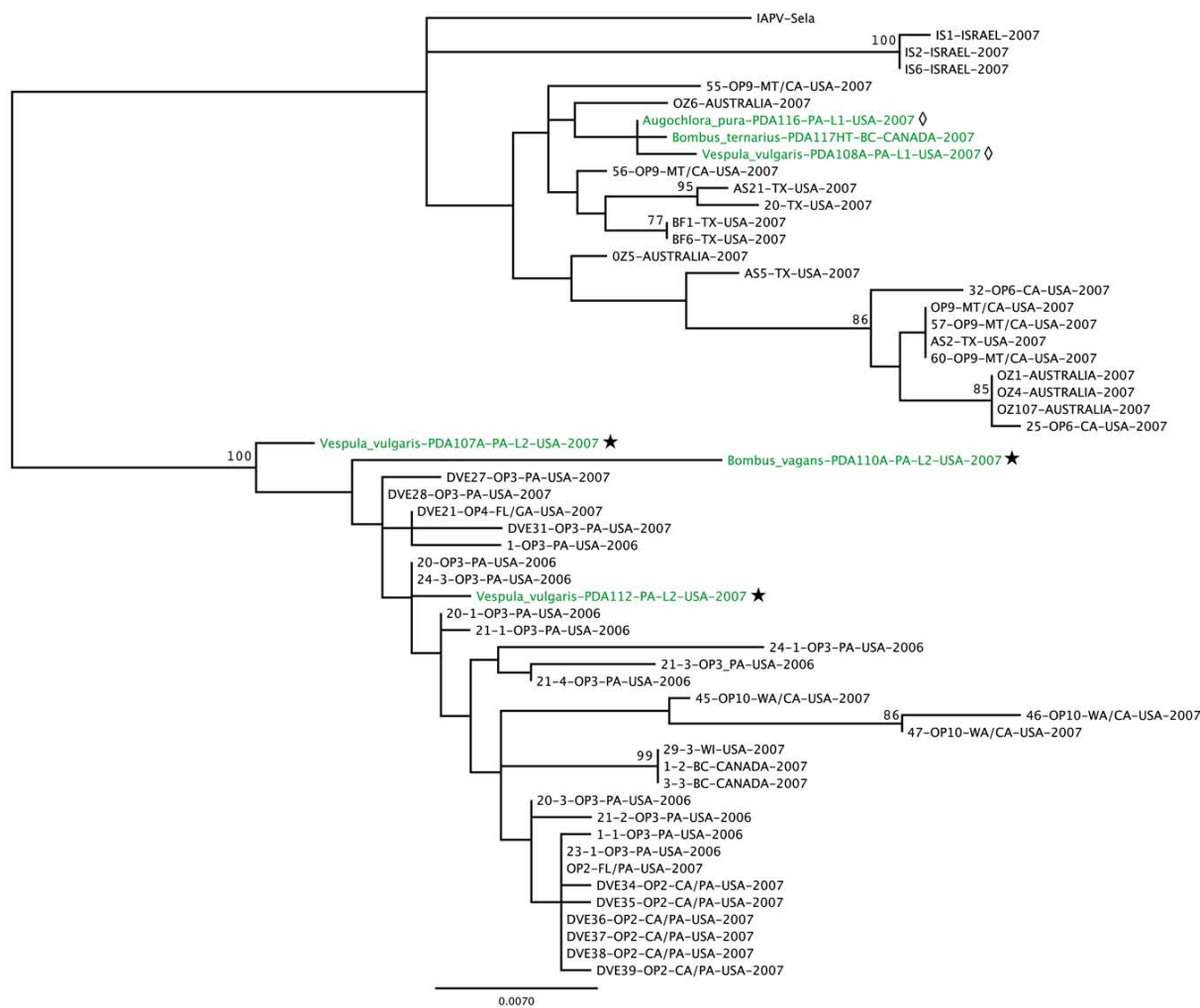
**Figure 5: Phylogenetic comparison of DWV sequences detected in honey bee foragers, pollen pellets, and non-*Apis* hymenopteran species.** An unrooted maximum likelihood phylogenetic tree of DWV (based on 1230-nt from the capsid) was generated using a region of the structural proteins of the virus. The support for the indicated branching topology was



evaluated by using bootstrap re-sampling of the sequences 1,000 times. Nodes supported by bootstrap values over 70 are given. Strains are annotated by genus, species, identification-label, country of isolation, and year of isolation. Blue= virus from honey bee, red= virus from pollen pellet, and green= virus from non-*Apis* hymenopteran species. Forager/pollen pellet pairs are indicated by common symbols following the sample label (●, ‡, ◇, ★, ▲, ☆).



**Figure 6: Phylogenetic comparison of BQCV sequences detected in honey bee foragers, pollen pellets, and non-*Apis* hymenopteran species.** An unrooted maximum likelihood phylogenetic tree of BQCV (based on 687-nt from Capsid/ 3'UTR) was generated using a region of the structural proteins of the virus. The support for the indicated branching topology was evaluated by using bootstrap re-sampling of the sequences 1,000 times. Nodes supported by bootstrap values over 70 are given. Strains were annotated by genus, species, identification label, country of isolation and year of isolation. Blue= virus from honey bee, red= virus from pollen pellet, and green= virus from non-*Apis* hymenopteran species. Forager/pollen pellet pairs are indicated by common symbols following the sample label (●, ‡, ◇, ★, ▲, ☆, ~).



**Figure 7: Phylogenetic comparison of IAPV sequences detected in honeybees and non-*Apis* hymenopteran species collected near IAPV(+) apiaries.** An unrooted maximum likelihood phylogenetic tree of IAPV (based on 771-nt for capsid region) was generated using a region of the structural proteins of the virus. The support for the indicated branching topology was evaluated by using bootstrap re-sampling of the sequences 1,000 times. Nodes supported by bootstrap values over 70 are given. Strains were annotated by genus, species, identification label, country of isolation and year of isolation. Green= virus from non-*Apis* hymenopteran pollinators; Black = virus sequences from original isolation and honey bees from CCD-affected operations

[16]. Non-*Apis* hymenopteran species collected from same local are indicated by common symbol ( $\diamond$ ,  $\star$ ).

**Table 1: Prevalence of RNA viruses in honey bee foragers and their corresponding pollen pellets collected from multiple hives and apiaries in central Pennsylvania from June to September 2007.**

<b><u>VIRUS</u></b> <b>DWV</b>	<b>% Infected Foragers</b>	<b>% Non-Infected Foragers</b>	<b>Total % Pollen Pellets</b>
<b>% Pollen Pellets (+) Virus</b>	44.6 (29)*	13.9 (9)	58.5 (38)
<b>% Pollen Pellets (-) Virus</b>	16.9 (11)	24.6 (16)	41.5 (27)
<b>Total % Foragers</b>	61.5 (40)	38.5 (25)	

<b>SBV</b>	<b>% Infected Foragers</b>	<b>% Non-Infected Foragers</b>	<b>Total % Pollen Pellets</b>
<b>% Pollen Pellets (+) Virus</b>	1.6 (1)	1.5 (1)	3.1 (2)
<b>% Pollen Pellets (-) Virus</b>	23.0 (15)	73.9 (48)	96.9 (63)
<b>Total % Foragers</b>	24.6 (16)	75.4 (49)	

<b>BQCV</b>	<b>% Infected Foragers</b>	<b>% Non-Infected Foragers</b>	<b>Total % Pollen Pellets</b>
<b>% Pollen Pellets (+) Virus</b>	30.8 (20)	0 (0)	30.8 (20)
<b>% Pollen Pellets (-) Virus</b>	67.7 (44)	1.5 (1)	69.2 (45)
<b>Total % Foragers</b>	98.5 (64)	1.5 (1)	

DWV: Deformed wing virus

SBV: Sacbrood virus

BQCV: Black queen cell virus

\* Number of individual foragers and their pollen pellets in category are indicated in parentheses

+ Positive for virus

- Negative for virus

**Table 2: RNA viruses detected in non-*Apis* hymenopteran species collected from flowering plants in Pennsylvania, New York and Illinois from May to October 2007.**

Collected near IAPV(-) honey bee apiaries in PA						
Species	IAPV	DWV	BQCV	SBV	KBV	Co-Infections*
<i>Andrena</i> sp. Mining bees (n=4)	-	+	-	-	-	
		(2)				
<i>Bembix</i> sp. Sand wasps (n=2)	-	+	+	-	-	DWV+BQCV
		(1)	(1)			
<i>Bombus impatiens</i> Eastern bumble bee (n=5)	-	+	+	-	-	DWV+BQCV
		(5)	(3)			
<i>Bombus</i> sp. Bumble bees (n=3)	-	+	+	+	+	DWV+BQCV+ SBV+KBV
		(3)	(1)	(3)	(3)	
<i>Ceratina dupla</i> Small carpenter bee (n=1)	-	+	-	-	-	
		(1)				
<i>Vespula vulgaris</i> Yellowjacket wasp (n=5)	-	+	+	-	-	DWV+BQCV
		(4)	(2)			
<i>Xylocopa virginica</i> Eastern carpenter bee (n=4)	-	+	+	-	-	DWV+BQCV
		(3)	(2)			

Collected near IAPV(+) honey bee apiaries in PA and NY						
Species	IAPV	DWV	BQCV	SBV	KBV	Co-Infections*
<i>Andrena</i> sp. Mining bees (n=4)	-	+	+	+	-	DWV+BQCV+SBV
		(3)	(1)	(1)		
<i>Augochlora pura</i> Sweat bee (n=1)	+	+	-	-	-	IAPV+DWV
	(1)	(1)				
<i>Bombus ternarius</i> Tricolored bumble bee (n=2)	+	+	+	+	-	IAPV+DWV+SBV
	(2)	(2)	(1)	(1)		
<i>Bombus vagans</i> Bumble bee (n=1)	+	+	+	+	-	IAPV+DWV+SBV+BQCV
	(1)	(1)	(1)	(1)		
<i>Ceratina dupla</i> Small carpenter bee (n=2)	-	+	-	-	-	
		(1)				
<i>Polistes fuscatus</i> Paper wasp (n=6)	-	+	-	-	-	
		(1)				
<i>Vespula vulgaris</i> Yellowjacket wasp (n=7)	+	+	+	+	-	IAPV+DWV+SBV+BQCV
	(5)	(7)	(6)	(4)		

Collected in IL						
Species	IAPV	DWV	BQCV	SBV	KBV	Co-Infections*
<i>Polistes metricus</i> Paper wasp (n=8)	-	+	+	+	-	DWV+SBV or SBV+BQCV
		(4)	(2)	(4)		

IAPV: Israeli acute paralysis virus

SBV: Sacbrood virus

KBV: Kashmir bee virus

NY: state of New York, USA

CCD: Colony collapse disorder

- Negative for virus

\* Represented by the individual detected with maximum number of co-infecting viruses

DWV: Deformed wing virus

BQCV: Black queen cell virus

PA: state of Pennsylvania, USA

IL: state of Illinois, USA

n= Total number of individuals tested

+ Positive for virus, (# of samples with virus)

### Chapter 3

## **Prevalence of RNA viruses in the commercial greenhouse bumble bee (*Bombus impatiens*), and the pathology, health impacts and transmission of Israeli acute paralysis virus (IAPV) in bumble bees and honey bees**

### **Abstract**

Widespread declines in bee populations have become a major cause of concern. RNA viruses, including recently described Israeli acute paralysis virus (IAPV), are linked to honey bee (*Apis mellifera*) losses worldwide; however, very little is known about the viral diseases in other managed bees. This study investigates the ecology and epidemiology of RNA viruses in the common eastern bumble bee, *Bombus impatiens*, the major pollinator of commercial greenhouse crops in the US, with the main focus on IAPV. Greenhouse experiments involving the commercial bumble and honey bee colonies were conducted to study the health impacts, pathology and inter-taxa transmission dynamics of IAPV. RNA viruses were prevalent in the newly purchased, commercial colonies of the common eastern bumble bee, with DWV being the most common virus. IAPV can potentially spread in bumble bees through various horizontal and vertical transmission routes. IAPV moved from infected honey bees to IAPV-free bumble bees and from infected bumble bees to honey bees within a week to 10 days, when bees were allowed to forage together on common flowering plants. There was no species barrier and directionality involved in the movement of RNA viruses, at least between honey bees and bumble bees. Weeks after feeding IAPV to bumble bee queens, the virus was detected in different tissues besides the mid-gut, indicating virus dissemination throughout the queen bee's body. The colony survival and the foraging activity of IAPV-infected bumble bees were lower than the control colonies. IAPV also resulted in heavy adult mortality and poor colony build up in infected honey bees. Overall, IAPV negatively impacted the colony health of both bumble bees and honey bees under



greenhouse conditions. The role of the IAPV and other RNA viruses needs to be studied in the recent declines in field populations of these pollinators.

## Introduction

Domestication of pollinators has revolutionized the production of numerous fruits, vegetables and field crops around the world [1]. The managed honey bee, *Apis mellifera*, has become an integral part of modern agriculture. Apart from the honey bee, bumble bees (*Bombus spp.*) are the predominant pollinator for a large number of wild native plants and agricultural crops, and yields of many field, fruit and seed crops are enhanced by their visitations [2,3]. Characteristics such as robust bodies, buzz-pollination behavior and ability to forage under low temperature and/or light conditions make bumble bees efficient and reliable pollinators both in the open fields as well as under greenhouse conditions. Commercial rearing of bumble bees has expanded over the last two decades with annual production of over a million colonies with estimated value of around \$80 million [4]. Bumble bee colonies are used extensively for pollination of high-value crops such as greenhouse tomatoes (*Solanum lycopersicum* L.) and peppers (*Capsicum annuum* L.) worth more than \$17 billion, and a variety of other fruits and vegetables. Among the five species of bumble bees under commercially production worldwide, the common eastern bumble bee, *Bombus impatiens* and the buff-tailed bumble bee, *Bombus terrestris* are the two major species reared in North America and Eurasia, respectively.

The widespread declines in pollinators, particularly managed honey bees, have raised serious concerns over the stability of global food production and natural plant communities [5-8]. Large-scale losses in honey bee (*Apis mellifera*) populations have been reported across the United States and several other countries in Europe and Middle East, attributed to a variety of factors including parasitic mite *Varroa destructor*, RNA viruses, pesticides and the microsporidians *Nosema ceranae* and *N. bombi* [9-11]. The honey bee losses, particularly in the

U.S. over the last five years, have been partially assigned to a syndrome named colony collapse disorder (CCD), which involves rapid loss of adult worker bees [11,12]. Epizootiological and metagenomic studies have revealed higher pathogen loads and co-infections in bees from CCD colonies, suggesting either an increased exposure to pathogens or a compromised ability of bees to fight diseases as a result of other stress factors such as pesticides, poor nutrition, etc. [13-15]. In addition, several once-common native bumble bee species have either locally disappeared or are declining across North America and Europe [16-18]. Bumble bee declines have been attributed to habitat degradation and fragmentation, pesticides, low genetic diversity and spread of parasites and pathogens [17]. A recent US study has revealed 96% reduction in abundance and up to 87% reduction in geographic ranges of four bumble bee species over the last few decades [19]. The declining populations had significantly higher infection levels of microsporidian pathogen (*Nosema bombi*) and lower genetic diversity as compared to populations of co-occurring stable species. Several other studies have also suggested the role of invasive pathogens and parasites in the decline of wild bumble bees and their impact on the commercial use of bumble bees, especially in the wake of pathogen spillover from commercial greenhouse bumble bees to wild populations [20-22].

Lately, the RNA viruses have become a major problem for bees, with more than 18 viruses plaguing honey bee populations around the world [23,24]. These obligate intracellular pathogens usually persist or co-exist with honey bees as asymptomatic covert infections and are transmitted both horizontally and vertically [25,26]. However, they can cause severe symptoms at the individual bee level and inflict heavy colony losses, especially in combination with a parasitic mite *V. Destructor* and other environmental factors that suppress the bee immune system [27-29]. Most of these viruses have a monopartite, positive-sense single-stranded RNA genome and have been categorized under the families *Dicistroviridae* and *Iflaviridae*, order Picornavirales, except the chronic bee paralysis virus (CBPV) and two newly discovered viruses that have a multipartite

genome organization and has not yet been assigned to any family or genus [24,30-32]. These so-called ‘honey bee viruses’ have been recently found to be far more widespread than the genus *Apis* [33]. At least six RNA viruses commonly found in honey bees have also been reported in bumble bees from across Europe and the United States: deformed wing virus (DWV), acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), black queen cell virus (BQCV), sac brood virus (SBV) and Israeli acute paralysis virus (IAPV) [33-36]. However, the impact of these viruses on the health and survival of bumble bees and the related pathology is largely unknown. So far, only DWV has been associated with any clinical symptoms in bumble bees [35].

Israeli acute paralysis virus (IAPV) has been found in many samples taken from honey bee colonies exhibiting CCD-like symptoms, although it is not believed to be the sole cause of CCD [12,13]. The causal relationship between IAPV and the pathogenesis of the disorder remains unknown; nonetheless, the capacity of IAPV to cause paralytic symptoms and adult mortality in honey bees has been demonstrated experimentally [37,38]. IAPV was first isolated and characterized from honey bees in Israel in 2002 [37]. Later, IAPV was also detected sporadically in honey bees from other parts of the world [33,39-41]. IAPV is a part of complex of closely related viruses from the family *Dicistroviridae*, which also includes KBV and ABPV. The RNA genome of these viruses is organized into two open reading frames (ORFs), separated by an intergenic region and flanked by non-translated regions [42]. These viruses are rapidly lethal to honey bee adults at elevated levels, and among these at least ABPV and KBV have been frequently implicated in colony losses in the past [43].

Due to its rather recent discovery, very little is known about the host range, geographical distribution, pathology and the epidemiology of IAPV. In fact, the basic physiology and molecular mechanisms underlying the pathology and the colony level impacts of most of the RNA viruses are poorly understood. The lack of any established bee cell line, and the difficulty in obtaining virus-free bee colonies and pure single-virus inoculum add to the complications in

experimental studies with RNA viruses. IAPV, being a relatively new virus, provides an opportunity to conduct such experimental studies, since IAPV-free honey bee and bumble bee colonies can be obtained. The rapid adult mortality associated with IAPV is especially interesting in the wake of CCD, which is characterized by the sudden disappearance of adult workers from the colony [11].

This study investigates the ecology and epidemiology of RNA viruses in the common eastern bumble bee, *B. impatiens*, with a major focus to understand the health impacts, pathology and inter-taxa transmission dynamics of IAPV. It involves a series of colony-level trials with commercial bumble bees and honey bees in the controlled greenhouse environment under quarantine conditions, along with bee dissections in the laboratory.

### **Material and methods**

In 2008, six bumble bee (*B. Impatiens*) colonies (a queen and approx. 100 workers) were obtained each from two major commercial bumble bee rearing facilities providing bumble bees to growers in the United States and Canada (the Koppert Biological Systems, Romulus, Michigan, US designated as a “source-A” throughout the manuscript and the Biobest Biological Systems, Leamington, Ontario, Canada designated as a “source-B”). Three honey bee colonies were established along with three bumble bee colonies from a single source in each of three rooms (16’X20’) in a quarantine greenhouse, while the fourth room had only three bumble bee colonies from source-B. All colonies of honey bees and bumble bees were tested for IAPV, DWV, BQCV, SBV, ABPV, KBV and CBPV when introduced into the greenhouse. For each bumble bee colony, five workers were tested upon arrival; for honey bee colonies, 20 workers and 10 egg samples were tested.

The rooms were shaded with shade cloth (70% blockage) and kept at 21-30° C with elevated humidity provided by continuous swamp coolers. In each room bees were allowed to

forage by providing common food sources outside the colonies; 50% sugar syrup in a common feeder; Megabee bee diet (Castle Dome Solutions, Yuma, Arizona) as a dry powder protein supplement in an aluminum tray; and blooming pollinator-friendly plants (4-6 each of blue spirea (*Caryopteris clandonensis*), blanket flower (*Gaillardia burgundy*), goldenrod (*Solidago* spp.) and sedum (*Sedum telephium*)). Bumble bees also had access to their internal sugar feeders that came installed in the hives, except when the colonies were fed with virus solution in Petri dishes.

One room was designated as IAPV+ and the other IAPV-; sampling, feeding, and observations of the bees were done carefully using separate bee suits, gloves and other equipment to minimize contamination. In the IAPV+ room, the honey bee colonies were each fed inside the colony with 2 ml semi-purified IAPV solution (approximately  $5-7 \times 10^9$  viral genome equivalents) in 30 ml 50% sugar syrup in a Petri dish; the IAPV- honey bee colonies were each fed inside the colony, 30 ml 50% sugar syrup in a Petri dish. Bees in each honey bee colony consumed 30 ml of sugar solution containing IAPV within a few hours. From the inside of each colony, 30 honey bee workers and 3 bumble bee workers were collected every 3 days for the first week and then weekly thereafter, frozen at  $-80^\circ \text{C}$ , and assayed for IAPV using RT-PCR. Positive reactions were sequenced for confirmation.

Two weeks after infecting the honey bee colonies, the bumble bee colonies in the IAPV+ room were also fed directly inside the colony with 3 ml semi-purified IAPV solution (approximately  $8-11 \times 10^9$  viral genome equivalents) in 30 ml 50% sugar syrup in a Petri dish; the IAPV- bumble bee colonies were each fed inside the colony, 30 ml 50% sugar syrup in a Petri dish. The health of each bumble bee colony was monitored weekly for the first 5 weeks and then every 3 days thereafter. Bumble bee colonies were opened to observe the status of the queen and other bees. Foraging strength of the colony was monitored, by counting the number of foragers exiting or entering the colonies for 10 min observation periods, between 10 am to 2 pm. Number of bees foraging on the flowers at the same time were also counted. The colony survival was

calculated by counting the number of days from the date the bees were brought into the greenhouse to the date when less than 5 individuals remained alive in the hive. Weight of each colony was measured in early morning or late afternoon when all the bees are inside the colony. Dead bees outside the colonies were counted weekly and removed.

This experiment was repeated in the summer of 2009, with more focus on the impact of IAPV on pollination efficiency of bumble bees. For this purpose important greenhouse plants were brought into the greenhouse including: 35 strawberry (*Fragaria × ananassa*), 6 tomato (*Lycopersicon esculentum*), 8 cucumber (*Cucumis sativus*) and 2 blueberry (*Vaccinium angustifolium*) potted plants along with other common ornamental flowering plants. Again, six bumble bee colonies (a queen and approx. 100 workers) were obtained from Koppert Biological Systems (Romulus, Michigan) and kept with honey bees and same experimental procedures were applied. Three established honey bee colonies were split prior to use. Each split was provided with a new queen and allowed to establish and build up in ten-frame nucleus hives in the field for six weeks, before installation in the greenhouse. The control and virus-infected room each had one of the sister honey bee colonies. Each bee colony was tested for viral infections and prevalence. Each honey bee colony tested negative for IAPV prior to installation in the greenhouse. In the IAPV+ room, the honey bee colonies were each fed with 4 ml semi-purified IAPV solution (approximately  $1-3 \times 10^{10}$  viral genome equivalents) in 30 ml 50% sugar syrup in a Petri dish. Honey bee colony health was monitored every 3-5 days by counting the number of foragers entering and exiting the hive per 5 min observation periods, between 11 am to 3 pm. Dead bees on the room floor were counted. Hive parameters including the number of frames covered with adult bees, bee brood, pollen and honey were also recorded.

Bee feces were also collected. To collect the feces, five 3 x 3 inch weighing papers were placed in each room and were collected one and two weeks after feeding virus or sugar water. Bumble bee feces were also collected from inside the plastic critter cages used to house the

bumble bees in one of the lab bioassays. Bee feces were dissolved in Trizol and analyzed for the presence of RNA viruses.

### **Virus detection:**

All samples were analyzed for the presence of seven RNA viruses: deformed wing virus (DWV), black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), sacbrood virus (SBV), Kashmir bee virus (KBV), acute bee paralysis virus (ABPV) and chronic bee paralysis virus (CBPV). The reverse transcriptase-polymerase chain reaction (RT-PCR) was used as a diagnostic tool, following the protocols of Singh et al. [33]. Total RNA was extracted from samples using TRIzol<sup>®</sup> reagent (Invitrogen). RNA concentrations were determined spectrometrically (Spectra Max 250, Molecular Devices), and cDNA was synthesized using random hexamers and M-MLV reverse transcriptase (Promega). RT-PCRs were performed with specific primer sets and PCR cycles, followed by agarose gel electrophoresis using SYBR<sup>®</sup> Safe DNA gel stain (Invitrogen). The different primer sets and the gene regions they amplify are listed in (Appendix C). Primers (actin-F: ATGAAGATCCTTACAGAAAG; actin-R: TCTTGTTTAGAGATCCACAT; GenBank accession no. BI504901) were used to amplify 514 bp of the honey bee actin gene, while the primers (bumble bee actin-F: GGAGAAACTTTGTTACGTCGCC; and R: CGCACTTCATGATCGAGTTG) were used to amplify 218bp of bumble bee actin gene, and served as an internal control for the quality of RNA extraction in honey bees and bumble bees respectively. Bumble bee actin primers were designed from *Bombus terrestris* mRNA sequence (GenBank accession no. JI045318). A negative control lacking template DNA and a positive cDNA control were performed for each PCR reaction. Positive identification was confirmed by sequencing the PCR products. PCR products were treated with ExoSAP-IT (USB) and sequenced on both strands. PCR products were also cloned using TOPO TA Cloning Kits with One Shot<sup>®</sup> Chemical Competent Cells (Invitrogen) and

several individual clones were sequenced for each amplification. Sequence data were aligned and analyzed using the MEGA package.

For the quantification of the virus in the stock solution, in bumble bee tissues and some bee samples, samples were analyzed using Power SYBR Green PCR Master mix and real time primers, on a 7500 Fast-Real Time PCR system. Standard curves for IAPV and DWV were run on each plate and actin was used as an internal control. Dissociation curves and melting temperatures were checked for the specificity of PCR amplifications.

#### **IAPV stock solution preparation:**

Virus solution was prepared by crushing IAPV-infected honey bees in 1ml per bee PBS (Phosphate buffer saline) buffer. The homogenate was then centrifuged at 2,000 g at 4° C for 3 min and passed through 0.2 µm NALGENE® syringe filters to remove bacterial or fungal contaminants and bee tissue. The resulting semi-purified virus stock solution was pooled and stored at -80° C until used. Two sub-samples (100 µl) from the stock solution were analyzed for seven RNA viruses using qualitative RT-PCR as described above. Agarose gel electrophoresis revealed a strong band of IAPV and weak bands of DWV and BQCV. IAPV and DWV concentrations in the stock virus solution were then quantified with quantitative RT-PCR. Quantitative RT-PCR showed very high concentration of IAPV ( $2-4 \times 10^8$  viral genome equivalents/100 µl of stock solution) and very low concentration of DWV ( $< 10^3$  viral genome equivalents/100 µl of stock solution) in the virus stock solution.

#### **Bumble bee dissections:**

Live bumble bee queens were anesthetized in a container with dry ice for 5-10 min (bees were kept separated from dry ice). After removing the legs, wings and antennae, they were restrained in a wax dissecting dish. Then, using a sterile surgical dissection kit and a dissecting



microscope, each bee was carefully dissected by cutting the ventral body wall to remove the digestive tract, ovaries, fat bodies, brain, salivary glands, thorax and abdominal exoskeleton. The digestive tract was further separated into the honey stomach, mid-gut, and hind-gut. To avoid potential contamination from the hemolymph, each body part/tissue was rinsed thrice in Bee Ringer's solution (0.156M NaCl, 0.003M KCl, 0.002M CaCl<sub>2</sub>), followed by three serial rinses of sterile nuclease-free water. The washing solution was changed every time for each tissue to prevent possible cross-contamination. A total of six IAPV-fed bumble bee queens obtained from source-A in 2008 were dissected. Eggs from queens were also analyzed for IAPV both before and after feeding virus to originally virus-free queens from source-A in 2008. Brain tissue and salivary glands from all the six bees were pooled together, while other dissected parts/tissues were analyzed separately for IAPV with RT-PCR.

Three bumble bees displaying paralytic symptoms were also dissected into two body regions: head+thorax and abdomen, and were analyzed for the IAPV.

**Statistical Analysis:** *JMP*<sup>®</sup> 8.0.2 statistical package (SAS Institute Inc. 2009) was used to analyze the data by unpaired t-test and ANOVA to compare the colony survival, foraging activity, and the number of living and dead adults and brood, between IAPV-infected and control bee colonies.

## **Results**

### **Virus presence in purchased bumble bee colonies:**

RT-PCR analysis revealed the presence of multiple RNA viruses in the all the commercial bumble bee colonies upon their arrival, irrespective of the source. DWV was consistently the most prevalent virus, detected in at least 90% of the bees from source A over the period of three years and 85% from source B, followed in prevalence by BQCV (Figure 2, 3).

Approximately, 2-4% bumble bees had various degrees of wing deformities. Out of five such deformed-winged bees analyzed for viral quantification, four were infected with high levels of DWV ( $10^7$ - $10^8$  viral genome equivalents). SBV, KBV and ABPV were also present at low levels but there was high variation in their prevalence over the years. CBPV was not detected in any of the bumble bees. The newly described virus, IAPV was detected in all the six bumble bee colonies from source-B (60% prevalence per colony) but not in a single bee from source-A in the first year (2008) (Figure 2). Interestingly, in the following year (2009), bees from all the six hives from source-A also came infected with IAPV (50% prevalence per colony) (Figure 3). However, the bees from the same source were again IAPV-free in the year 2010. Further, sequencing and bioinformatic analysis revealed the presence of two different IAPV-strains in the bumble bees; 'IAPV-eastern strain' in 2008 source-B colonies and 'IAPV-western strain' in 2009 source-A bumble bee colonies.

#### **Inter-genera virus transmission:**

In the experimental greenhouse rooms both bumble bees and honey bees were frequently observed visiting the flowering plants for pollen and nectar, often foraging at the same time. Megabee trays and sugar feeders attracted much honey bee activity; however, no bumble bees were observed to collect powdered Megabee diet and they rarely collected sugar water at the common feeders. Bumble bees collected Megabee powder only when it was sprinkled over the potted flowers. In the 2008 greenhouse experiment, one week after feeding IAPV (eastern strain) to the honey bee colonies, 60-80% of worker bees from those colonies had detectable IAPV, while colonies fed only sugar water in the control room remained IAPV-free throughout the experiment. Out of the three bumble bee colonies from source-A co-existing and co-foraging with the IAPV-infected honey bees, workers from one colony tested positive for IAPV on week two onwards; whereas, no IAPV was detected in any of the worker bumble bees from the three

colonies in the IAPV-free room (Figure 4). On the other hand, in the bumble bees colonies from source-B, all colonies remained infected but IAPV prevalence decreased from 60% at the time of colony arrival to 20% by the end of the experiment in the control room. In contrast, when in IAPV+ rooms and interacting with IAPV-fed honey bee colonies, the prevalence of IAPV slightly increased within each of bumble bee colony from Source B (Figure 5).

The 2009 greenhouse trial (GH-2009) provided more evidence of virus transmission between the two bee species. The western strain of IAPV moved from infected bumble bees into two of the six IAPV-free honey bee hives (30 and 40% prevalence levels) after 10 days of foraging together in the same rooms (Figure 17). One week after feeding the eastern strain of IAPV to honey bees, 80% of the honey bees in the virus room were IAPV positive. The eastern strain also moved from honey bees into all the three bumble bee hives (8 out of nine bumble bees tested positive) within a week; whereas, none of the bumble bees in the control room (without eastern strain of IAPV being fed) tested positive for the eastern strain of IAPV throughout the experiment. While the IAPV levels in bumble bee colonies in the control room slowly decreased over the course of the experiment, IAPV increased in prevalence in the bumble bee colonies in the virus room after feeding virus to honey bees (Figure 6).

#### **Impact of IAPV infection on bumble bee colonies:**

**2008 Greenhouse experiment (GH-2008); source-A bumble bees:** Four days after the bumble bees were directly given IAPV in their colonies, approximately 90% of bumble bees were infected with IAPV, while none of the sugar-fed bumble bees had IAPV (Figure 4). The longevity of the IAPV-infected bumble bee colonies ( $41 \pm 4.8$  days) was significantly less than those which were free from IAPV ( $54 \pm 1.5$  days) (unpaired t-test,  $p=0.05$ ) (Figure 7). IAPV infection also impacted the foraging activity of the bumble bee colonies over time. Fewer bees were observed foraging on the flowers in the IAPV room (Figure 8), and entering or leaving the hive in the

infected colonies after the bumble bees were given the eastern strain of IAPV as compared to sugar-fed bees (before virus  $F = 0.44$ ,  $p = 0.5205$ ; after virus  $F = 27.22$ ,  $p < 0.0001$ ) (Figure 7). However, neither any difference nor any specific pattern was observed in terms of number of dead bees on the floor, between the virus and the control rooms (Figure 8). Paralytic symptoms were occasionally observed in IAPV-infected bumble bees both inside and out-side the colonies.

***GH-2008; source-B bumble bees:*** All of the bumble bee colonies from source-B arrived infected with an average of 60% prevalence of IAPV infection. Infection prevalence per colony increased four days after feeding IAPV directly to the bumble bees, with IAPV prevalence approximating 100% in virus-fed colonies as compared to only 33% in sugar-fed colonies (Figure 5). The colony survival data was not available for these bumble bee colonies because they had to be terminated due to the time restrictions on the availability of greenhouse. However, significant differences were observed in the health parameters of IAPV-fed and sugar-fed colonies at the time of colony termination (Figure 9). The number of living adult bees, larvae and pupae was significantly higher in the sugar-fed colonies as compared to IAPV-fed colonies (adults  $F = 9.02$ ,  $d.f. = 5$ ,  $p = 0.0398$ ; larvae  $F = 37.79$ ,  $d.f. = 5$ ,  $p = 0.0036$ ; pupae  $F = 7.03$ ,  $d.f. = 5$ ,  $p = 0.056$ ). More bees died inside the IAPV-fed colonies ( $F = 5.26$ ,  $d.f. = 5$ ,  $p = 0.0835$ ) than the sugar-fed colonies. Similar to findings with source-A bumble bees, IAPV-feeding reduced the colony foraging activity (before virus  $F = 0.67$ ,  $p = 0.4298$ ; after virus  $F = 28.31$ ,  $p < 0.0001$ ) (Figure 9), while no difference was observed in terms of bee drop rate on the room floor (Figure 10).

***(GH-2009); source-A bumble bees:*** In 2009, IAPV was never fed-directly to bumble bee colonies, although all colonies arrived infected with the western strain of IAPV with an approximate 50% prevalence. Only the honey bee hives co-foraging with bumble bees in the virus room were fed with IAPV. In this greenhouse trial, abrupt termination of bumble bee colonies resulted when the bumble bee colonies were suddenly raided and robbed by honey bees (these colonies were much larger and stronger than in previous trials. Robbing activity started in

the control room about a week earlier than in the virus room, reflecting the healthier and stronger honey bee colonies in the control rooms. Although IAPV prevalence increased in the bumble bee colonies co-existing with virus-fed honey bees (Figure 6), no difference between colonies in virus versus control rooms was observed for any of the bumble bee health parameters including foraging activity (before virus  $F = 1.12$ ,  $p = 0.3049$ ; after virus  $F = 0.40$ ,  $p = 0.5359$ ), colony weight (d.f. = 23,  $F = 0.08$ ,  $p = 0.7858$ ) and number of dead bees on the room floor (Figure 11 & 12). Small (statistically insignificant; alive bees  $F = 2.53$ ,  $p = 0.1868$ ; dead bees  $F = 0.95$ ,  $p = 0.3855$ ; eggs  $F = 0.28$ ,  $p = 0.6242$ ; larvae  $F = 0.24$ ,  $p = 0.6530$ ) differences observed in the colony health status at the time of termination (Figure 12), are probably the result of a difference in the timing of colony termination.

#### **Virus tissue specificity:**

Both IAPV and DWV were detected in the digestive tract, ovaries, abdominal exoskeleton, fat bodies and thorax (Figure 1). IAPV was also detected in the brain tissue but was not detected in the salivary glands; whereas, DWV was absent in the brain tissue but was detected in the salivary glands. The highest levels of IAPV concentration were observed in the mid-gut tissue ( $9.55 \times 10^7$  viral genome equivalents) followed by the hind-gut, thorax, honey stomach, abdominal exoskeleton, fat bodies and brain tissue ( $2.43 \times 10^3$  viral genome equivalents). None of the bumble tissue had DWV levels above  $10^3$  viral genome equivalents. Before feeding virus, bumble queens were laying IAPV-free eggs; however, one week after feeding virus, IAPV was detected in the surface sterilized eggs laid by the IAPV-fed queens.

Feces collected from greenhouse rooms in which both bumble and honey bees were kept together tested positive for IAPV, DWV, BQCV and SBV. Bumble bee feces had IAPV and DWV but no BQCV or SBV.

Further, the head+thorax and abdomen regions of three bumble bees showing paralytic symptoms had high titers of IAPV ( $> 10^9$  viral genome equivalents). These bees also tested positive for DWV and BQCV.

### **Impact of IAPV infection on honey bee colonies in 2009:**

At the time of installation all honey bee mini hive (10 frame hives in two hive bodies) were equally strong with 4-5 frames of adult bees, 2-3 frames of brood, 2-3 frames of honey and 1-2 frames of pollen. IAPV infected colonies were less healthy than IAPV-free honey bee colonies at the time of colony termination (Figure 13). IAPV infected honey bee colonies had significantly fewer adult bees ( $F = 10.29$ , d.f. = 5,  $p = 0.0327$ ) and capped brood ( $F = 12.62$ , d.f. = 5,  $p = 0.0237$ ) as compared to control colonies. However, the number of frames of stored pollen ( $F = 2.12$ , d.f. = 5,  $p = 0.2193$ ) and honey ( $F = 4.95$ , d.f. = 5,  $p = 0.0902$ ) were not statistically different between the two treatments. IAPV infection resulted in higher adult mortality as revealed by the higher number of dead bees on the floor of the room housing IAPV infected colonies as compared to the control room four days after feeding IAPV (Figure 14). Paralytic symptoms were observed in the IAPV-infected honey bees. Initially the infected bees lost their flight ability and were seen crawling on the floor in circles. Such bees soon became paralytic, twitching their legs, unable to move, and eventually died within a few hours. Adult mortality remained higher in the virus room for about a week, after which more bees were dying in the control room.

There was no difference between the foraging strength of the colonies in the two rooms before feeding IAPV (incoming forager rate  $F = 0.16$ , d.f. = 5,  $p = 0.7067$ ; outgoing forager rate  $F = 0.08$ , d.f. = 5,  $p = 0.7946$  (Figure 15). The forager numbers peaked in both rooms about three weeks after installation, but the IAPV-infected colonies had significantly fewer foragers than the IAPV-free colonies. From around ten days after feeding IAPV, both the incoming forager rates

(on day 23  $F = 8.56$ ,  $p = 0.0430$ ; on day 28  $F = 21.27$ ,  $p = 0.0099$ ) and outgoing forager rates (on day 23  $F = 6.08$ ,  $p = 0.0693$ ; on day 28  $F = 11.53$ ,  $p = 0.0259$ ) were significantly lower in IAPV infected bees compared to uninfected control hives (Figure 14). Foraging activity rates (that includes both the incoming and outgoing bees) of infected sister colonies was lower than uninfected (Figure 16). Lower activity was observed in infected colonies except on day 39 when two of the infected honey bee colonies (Hive A & B) showed unusually high activity. The general decline observed in honey bee forager numbers in both the virus and control colonies after three weeks correlated with the exhaustion of stored pollen resources that were present at the time of hive installation. Although Megabee diet was provided in the greenhouse, it only served as a protein supplement but was not an alternative to plant pollen. The pollen availability from the potted plants was not enough to sustain the growth of colonies for prolonged periods. The same is true for the bumble bees, which did not get substantial amounts of protein for brood rearing, especially since they did not accept the protein supplement Megabee diet provided as dry powder.

No differences were observed in the pollination success of tomatoes, strawberries, cucumbers and blueberries between the virus and the control rooms as measured in terms of total number of fruits set, fruit weight and the proportion of deformed fruits (data not shown). High bee to flower ratio in the greenhouse might have masked any negative impact of IAPV infection on the pollination efficiency of the bees.

## **Discussion**

Infectious diseases are a serious threat to managed bee populations in which unnaturally high densities and environmental stresses can increase exposure and susceptibility to disease organisms [44,45]. RNA viruses, including recently described Israeli acute paralysis virus (IAPV), are responsible for honey bee (*Apis mellifera*) losses around the world and can be a potential problem in other managed bees also.

This is the first comprehensive study of the full spectrum of RNA viruses in the commercial greenhouse bumble bee, *B. impatiens*, and the pathology and epidemiology of IAPV. Detection of multiple RNA viruses in bumble bees in this study suggests a very broad occurrence of RNA viruses in this economically important managed pollinator. Our study corroborates the previous reports of sporadic detection of RNA viruses in different bumble bee species [33-35]. In the present study, DWV was the most prevalent virus in bumble bees with very little variation between the colonies from different origins or over the years, followed by BQCV. Further, the detection of high titers of DWV in deformed-winged bees in the commercial colonies is in agreement with the previous report of association of DWV with such symptoms in two bumble bee species, *B. terrestris* and *B. pascuorum* in Europe [35]. In general the prevalence and spread of RNA viruses in bumble bees follows similar trends observed in honey bees in the United States [25,46]. Since bumble bee colonies are shipped across the nation, my findings reflect a real threat for the spread of these pathogens.

A recent phylogenetic study [41] has revealed the existence of at least three distinct IAPV lineages, with two of them circulating in United States honey bee populations. IAPV group 1 (the western strain) was found in samples from operations in the western United States, as well as from bee packages imported from Australia; IAPV group 2 includes sequences from Israel; and IAPV group 3 (the eastern strain) includes sequences from three operations in the eastern United States and one operation in Canada. Both the eastern and the western strains of IAPV have been detected in commercial bumble bees in this study.

Bee-collected pollen can potentially lead to long distance spread of RNA viruses without the movement of pollinators themselves. Both commercial suppliers of bumble bees reported that honey bee-collected pollen purchased from beekeeping operations in the US and Canada was used in rearing the bumble bees; in fact, > 200 tons of honeybee-collected and preferably freshly-frozen pollen is used annually for this purpose worldwide [4]. We surmise that the IAPV-



contaminated pollen served as the vehicle for viral transmission into these colonies. It has previously been shown that pollen itself can carry RNA viruses [33] and other plant viruses [47,48]. The pathogenicity of RNA viruses in pollen (bee bread) has been experimentally demonstrated even after six months of storage under ambient conditions [33], and viruses may survive even longer in frozen pollen. In addition, other picornaviruses, such as poliovirus and foot and mouth virus are known to survive prolonged exposures to sunlight, desiccation and temperature fluctuations [49,50]. Risk mitigation strategies for rational use of pollen in bee rearing should be investigated. Gamma irradiation of pollen provisions has been successfully used against a fungal disease (chalkbrood) in alfalfa leaf cutting bees [51]. However, dosage and exposure timing for gamma irradiation and other methods that can effectively decontaminate pollen against RNA viruses without adversely impacting its nutritional and other physiochemical properties need to be investigated.

An in-depth understanding of pathogen transmission dynamics is critical in designing strategies to control the pathogen spread and to predict and avoid disease epidemics. Our greenhouse experiments involving IAPV have demonstrated that RNA viruses could be transmitted from one bee species to another when they forage together on common flowering plants. Different strains of IAPV moved from infected honey bees to bumble bees and from infected bumble bees to honey bees within a week to 10 days, indicating that there is no species barrier and no directionality involved in the virus movement at least between honey bees and bumble bees.

Further, the detection of IAPV in ovaries, eggs and feces of bumble bees, suggest multiple potential routes of RNA virus transmission in bumble bees. Vertical transmission possibly occurs when an infected queen passes an infection to offspring transovarially. Transmission can also occur through infection of young gynes (future queens) within the nest via virus shed in the bee feces or contamination of the hive food. The infectivity of virus in bee feces

has previously been proven [52]. Bumble bees have an annual life cycle, where a single queen starts the colony in spring, which then expands in worker numbers throughout the summer [3]. Males and future queens (larger workers) are produced late in the season and only the mated young gynes hibernate over the winter, starting their own colonies the following spring. So, unlike honey bees where pathogens can spread along with the natural colony reproduction that occurs through a swarming event, bumble bee colony multiplication and therefore the vertical pathogen spread totally depends on future queens.

The horizontal transmission of IAPV and other viruses in natural bumble bee populations possibly occurs through the shared use of flowers, much like the intestinal protozoan *Crithidia bombi*. *C. Bombi* is heavily shed in the bee feces, like these RNA viruses. The transmission of *Crithidia* within the bumble bee population through the shared use of flowers, has been experimentally demonstrated [53]. Yet another study suggests the potential role of honey bees in the epidemiology of *C. bombi* in its bumble bee host [54]. The risks of specific or non-specific host-pathogen interactions are very high between honey bees and bumble bees due to their geographical proximity, close relatedness and frequent sharing of floral resources. More research is thus needed to understand the disease dynamics and potential health impacts of multi-host parasites and pathogens. There is an additional need to study the interaction of RNA viruses with other intestinal parasites of bumble bees such as *Crithidia* and *Nosema*. These parasites may significantly impact the ecology of viruses in bumble bees, especially because in bumble bees the gut seems to be the most common route of infection. Unlike honey bees, bumble bees are not parasitized by varroa which is a major vector of viruses in honey bees.

Detection of IAPV in various tissues of virus-fed bumble bees also provides evidence of active infection and virus dissemination from the gut throughout the bee body. Previously, other picornaviruses such as poliovirus, cricket paralysis virus and *Solenopsis invicta* virus, and recently DWV in the bumble bees were known to infect a variety of tissues, with dissemination

from the gut or site of infection affected by host conditions [55-57]. Further, the detection of highest levels of IAPV in the mid-gut tissue even weeks after feeding virus, suggests IAPV replication in the mid-gut, thus corroborating the previous studies suggesting the mid-gut as the main site of RNA virus replication [58,59]. However, more research is needed to provide support to this inference.

This is a first study that provides comprehensive data on the colony level impacts of IAPV in bumble bees and honey bees, the two most widely managed pollinators in the world. Cumulative data from our greenhouse trials indicate negative impacts of IAPV infection on the health of both bumble bee and honey bee colonies. Like other insect-infecting RNA viruses [26], IAPV seems to be asymptomatic at low levels, but highly virulent at higher infection levels. Adult mortality from IAPV infection observed especially in honey bees in our trials, corroborates the previous study that reported up to 80% mortality in adult honey bees infected orally with IAPV in the laboratory, within a week of infection [38]. In addition, the two other closely related viruses, KBV and ABPV, are known to be rapidly lethal to honey bee adults in the laboratory when injected to pupae or adults at low doses or when fed to newly emerged adults at larger doses [43]. Sharp declines in adult honey bee populations have been associated with severe ABPV and KBV infections in the past [43,60]. Along with the adult mortality in honey bees, IAPV seems to have an impact on the developing brood, indicated by poor population build-up in infected colonies in our study. However, our inference of negative impact of IAPV on honey bee brood development, may just be reflecting the poor brood rearing ability of infected colonies due to the lack of enough nurse bees.

While honey bees exhibited typical paralytic symptoms and predominantly left the colony to die or were removed from the colony by undertaker bees to be found dead on the greenhouse floor, only few bumble bees were found dead on the floor. Predominantly, bumble bee death occurred within the colonies, with some bumble bees exhibiting paralytic seizures. This

suggests the possible impact of host sociality on virus ecology. Honey bees being eusocial insects, are known for their ability to sense and remove the diseased/dead individuals from the colony and their general hygienic behavior [61,62]. There is even an indication that altruistic or sacrificial behavior is involved, where the infected or sick individuals leave the nest to die [63,64]. Detection of IAPV in the bee nervous tissue or brain provides the initial clue to the paralytic symptoms associated with the virus, however, more research is needed to understand the physiological and molecular basis of such behavior. Reduction in foraging activity and colony survival of *B. impatiens* after experimental infection with IAPV, provides an initial indication of the potential impact these RNA viruses can have on the health of the pollinator community in general. Further research is needed to study the impacts of these viruses on specific species of native pollinators and to determine if IAPV or other RNA viruses are linked to declines in other pollinator species.

In conclusion, RNA viruses are widespread in the commercial colonies of the common eastern bumble bee, *B. impatiens*. IAPV can spread in bumble bees through various horizontal and vertical routes and it shows no species barrier and directionality at least between honey bees and bumble bees. IAPV negatively impacts the health of both the bumble bee and honey bee colonies under greenhouse environment. However, the role of IAPV and other RNA viruses needs to be studied in the recent declines in field populations of different pollinators. The risks of such intergeneric disease transmission calls for a broader epidemiological approach in future and to study the disease dynamics of pollinator community as a whole instead of dealing on individual species basis.

### **Acknowledgments**

I thank Jeremy Fitzgerald for help in setting up greenhouse experiments, and Michael Williams and Michael Freiberg in greenhouse data collection. The help of Amanda Mahoney in

processing samples for virus analysis is also greatly appreciated. I also like to thank Maryann Frazier and Nancy Ostiguy, in providing honey bee colonies and queens used in this study.

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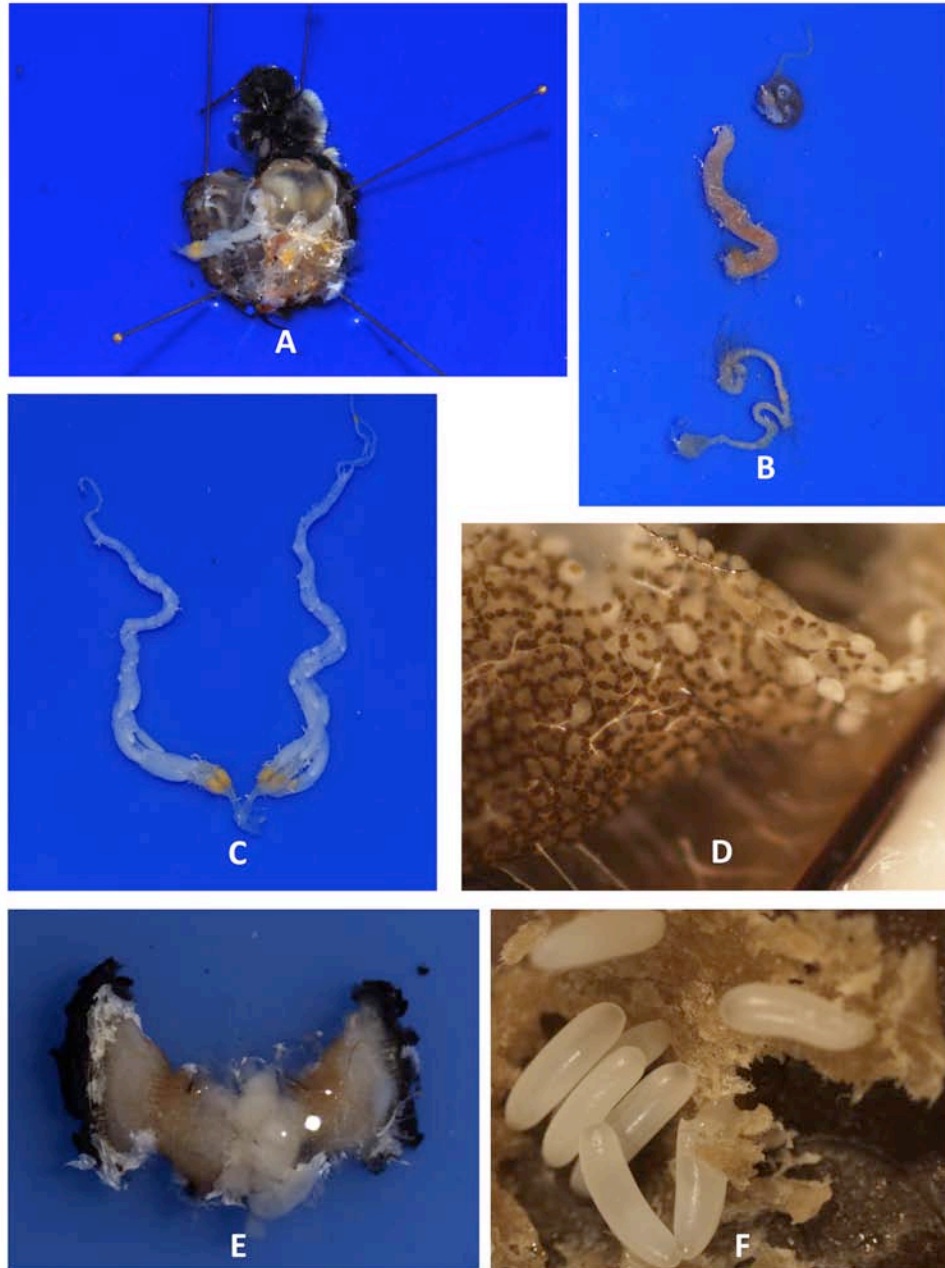


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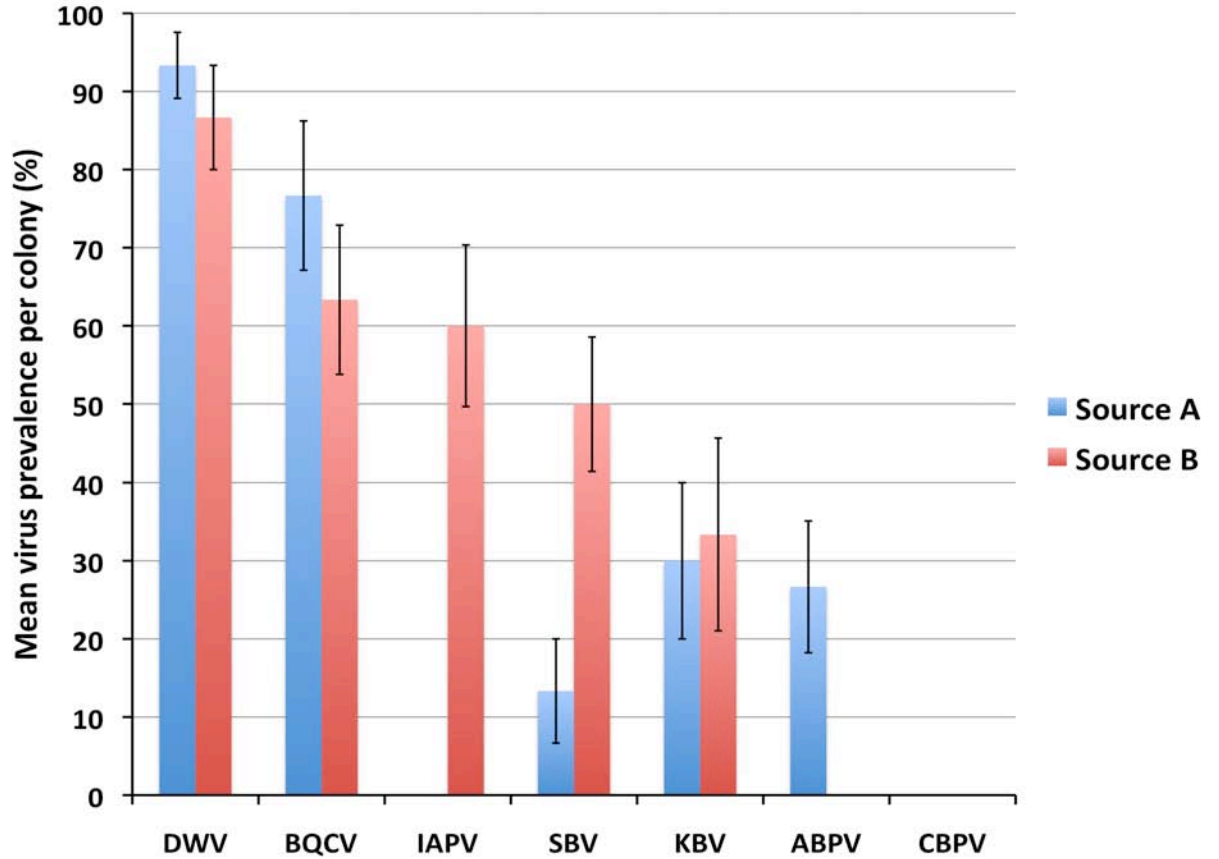
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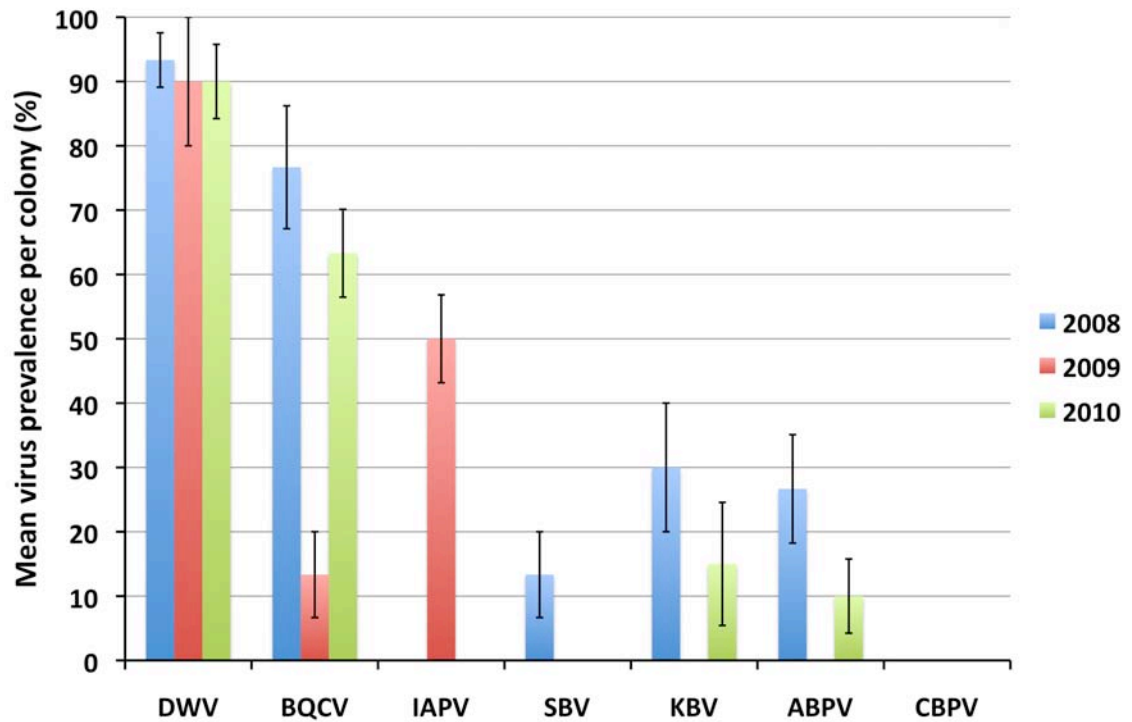


**Figure 1: Pictures depicting various tissues and the eggs of the bumble bee, *B. impatiens*, queen.** Six IAPV-fed bumble bee queens were dissected to remove various tissues. A = a dissected bumble bee with the ventral abdominal wall flipped over to expose

the digestive tract, reproductive system and other tissues and organs; B = the digestive tract divided into three regions- the honey stomach, mid-gut and the hind-gut; C = ovaries; D = fat bodies; bee brain; eggs.



**Figure 2: Prevalence of RNA viruses in the bumble bee, *B. impatiens*, colonies obtained from two commercial sources in the year 2008.** Six colonies were obtained from each commercial rearing facility and five workers were analyzed per colony. Results are represented as Mean  $\pm$  SE. Source A = Koppert Biological Systems, Romulus, Michigan, US; Source B = Biobest Biological Systems, Leamington, Ontario, Canada.



**Figure 3: Temporal variation in the prevalence of RNA viruses in the bumble bee, *B. impatiens*, colonies obtained from a single commercial source, over a three year period.** Six colonies were obtained each year from a commercial rearing facility over three consecutive years. Five workers were analyzed per colony. Results are represented as Mean  $\pm$  SE. Source = Koppert Biological Systems, Romulus, Michigan, US.



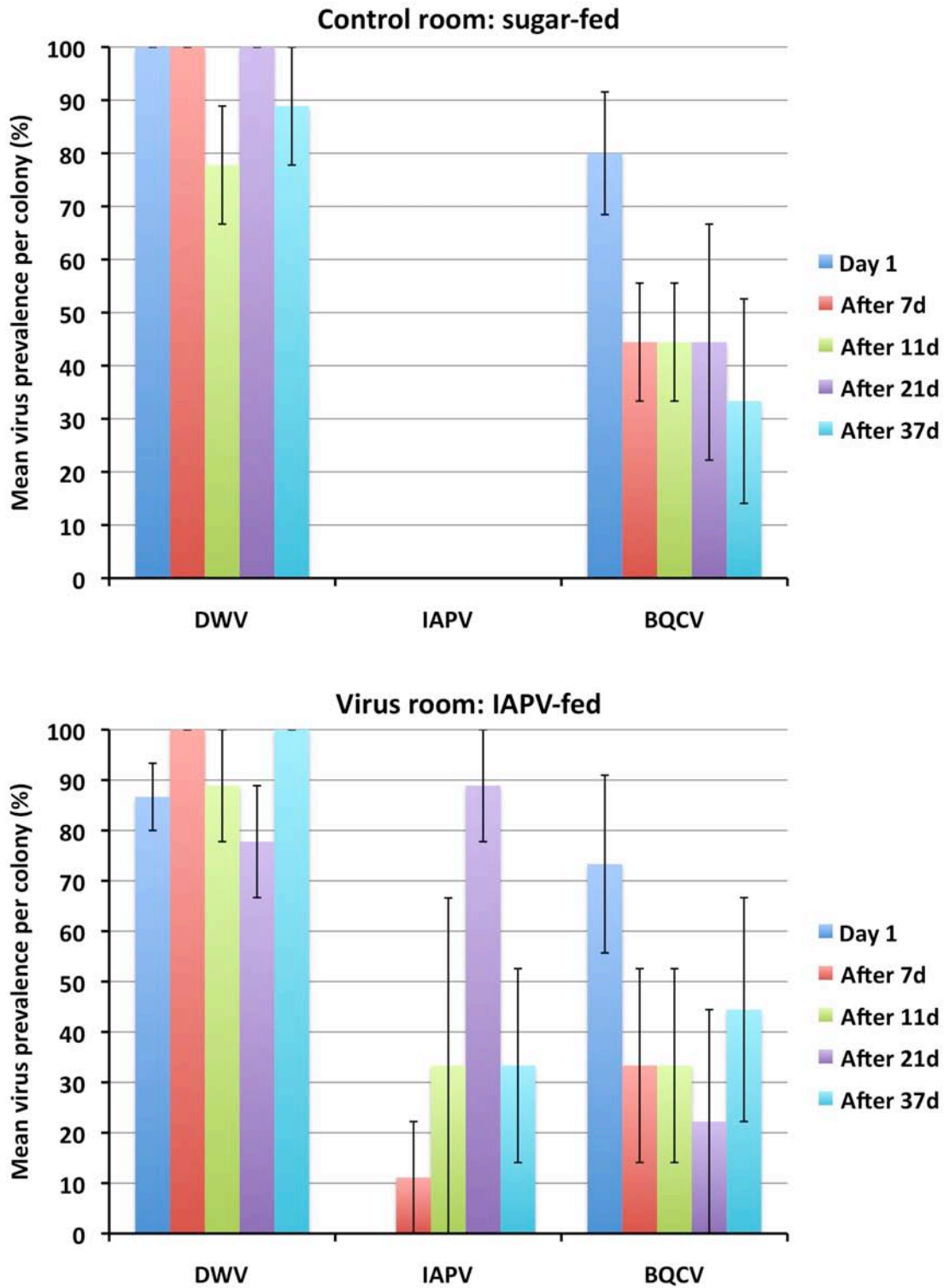


Figure 4: Variation in the prevalence of RNA viruses in the bumble bee (*B. impatiens*) colonies, obtained from source-A, over the course of the 2008 greenhouse experiment. Three

bumble bee and three honey bees colonies were installed in each greenhouse room. In the IAPV room, each honey bee colony was fed with 2 ml semi-purified IAPV solution (approximately  $5-7 \times 10^9$  viral genome equivalents) on day 3 and each bumble bee colony was fed with 3 ml semi-purified IAPV solution (approximately  $8-11 \times 10^{10}$  viral genome equivalents) on day 17. Initial data are based on five workers/colony while the remaining data on three workers/colony. Results are represented as Mean  $\pm$  SE. Source of bumble bees: Koppert Biological Systems, Romulus, Michigan, US.

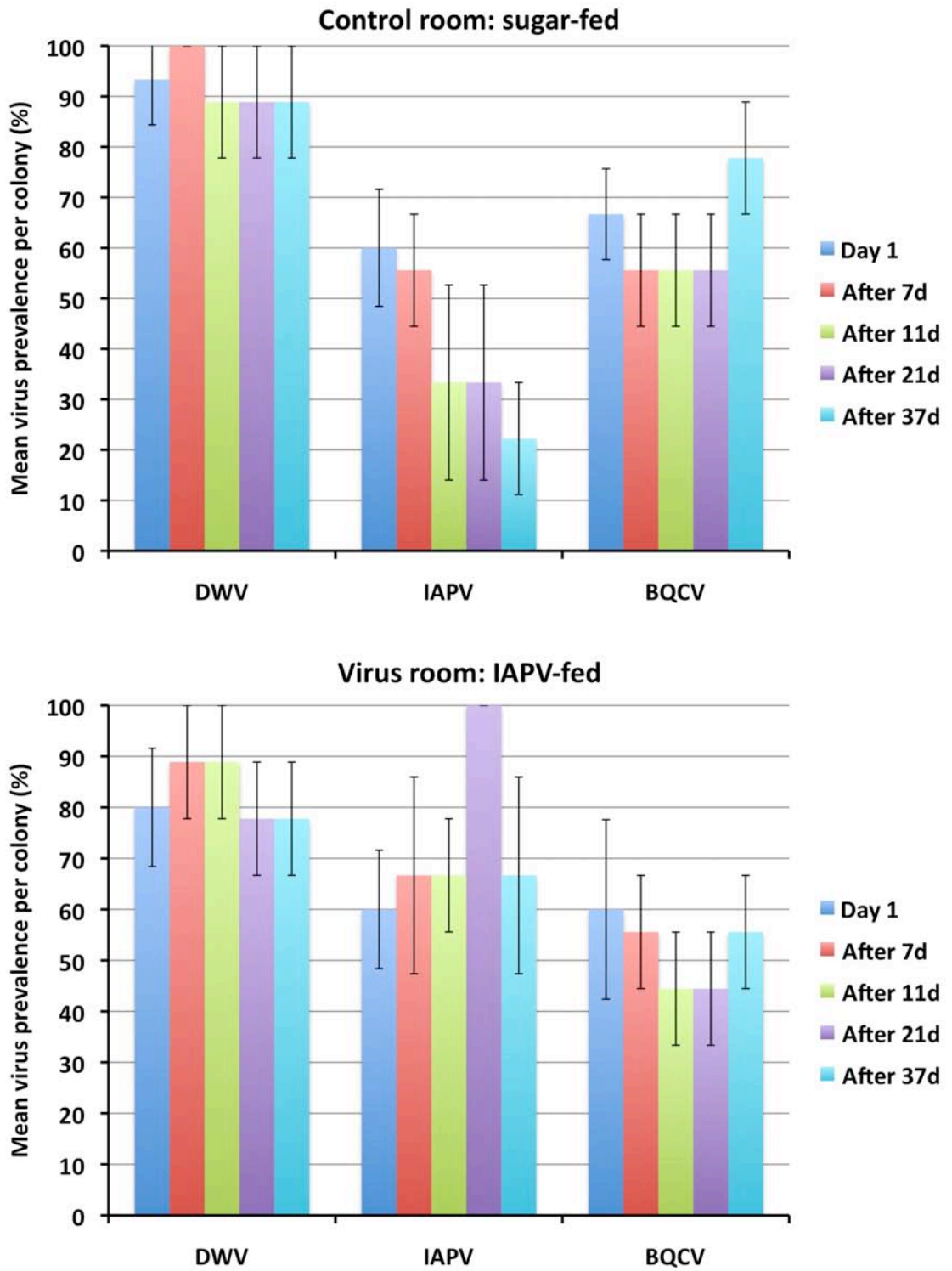


Figure 5: Variation in the prevalence of RNA viruses in the bumble bee (*B. impatiens*)

**colonies, obtained from source-B, over the course of the 2008 greenhouse experiment.** In the IAPV room, each honey bee colony was fed with 2 ml semi-purified IAPV solution (approximately  $5-7 \times 10^9$  viral genome equivalents) on day 3 and each bumble bee colony was fed with 3 ml semi-purified IAPV solution (approximately  $8-11 \times 10^{10}$  viral genome equivalents) on day 17. Initial data are based on five workers/colony while the remaining data on three workers/colony. Results are represented as Mean  $\pm$  SE. Source of bumble bees: Biobest Biological Systems, Leamington, Ontario, Canada.

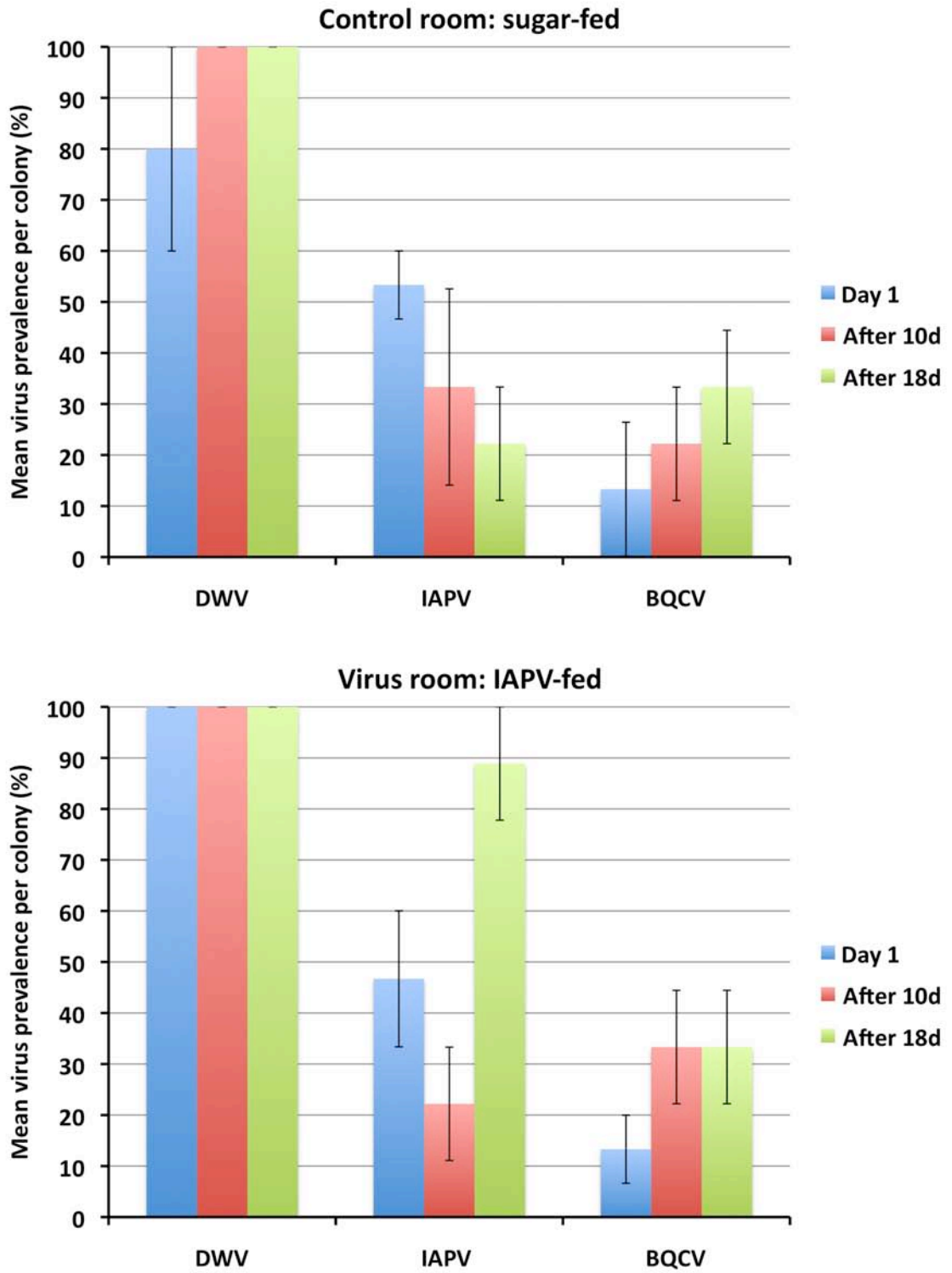
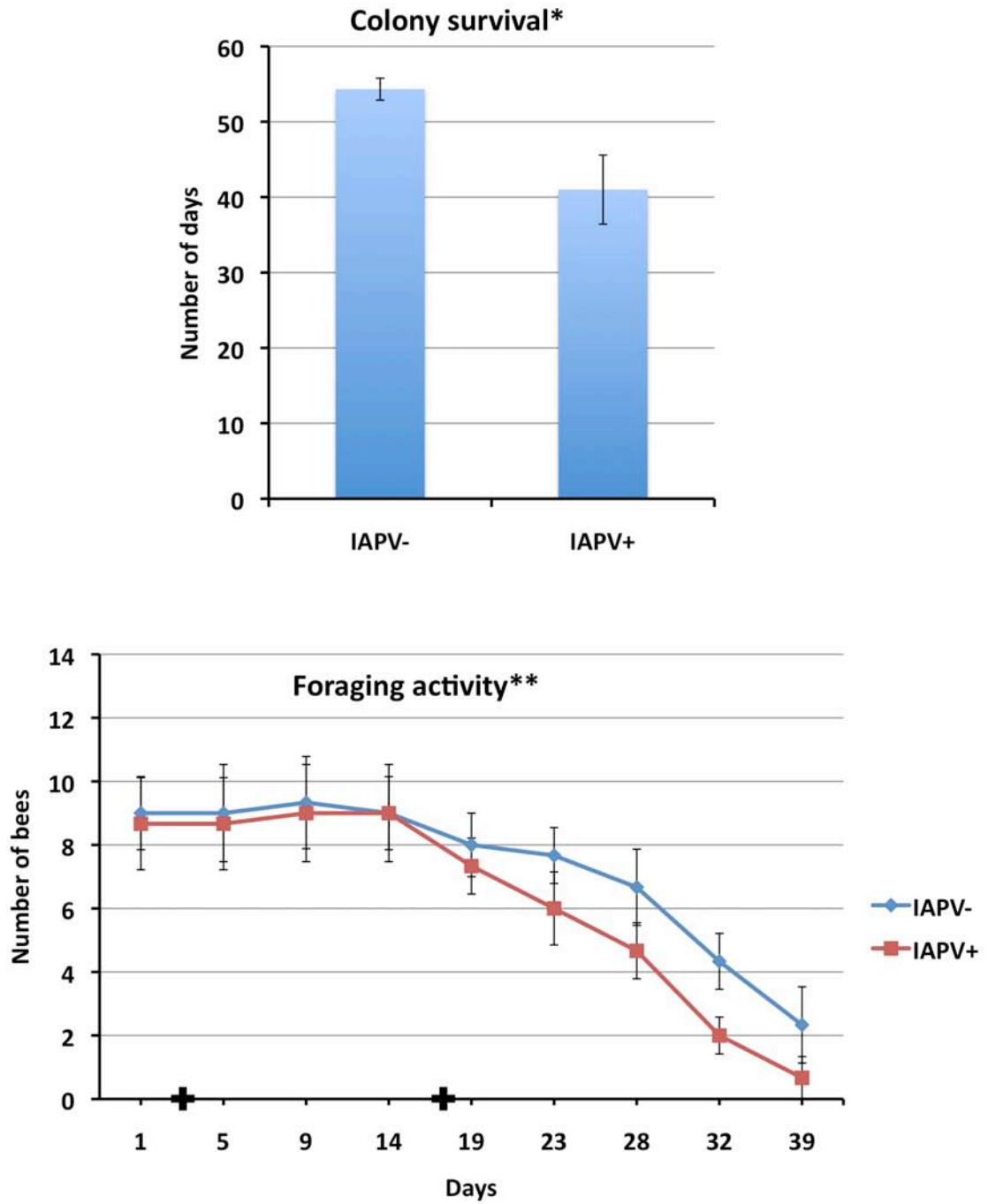


Figure 6: Variation in the prevalence of RNA viruses in the bumble bee (*B. impatiens*) colonies, obtained from source-A, over the course of the 2009 greenhouse experiment. Three

bumble bee and three honey bees colonies were installed in each greenhouse room. Each honey bee colony in the IAPV room was fed with 4 ml semi-purified IAPV solution (approximately  $1-3 \times 10^{10}$  viral genome equivalents) on day 11. Bumble bee colonies never received IAPV directly. Initial data are based on five workers/colony while the remaining data on three workers/colony. Results are represented as Mean  $\pm$  SE. Source of bumble bees: Koppert Biological Systems, Romulus, Michigan, US.

**GH-2008; Bumble bee source A**

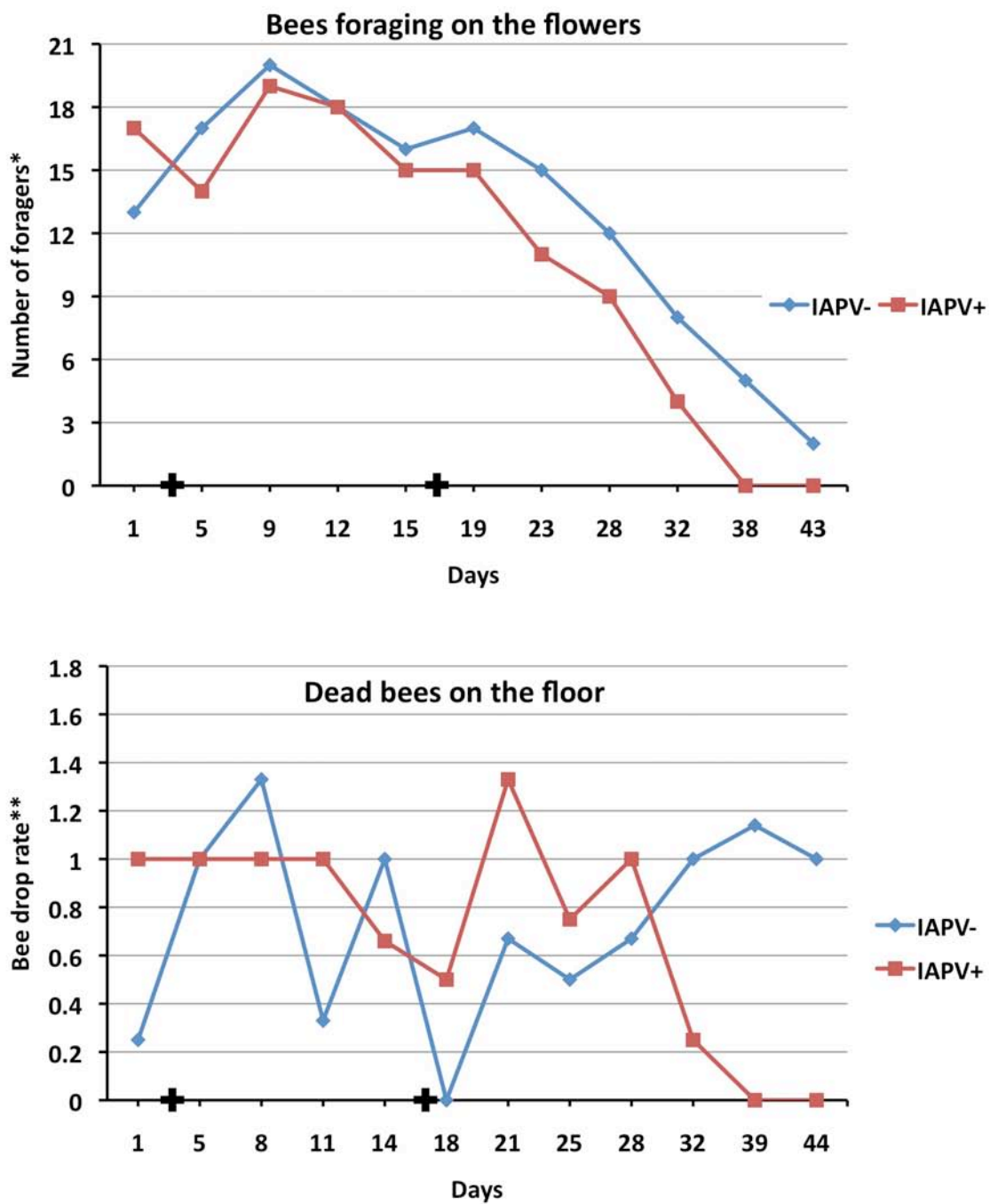


**Figure 7: Impact of Israeli acute paralysis virus (IAPV) infection on the bumble bee (*B. impatiens*) colonies obtained from source-A in 2008. Three bumble bee and three honey bees**

colonies were installed in each greenhouse room. In the IAPV room, each honey bee colony was fed with 2 ml semi-purified IAPV solution (approximately  $5-7 \times 10^9$  viral genome equivalents) on day 3 and each bumble bee colony was fed with 3 ml semi-purified IAPV solution (approximately  $8-11 \times 10^{10}$  viral genome equivalents) on day 17. Days when virus was fed are denoted with **+** symbol. Results are represented as Mean  $\pm$  SE. Source of bumble bees: Koppert Biological Systems, Romulus, Michigan, US. \* Number of days from the day of colony installation to the day when less than five living bees were left in the colony (unpaired t-test; d.f. = 5, F = 7.6923, p = 0.050). \*\* Number of bees entering or leaving the hive per 10 min observation period (ANOVA; before virus F = 0.44, p = 0.5205; after virus F = 27.22, p < 0.0001).



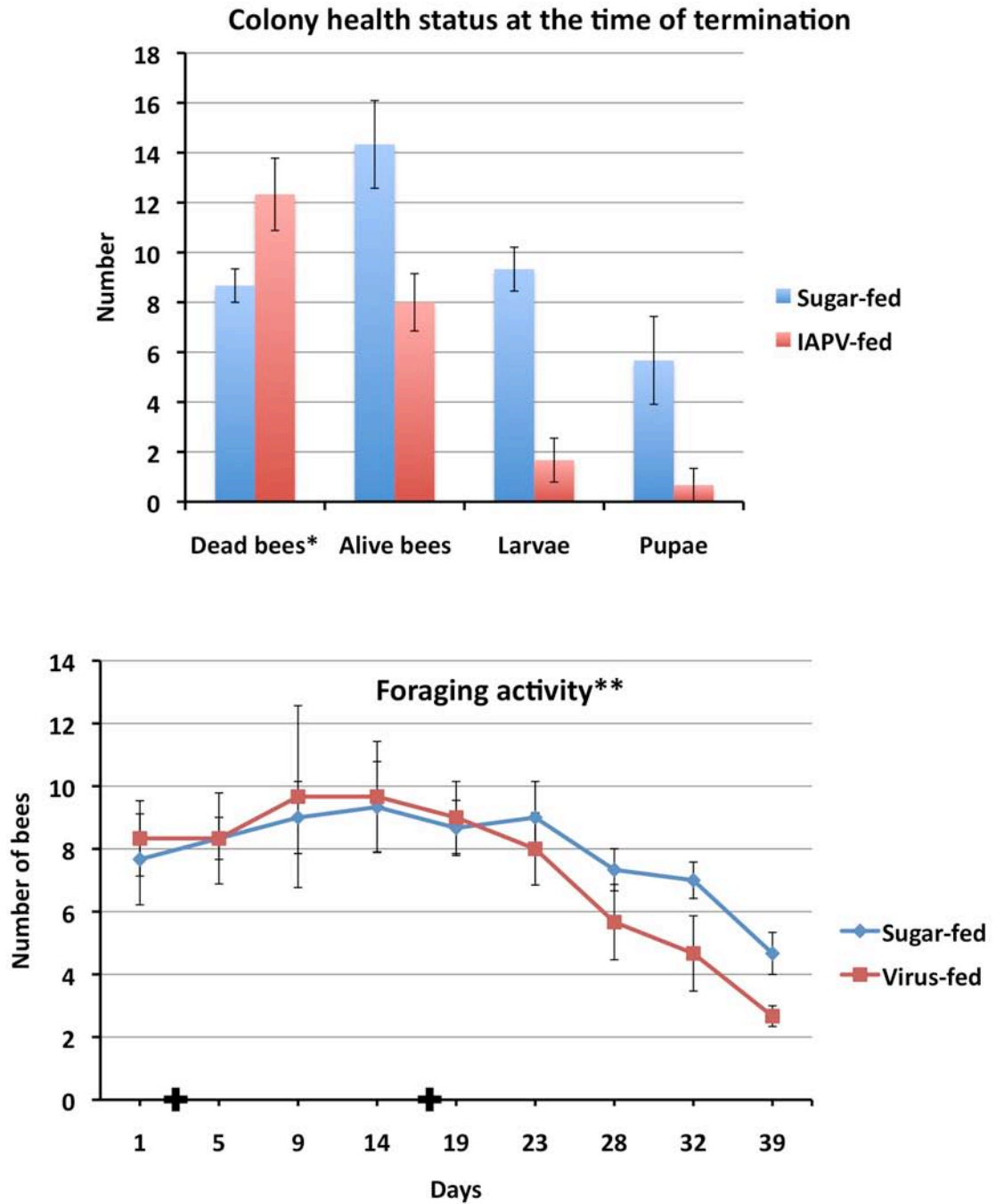
**GH-2008; Bumble bee source A**



**Figure 8: Impact of Israeli acute paralysis virus (IAPV) infection on the bumble bee (*B. impatiens*) colonies obtained from source-A in 2008. Three bumble bee and three honey bees**

colonies were installed in each greenhouse room. In the IAPV room, each honey bee colony was fed with 2 ml semi-purified IAPV solution (approximately  $5-7 \times 10^9$  viral genome equivalents) on day 3 and each bumble bee colony was fed with 3 ml semi-purified IAPV solution (approximately  $8-11 \times 10^{10}$  viral genome equivalents) on day 17. Days when virus was fed are denoted with **+** symbol. Source of bumble bees: Koppert Biological Systems, Romulus, Michigan, US. \* Number of bees foraging on the potted flowers in the room at a given moment.  
\*\* Number of bees found dead on the room floor per day.

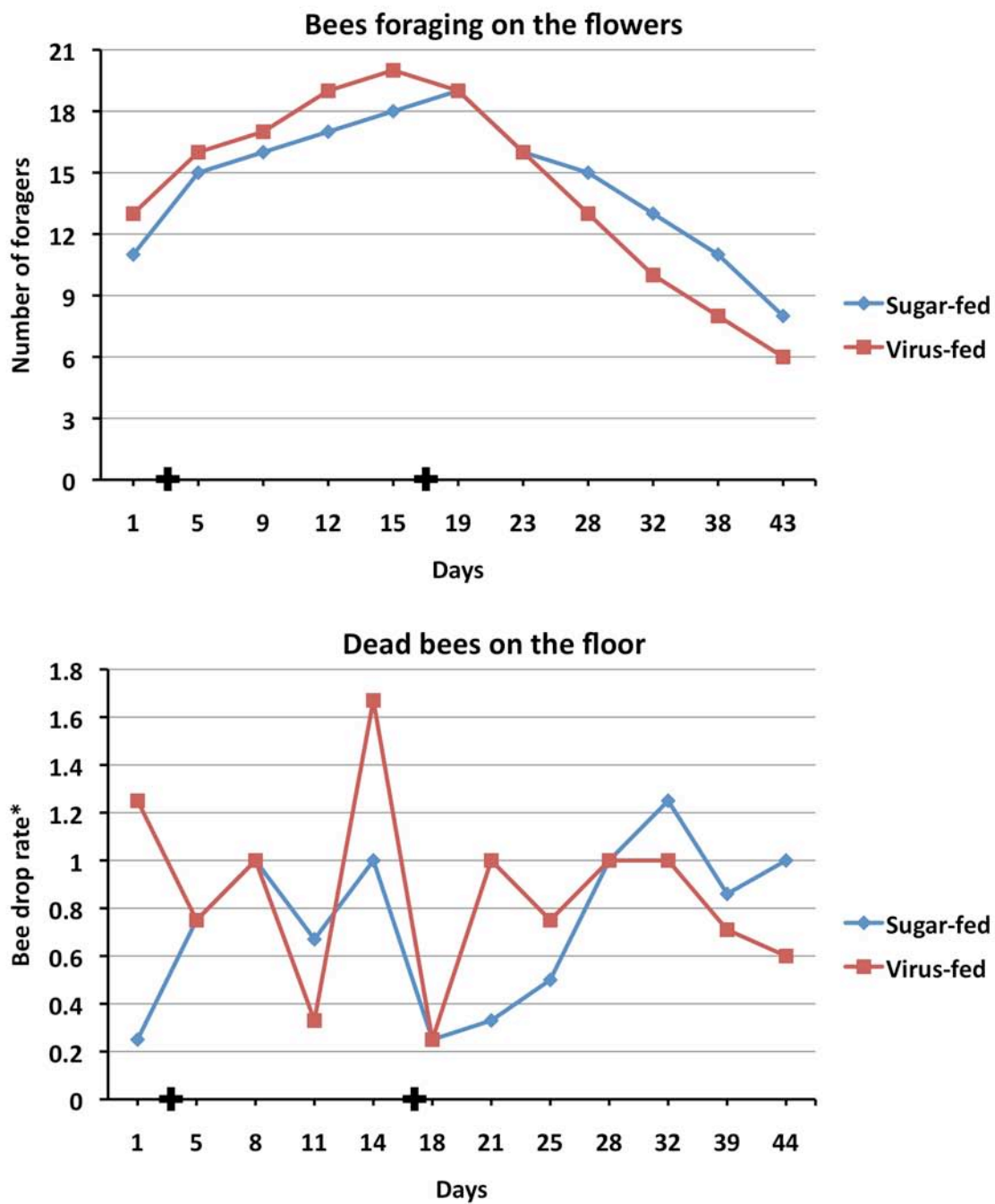
**GH-2008; Bumble bee source B**



**Figure 9: Impact of Israeli acute paralysis virus (IAPV) infection on the bumble bee (*B. impatiens*) colonies obtained from source-B in 2008. In the IAPV room, each honey bee colony**

was fed with 2 ml semi-purified IAPV solution (approximately  $5-7 \times 10^9$  viral genome equivalents) on day 3 and each bumble bee colony was fed with 3 ml semi-purified IAPV solution (approximately  $8-11 \times 10^{10}$  viral genome equivalents) on day 17. Days when virus was fed are denoted with **+** symbol. Results are represented as Mean  $\pm$  SE. Source of bumble bees: Biobest Biological Systems, Leamington, Ontario, Canada. \* Number of dead bees inside the hive counted at the time of colony termination. This may not be an absolute number because some dead bees were badly dried out and dismembered. (ANOVA; dead bees,  $F = 5.26$ , d.f. = 5,  $p = 0.0835$ ; alive bees,  $F = 9.06$ , d.f. = 5,  $p = 0.0398$ ; larvae,  $F = 37.79$ , d.f. = 5,  $p = 0.0036$ ; pupae,  $F = 7.03$ , d.f. = 5,  $p = 0.0569$ ). \*\* Number of bees entering or leaving the hive per 10 min observation period. (ANOVA; before virus  $F = 0.67$ ,  $p = 0.4298$ ; after virus  $F = 28.31$ ,  $p < 0.0001$ ).

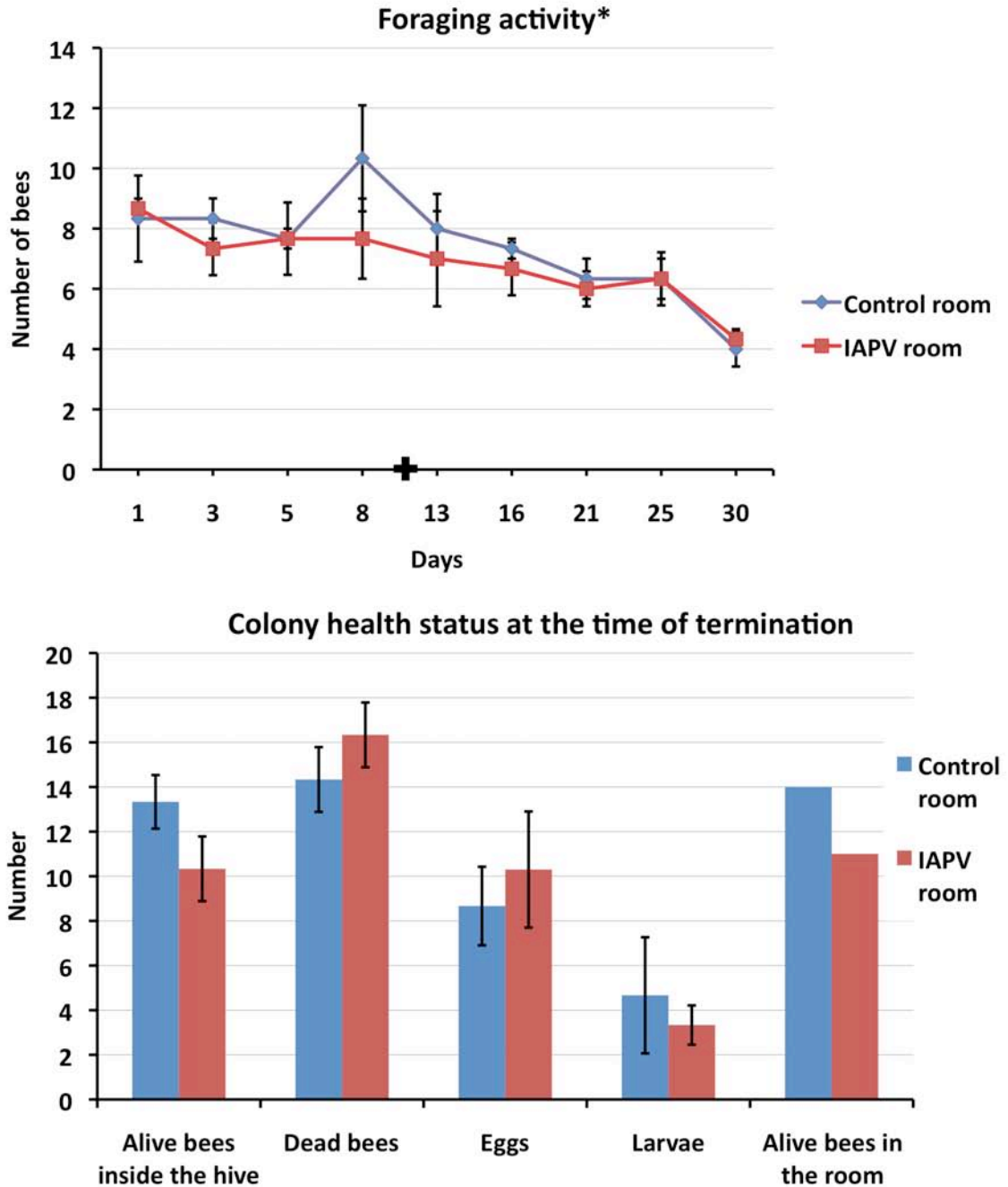
### GH-2008; Bumble bee source B



**Figure 10: Impact of Israeli acute paralysis virus (IAPV) infection on the bumble bee (*B. impatiens*) colonies obtained from source-B in 2008. In the IAPV room, each honey bee colony**

was fed with 2 ml semi-purified IAPV solution (approximately  $5-7 \times 10^9$  viral genome equivalents) on day 3 and each bumble bee colony was fed with 3 ml semi-purified IAPV solution (approximately  $8-11 \times 10^{10}$  viral genome equivalents) on day 17. Days when virus was fed are denoted with **+** symbol. Source of bumble bees: Biobest Biological Systems, Leamington, Ontario, Canada. \* Number of bees foraging on the potted flowers in the room at a given moment. \*\* Number of bees found dead on the room floor per day.

**GH-2009; Bumble bee source A**

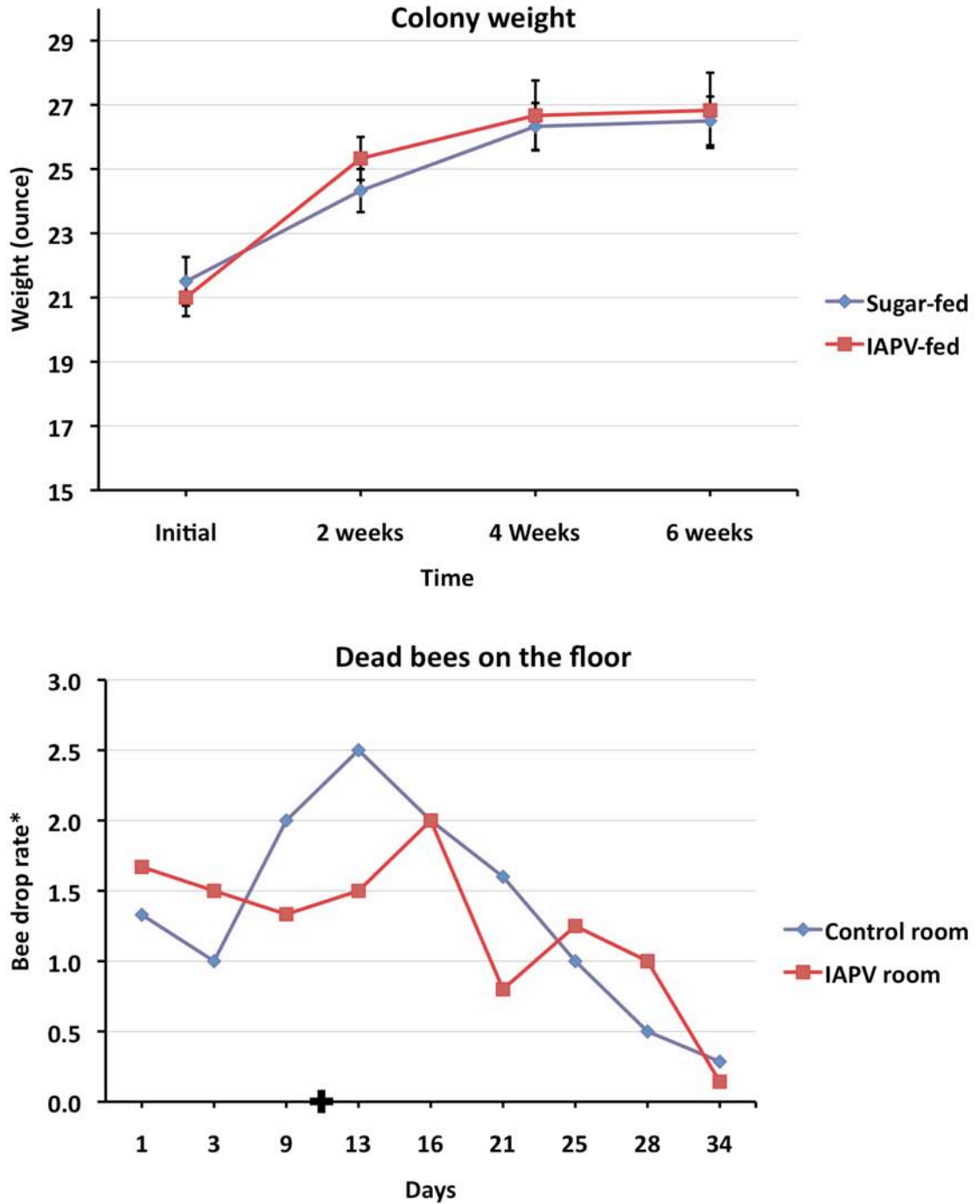


**Figure 11: Impact of Israeli acute paralysis virus (IAPV) infection on the bumble bee (*B. impatiens*) colonies obtained from source-A in 2009. Three bumble bee and three honey bees**

colonies were installed in each greenhouse room. Each honey bee colony in the IAPV room was fed with 4 ml semi-purified IAPV solution (approximately  $1-3 \times 10^{10}$  viral genome equivalents) on day 11 denoted with **+** symbol. Bumble bee colonies never received IAPV directly. Results are represented as Mean  $\pm$  SE. Source of bumble bees: Koppert Biological Systems, Romulus, Michigan, US. \* Number of bees entering or leaving the hive per 10 min observation period (ANOVA; before virus  $F = 1.12$ ,  $p = 0.3049$ ; after virus  $F = 0.40$ ,  $p = 0.5359$ ). Colony health: (ANOVA; alive bees inside the hive  $F = 2.53$ ,  $p = 0.1868$ ; dead bees  $F = 0.95$ ,  $p = 0.3855$ ; eggs  $F = 0.28$ ,  $p = 0.6242$ ; larvae  $F = 0.24$ ,  $p = 0.6530$ ).



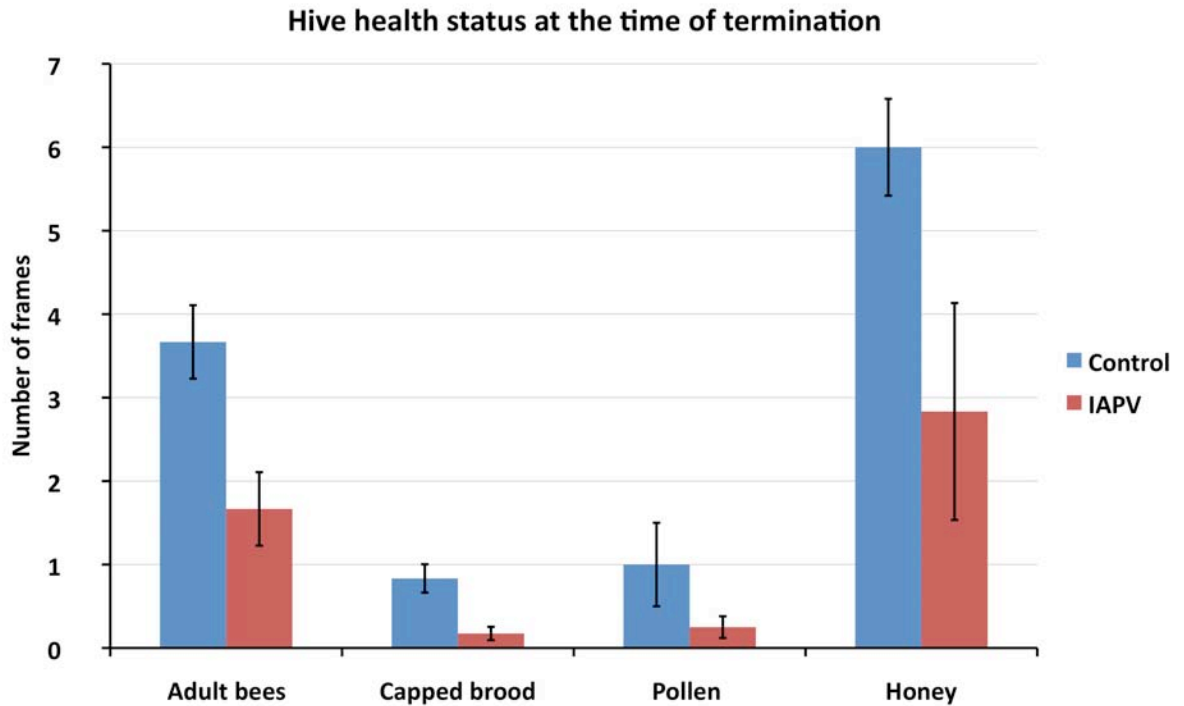
**GH-2009; Bumble bee source A**



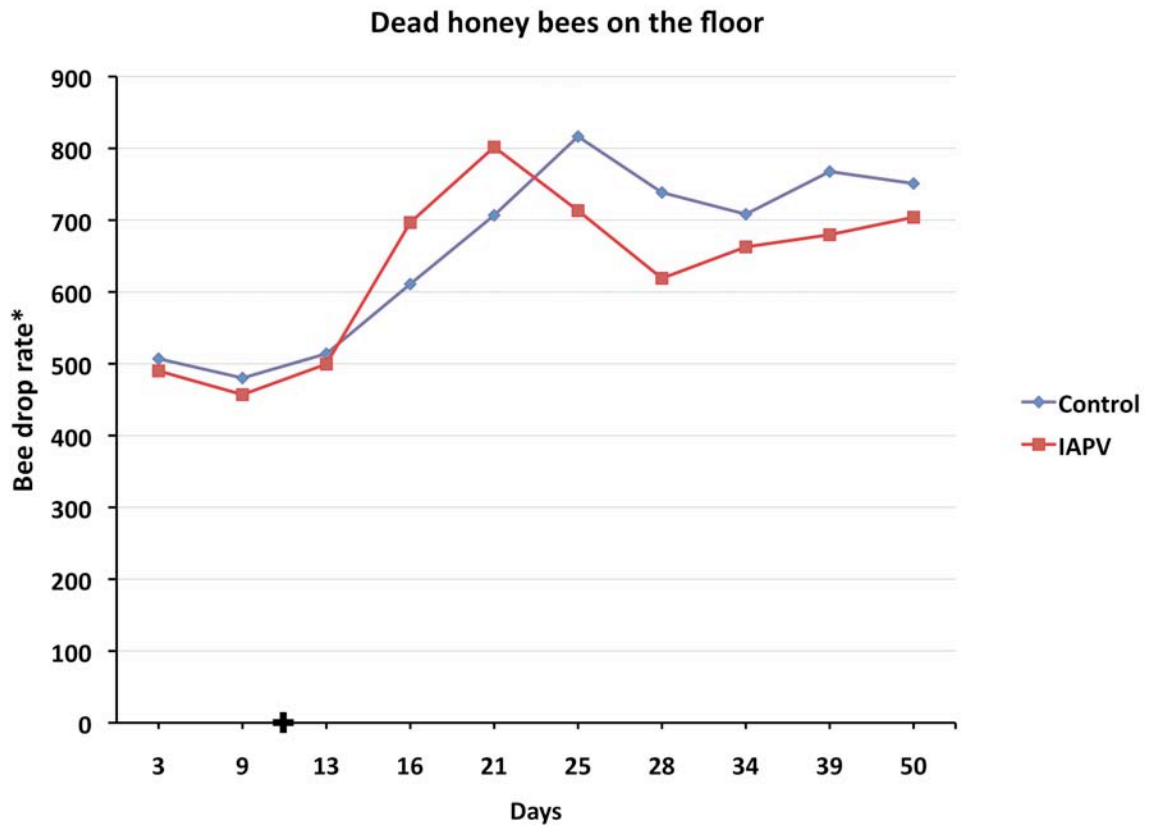
**Figure 12: Impact of Israeli acute paralysis virus (IAPV) infection on the bumble bee (*B. impatiens*) colonies obtained from source-A in 2009. Three bumble bee and three honey bees**

colonies were installed in each greenhouse room. Each honey bee colony in the IAPV room was fed with 4 ml semi-purified IAPV solution (approximately  $1-3 \times 10^{10}$  viral genome equivalents) on day 11 (denoted with **+** symbol). Bumble bee colonies never received IAPV directly. Source of bumble bees: Koppert Biological Systems, Romulus, Michigan, US. \* Number of bees found dead on the room floor per day. (Colony weight ANOVA; d.f. = 23, F = 0.08, p = 0.7858).

## GH-2009; Honey bees



**Figure 13: Impact of Israeli acute paralysis virus (IAPV) infection on the honey bee (*A. mellifera*) hive health parameters in 2009.** Three bumble bee and three honey bees colonies were installed in each greenhouse room. While installing honey bee splits in the greenhouse it was ensured that one sister hive from each parent hive is installed in the virus and one in the control room. Each honey bee colony in the IAPV room was fed with 4 ml semi-purified IAPV solution (approximately  $1-3 \times 10^{10}$  viral genome equivalents) on day 11. Results are represented as Mean  $\pm$  SE. (ANOVA; adult bees  $F = 10.29$ , d.f. = 5,  $p = 0.0327$ ; capped brood  $F = 12.62$ , d.f. = 5,  $p = 0.0237$ ; pollen  $F = 2.12$ , d.f. = 5,  $p = 0.2193$ ; honey  $F = 4.95$ , d.f. = 5,  $p = 0.0902$ ).



**Figure 14: Impact of Israeli acute paralysis virus (IAPV) infection on the honey bee, *A. mellifera*, adult mortality in 2009.** Three bumble bee and three honey bees colonies were installed in each greenhouse room. While installing honey bee splits in the greenhouse it was ensured that one sister hive from each split is installed in the virus and one in the control room. Each honey bee colony in the IAPV room was fed with 4 ml semi-purified IAPV solution (approximately  $1-3 \times 10^{10}$  viral genome equivalents) on day 11 (denoted with + symbol). \* Number of bees found dead on the room floor per day.

### GH-2009; Honey bees

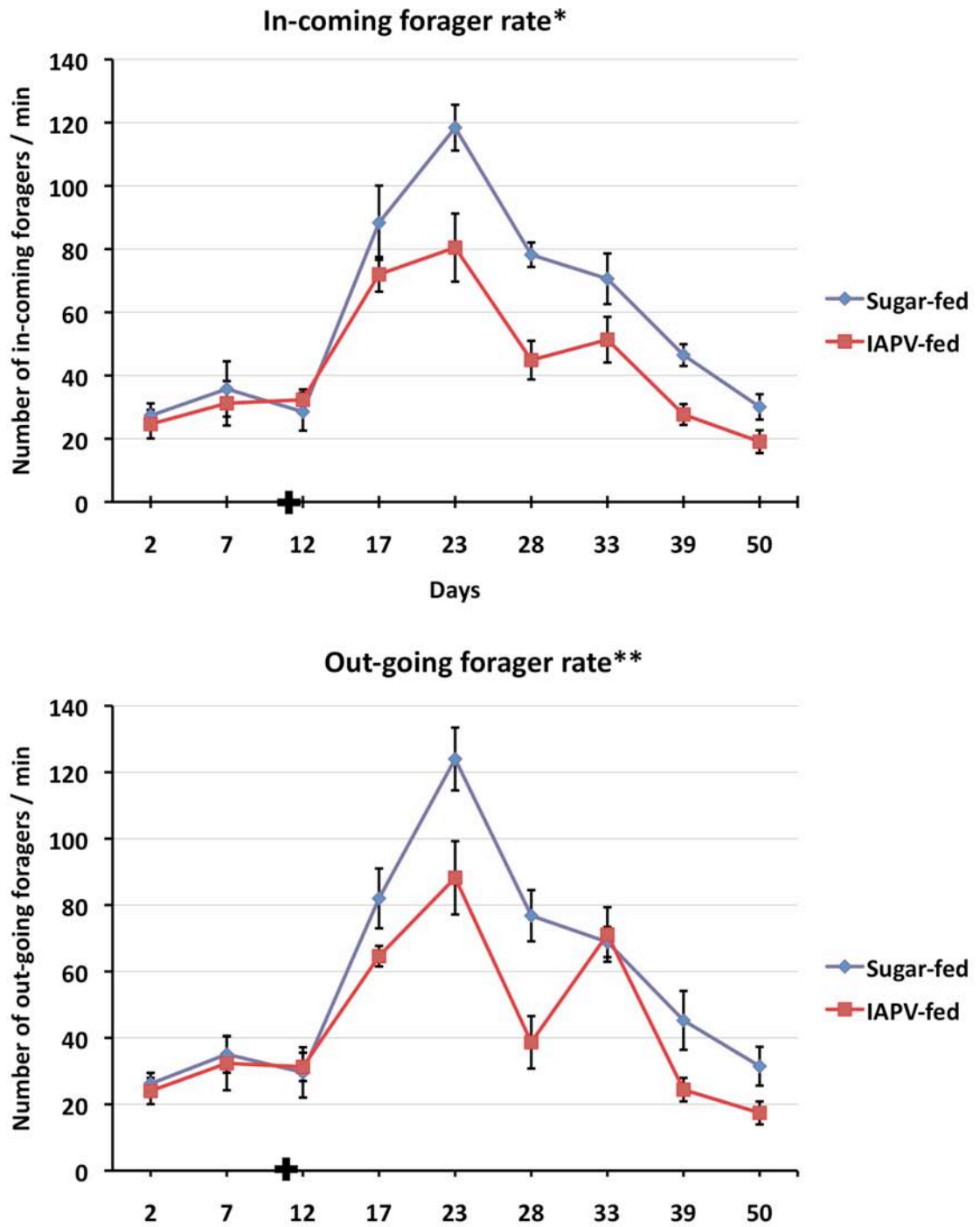


Figure 15: Impact of Israeli acute paralysis virus (IAPV) infection on the honey bee, *A.*

***mellifera*, foraging activity in 2009.** Three bumble bee and three honey bees colonies were installed in each greenhouse room. While installing honey bee splits in the greenhouse it was ensured that one sister hive from each split is installed in the virus and one in the control room. Each honey bee colony in the IAPV room was fed with 4 ml semi-purified IAPV solution (approximately  $1-3 \times 10^{10}$  viral genome equivalents) on day 11 (denoted with  $\blackspadesuit$  symbol). Results are represented as Mean  $\pm$  SE. Observations on incoming and outgoing foragers were made for 5 min each. \* Number of foragers entering the hive per min (ANOVA; before feeding IAPV F = 0.16, d.f. = 5, p = 0.7067; after feeding IAPV, on day 23 F = 8.56, p = 0.0430; day 28 F = 21.27, p = 0.0099). \*\* Number of foragers leaving the hive per min (ANOVA; before feeding IAPV F = 0.08, d.f. = 5, p = 0.7946; after feeding IAPV, on day 23 F = 6.08, p = 0.0693; day 28 F = 11.53, p = 0.0259).

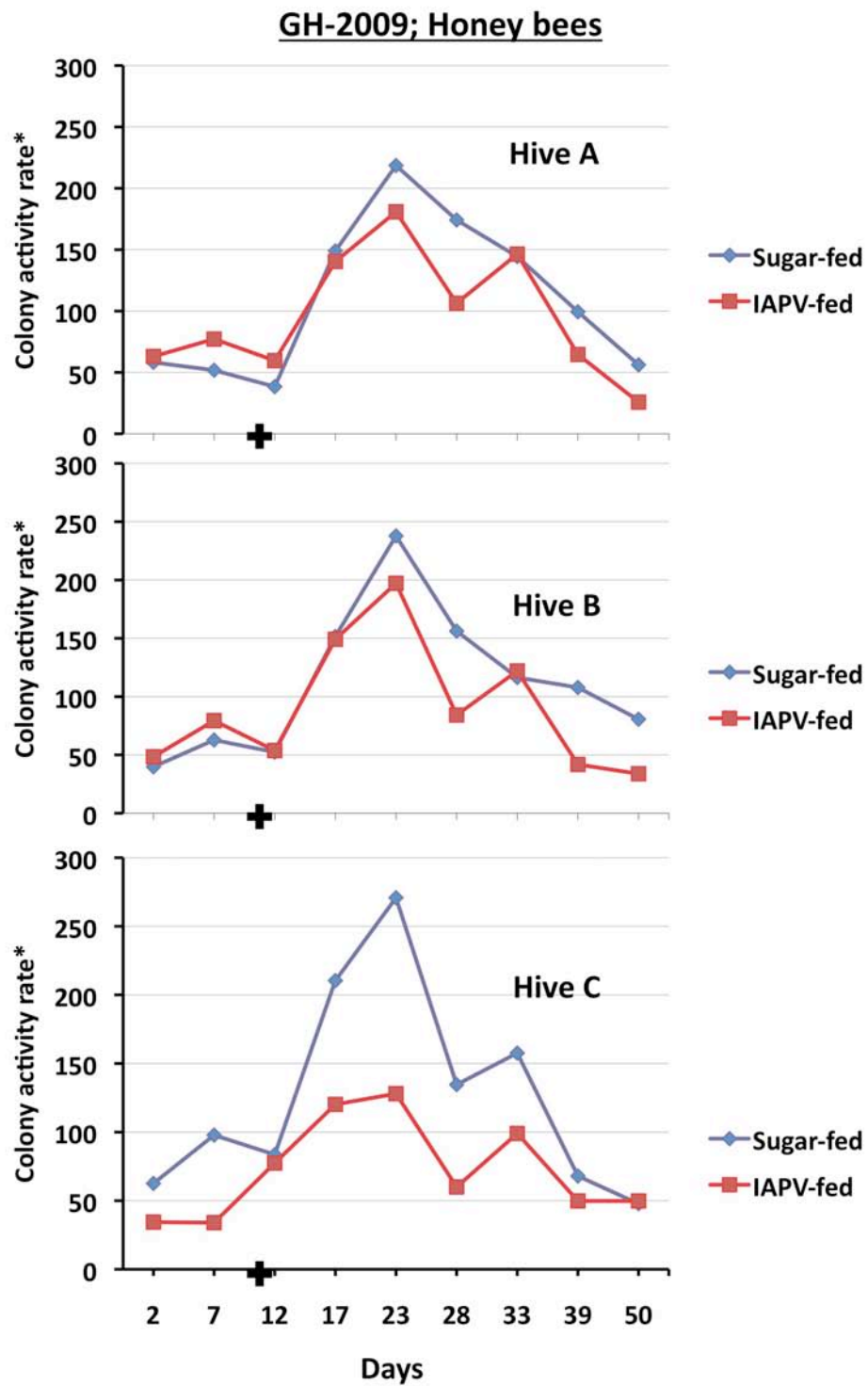
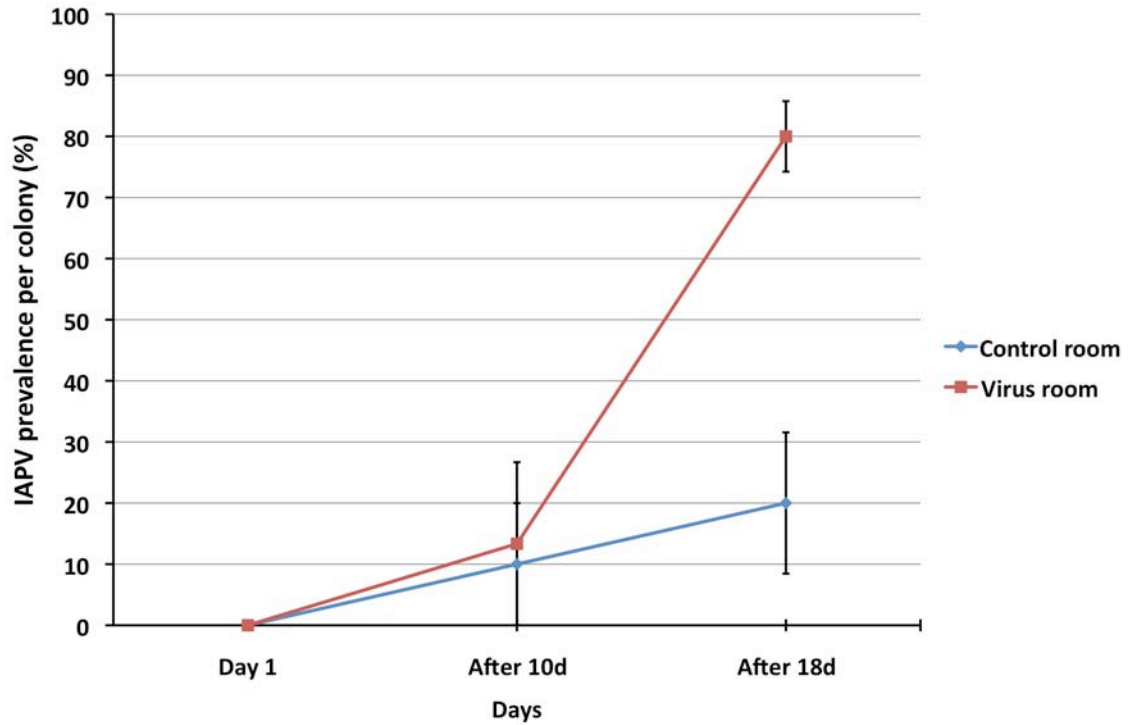


Figure 16: Pair-wise comparison between the foraging activity of the honey bee (*A. mellifera*) sister hives in the virus and control rooms. Three bumble bee and three honey bees

colonies were installed in each greenhouse room. While installing honey bee splits in the greenhouse it was ensured that one sister hive from each split is installed in the virus and one in the control room. Each honey bee colony in the IAPV room was fed with 4 ml semi-purified IAPV solution (approximately  $1-3 \times 10^{10}$  viral genome equivalents) on day 11 (denoted with **+** symbol). Observations on incoming and outgoing foragers were made for 5 min each. \* Number of foragers entering plus the number of foragers leaving the hive per min.





**Figure 17: Prevalence of Israeli acute paralysis virus (IAPV) in the honey bee, *A. mellifera*, hives over the course of 2009 greenhouse experiment.** Three bumble bee and three honey bees colonies were installed in each greenhouse room. Bumble bees colonies were already infected with IAPV at the beginning. Each honey bee colony in the IAPV room was fed with 4 ml semi-purified IAPV solution (approximately  $1-3 \times 10^{10}$  viral genome equivalents) on day 11 (denoted with  $\oplus$  symbol). Results are represented as Mean  $\pm$  SE.

## Chapter 4

### **Prevalence and Impact of RNA Viruses in Non-*Apis* Commercially Managed Bees Essential for Alfalfa Seed Production**

#### **Abstract**

Bees are intensely managed animals, given their pollination services; however, the stresses of intense management may expose them to a variety of infectious diseases. In honey bees (*Apis mellifera*), RNA viruses have emerged as a major problem over the last couple of decades and are linked to colony losses worldwide, but our knowledge of viral diseases in other managed bee species is very limited. A survey of the alfalfa leafcutting bee (ALCB) (*Megachile rotundata*) and the alkali bee (*Nomia melanderi*), the two major pollinators of the alfalfa seed crop in the U.S., was conducted in Utah and Washington State. Molecular diagnosis, using reverse transcriptase-polymerase chain reaction (RT-PCR) for seven different RNA viruses, revealed the presence of deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), black queen cell virus (BQCV) and sacbrood virus (SBV). When IAPV was fed to virus-free ALCB larvae, infections occurred but did not affect larval survival and development to the pre-pupal stage; however, the infections did disrupt diapause. A greater percentage of IAPV-fed larvae pupated without entering diapause and emerged as second-generation adults, than in the control treatment. IAPV infection significantly reduced the ALCB adult survivorship in both males and females, when acquired as newly emerged adults. IAPV was confirmed to be replicating in both the adult and larval stages, and the infection was confined to the gut in larvae. These results indicate that RNA viruses can infect and be detrimental to bee populations essential for alfalfa pollination, threatening the multi-million dollar seed industry. To our knowledge, this is the first report on the impact of IAPV infections on non-*Apis* pollinators.

## Introduction

Bees (Hymenoptera:Apiformes) are indispensable to our agricultural and natural ecosystems as they provide pollination services to the majority of the world's flowering plants that benefit from animal-mediated pollination [1]. The value of bees to North American agriculture in terms of pollination benefits to fruits, vegetables, nuts, oilseed and legumes, is estimated to be \$20 billion annually [2-4]. Although, honey bees (*Apis mellifera*) remain the species of choice for pollinating most of our crop monocultures, several other pollinators are far more efficient in particular cropping systems [5-7]. Moreover, the recent declines in honey bees and bumble bee (*Bombus spp.*) populations have further highlighted the need to diversify our pollination workforce [8-10].

Alfalfa, *Medicago sativa* L. (Fabaceae), is the third largest crop produced in the United States and a leading forage legume in the world, used primarily as hay to feed dairy cows and horses [11]. Although pollination is not important in hay production, the multi-million dollar, alfalfa-seed production industry that provides seed to hay growers depends heavily on bees for pollination. The alfalfa leafcutting bee (ALCB), *Megachile rotundata* F. (Apoidea: Megachilidae), and the alkali bee, *Nomia melanderi* Cockerell (Apoidea: Halictidae) are the two most effective commercially-managed pollinators for alfalfa seed production. While females of these two species pollinate up to 80% of visited flowers, other bees including honey bees are not as efficient because they gather nectar from the side of the flower to avoid being struck by the spring-like staminal column, bypassing pollen transfer [5].

The ALCB is native to Eurasia and was accidentally introduced into the United States, while the alkali bee is native to arid and semi-arid regions of the U.S., west of the Rocky Mountains. ALCB females use existing, above ground, natural or man-made cavities (e.g. holes drilled in wooden or polystyrene boards) to build linear nests with multiple cells made from leaf cuttings. Owing to its commercial use in alfalfa, this efficient pollinator has become the most

intensively managed solitary bee in the world and is valued second only to honey bees in field crop pollination [12]. The ALCB has also been used to pollinate other crops, such as canola and carrots for hybrid seed production, lowbush blueberries, and cranberries with varying degree of success. On the contrary, the once extensively used alkali bee, because of its special nesting requirements and other limiting factors, is now restricted to a few nesting sites, primarily in Washington and sparsely in some neighboring states [11,13]. Nonetheless, the alkali bee is still ecologically important and is the only ground-nesting bee in the world that is managed.

The ALCB revolutionized the alfalfa seed industry in the northwestern United States and central Canada. However, inundation of fields with unnaturally high bee densities (100,000-150,000 bees/ha) and gregarious nesting in man-made bee shelters [14], have resulted in intense interactions among the bees, potentially contributing to the spread and evolution of infectious pathogens [15]. The ALCB suffers heavily from chalkbrood, a disease caused by a fungus (*Ascosphaera aggregata*) that can result in up to 60% mortality in developing bee brood [16-18]. In addition, heavy (>50%) unexplained losses have been reported as broodless cells, cells with collapsed eggs, and dead larvae; the suspected causes include unidentified viruses and other pathogens [12].

Great strides have been made in understanding honey bee virology during the last few decades, but little is known of viral diseases of other managed bees. More than 18 viruses are known to infect honey bees, with the majority being positive-sense, single-stranded RNA viruses from the families *Dicistroviridae* and *Iflaviridae*, order Picornavirales [19-21]. RNA of these viruses serves both as a genome and as mRNA that is translated into polyproteins, which are cleaved into functional proteins at conserved proteolytic cleavage sites. These RNA viruses usually persist in strong healthy colonies as seemingly benign, persistent infections but multiply rapidly in immunologically challenged honey bees, leading to a manifestation of disease and population losses [22-24]. Particularly after the arrival of the parasitic mite (*Varroa destructor*),

RNA viruses have emerged as a major threat to honey bee health [25]. Elevated titers of multiple RNA viruses, including deformed wing virus (DWV), black queen cell virus (BQCV), Kashmir bee virus (KBV), and the recently described Israeli acute paralysis virus (IAPV), are found in honey bee colonies affected by colony collapse disorder [9,26,27]. There are increasing reports of bumble bees being infected with these RNA viruses originally thought to be associated only with honey bees [28,29]. Recent molecular analysis detected RNA viruses in as many as eleven non-*Apis* hymenopteran species in the U.S. [30]. The commonly-used phrase “honey bee viruses” seems to be a misnomer, since these RNA viruses are not restricted to *Apis*. The prevalence and impact of these viruses in specific crop-pollinator systems is currently unknown. The focus of this study was to determine the prevalence of RNA viruses in two commercially managed solitary bees, the ALCB and the alkali bee, and to study the impact of IAPV on the health of ALCBs. More specifically, we addressed the following questions: (i) Which RNA viruses infect ALCBs and alkali bees, and how prevalent are they in commercial field populations? (ii) Does IAPV replicate actively in ALCBs? (iii) Does IAPV have any impact on ALCBs when fed to larvae? (iv) Does the IAPV infection remain localized or does it become systemic in ALCB larvae? (v) What is the impact of IAPV on ALCBs when the virus was acquired as newly-emerged adults?

## **Materials and Methods**

### **Sample collection to determine virus prevalence:**

Thirty ALCB adults per field were collected from four separately owned alfalfa seed farms in Box Elder County, UT, USA, July 2010. Fifteen ALCB eggs, 15 larvae and five pollen provisions were also collected from one alfalfa field. Adults were collected with an insect collecting net, while eggs, larvae and pollen provisions were collected from nest cells from the field. Eggs and larvae were surface sterilized (procedures explained below in the larval bioassays section). Honey bees were collected from four commercial apiaries scattered in and around the

Box Elder and Cache Counties, UT, USA, with 50 bees removed from each of three hives per apiary. Honey bee samples were collected from inside the hives and were comprised mostly of adult workers. All adult bees were washed with sterile water, to remove any visible pollen, prior to RNA extraction from the whole bee (including the gut). Twenty ALCB adults per field, and 20 honey bee workers per hive, were analyzed individually. The remaining 10 ALCBs per field, and two batches of 15 honey bees per hive, were analyzed as pooled samples. ALCB eggs, larvae and pollen provisions were also individually analyzed for viruses. Alkali bee samples collected from bee beds in Walla Walla County, WA, USA, were provided by Douglas Walsh (Washington State University, Pullman, WA). In all, 20 adult and 15 larval alkali bees collected from four different farms were individually analyzed for viruses. All the samples were immediately frozen after collection and kept at -80° C until virus analysis, except the alkali bees. Dead alkali bee adults were stored at room temperature for 1-2 weeks before freezing and the larvae were stored in 70% ethanol. Ideally, samples should be frozen immediately when using a molecular diagnosis; however, RNA viruses can be successfully detected from bee samples stored in 70% ethanol or air-dried for several months (Singh and Nguyen, unpublished data).

#### **Virus detection:**

All samples were analyzed for the presence of seven RNA viruses: deformed wing virus (DWV), black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), sacbrood virus (SBV), Kashmir bee virus (KBV), acute bee paralysis virus (ABPV), and chronic bee paralysis virus (CBPV). The reverse transcriptase-polymerase chain reaction (RT-PCR) was used as a diagnostic tool, following the protocols of Singh et al. [30]. Total RNA was extracted from samples using TRIzol<sup>®</sup> reagent (Invitrogen). RNA concentrations were determined spectrometrically (Spectra Max 250, Molecular Devices), and cDNA was synthesized using random hexamers and M-MLV reverse transcriptase (Promega). RT-PCRs were performed with

specific primer sets and PCR cycles, followed by agarose gel electrophoresis using SYBR<sup>®</sup> Safe DNA gel stain (Invitrogen). The different primer sets and the gene regions they amplify are listed in Appendix D. Primers (bumble bee actin-F: GGAGAAACTTTGTTACGTCGCC; bumble bee actin-R: CGCACTTCATGATCGAGTTG) were used to amplify 218bp of actin gene, which served as an internal control for the quality of RNA extraction in all the samples. These actin primers were designed from *Bombus terrestris* mRNA sequence (GenBank accession no. J1045318) and have worked equally well for ALCBs, alkali bees and honey bees. A negative control lacking template DNA and a positive cDNA control were performed for each PCR reaction. Positive identification was confirmed by sequencing the PCR products.

#### **IAPV stock solution preparation:**

Semi-purified IAPV solution was prepared by crushing IAPV-infected honey bees in Bee Ringer's solution (0.156M NaCl, 0.003M KCl, 0.002M CaCl<sub>2</sub>) using 1 ml per bee. The homogenate was centrifuged at 2,000 g at 4° C for 3 min and passed through 0.2 µm NALGENE<sup>®</sup> syringe filters to remove bacterial or fungal contaminants and bee tissue. The filtrate was then passed through Amicon<sup>®</sup> Bioseparations- Microcon<sup>®</sup> Centrifugal Filter devices (Model YM-30, Regenerated Cellulose 30,000 MWCO) in batches of 500 µl, by spinning at 13,800 g at room temperature for 1 h, to remove any RNA fragments smaller than the intact virions and to concentrate the virus. The Microcon filters were then inverted in new tubes and washed by adding 50 µl of Bee Ringer's solution followed by spinning at 750 g for 2 min. The resulting semi-purified virus stock solution was pooled and stored at -80° C until used. Two sub-samples (100 µl) from stock solution were analyzed for seven RNA viruses using qualitative RT-PCR as described above. Agarose gel electrophoresis revealed strong band of IAPV and weak bands of DWV and BQCV, whereas no other virus was present. IAPV and DWV concentrations in the stock virus solution were then quantified with quantitative RT-PCR [26]. Quantitative RT-PCR

showed very high concentration of IAPV ( $1-2 \times 10^9$  viral genome equivalents/100  $\mu$ l of stock solution) and very low concentration of DWV ( $< 10^3$  viral genome equivalents/100  $\mu$ l of stock solution) in the virus stock solution.

#### **ALCB larval bioassays:**

ALCB eggs and pollen provisions were collected by placing nesting boards made of polystyrene blocks with multiple cavities lined with special paper straws (5.5 mm in diameter), in a nesting shelter in an alfalfa seed field in Box Elder County, UT, USA. After the bees were allowed to nest in the boards for 3-4 d, the nesting boards were removed and replaced. Straws were withdrawn from the boards and unwrapped to collect the cells. After removing the eggs, pollen provisions were transferred from the cells into 50 ml plastic tubes and sterilized to protect against chalkbrood disease, using  $\gamma$ -irradiation at a dose of 28 kGy for 12 h (courtesy of G. Hallman, USDA-ARS Subtropical Agricultural Research Center at Weslaco, TX, USA). Eggs were washed with a sterile disinfectant (Aquify<sup>TM</sup>, Novartis Co., Duluth, GA), followed by three serial rinses with a sterile saline solution (Saline Solution<sup>TM</sup>, Western Family Foods, Inc., Portland, OR), and then placed on sterile agar media until transferred to the  $\gamma$ -irradiated pollen provisions in 96-well tissue culture plates [31,32].

IAPV was administered to larvae by mixing 2.25 ml semi-purified IAPV solution (approximately  $3-5 \times 10^{10}$  viral genome equivalents or the viral load of approximately 12 honey bees) into 50g irradiated pollen provision. The virus-laden pollen provision mix was then distributed into sterile 96-well tissue culture plates (125-150 mg/well containing approximately  $7.5-12.5 \times 10^7$  IAPV genome equivalents), using a 20ml syringe. At the same time, another set of 96-well plates received a mixture of irradiated pollen and 2.25 ml sterile Bee Ringer's solution without virus, as a control. Three 96-well plates were established per treatment, with each plate serving as a replicate. A single disinfected egg was then carefully paced on the surface of pollen



in each well. The plates were incubated at 29°C and approximately 70% relative humidity, which was maintained by placing a water-filled beaker in the incubator. After 24 h, all eggs were examined under a dissection microscope, and any collapsed eggs were replaced. Data on larval mortality, cocoon formation, adult emergence and disease symptoms were recorded.

Five living larvae were collected at random from each replicate at 5 and 10 d post-treatment, for molecular analysis to track viral infection. Ten-day old larvae, which were mostly late fifth-instars, were shifted to sterile artificial diet for 24 h, to clear their guts of virus-laden pollen. All these larvae were surface sterilized with 1% chlorine solution (sodium dichloro-s-triazinetriane dihydrate, Quantum Biochemical, Alpharetta, GA), followed by three rinses in sterile distilled water [33] and frozen at -80° C until analyzed.

#### **ALCB adult bioassays:**

For adult bioassays, ALCB cocoons were obtained from a local alfalfa seed producer in Box Elder County, Utah, USA and were incubated at 29° C. Newly emerged adults were separated daily by sex and randomly assigned into groups of 21 bees in feeding cages made from paper cups [34]. Replicate cages (for males eight replicate cages each in virus treatment and control, and for females six replicate cages each in virus treatment and control) were established over time, with each replicate comprised of a cohort of bees that emerged on the same day. In the virus treatment, 370 µl of semi-purified IAPV solution (approximately 5-8 x 10<sup>9</sup> viral genome equivalents or the viral load of approximately two infected honey bees) was fed to the adults in each cage in 9 ml of 50:50 sucrose:water solution. Bees were allowed to feed on the virus-laden sucrose solution for 10 days and then shifted to a clean sucrose solution. The control consisted of bees fed on 370 µl sterile Bee Ringer's solution without virus in 9 ml 50% sucrose solution for 10 days, followed by sucrose solution only. The cages were kept at 29°C, 70% relative humidity, and a 12:12 h (L:D) photoperiod. Five days post-treatment, three living bees were collected from each

cage for viral analysis. Bees were observed over a period of two months, recording daily mortality, any disease symptoms and unusual behavior. Dead bees were collected daily.

#### **Selective RT-PCR for confirmation of active infection:**

To determine if IAPV was actively multiplying in ALCBs, selective RT-PCR was performed both on larvae and adults from the bioassays, following Ongus et al. [35], with modification. cDNA was synthesized from negative-sense RNA using only a forward IAPV primer, instead of the random primers used in our RT-PCR described above. In the PCR reaction to detect the IAPV structural polyprotein gene, two different sets of primers were used, the short IAPV Capsid primers that amplify a 368 bp fragment and the IAPV Capsid1 primers that amplify 840 bp fragment (Appendix C). Both sets of primers worked equally well, but only the data for short primers are shown. Along with standard DNA template controls (both positive & negative), each sample had a no-RT control where the reverse transcription step was omitted. Using the same RNA sample, a regular cDNA synthesis with random hexamers was performed and actin mRNA detected as described above to check the RNA integrity.

When RNA samples were treated with DNase prior to RT-PCR using RQ1 RNase-free DNase (Promega), according to manufacturer's protocol, detection sensitivity was decreased; therefore, the RT-PCR was conducted without DNase treatment. No differences were observed in the presence/absence in the negative strand bands, only the band brightness was greatly reduced after DNase treatment (data not shown).

#### **Larval dissections:**

Fifth-instar ALCBs were dissected to determine whether IAPV infection was restricted to the gut or if the virus had disseminated throughout the body, i.e. became systemic. Thin slices of frozen larval tissue (Figure 3) were carefully removed using sterilized surgical scalpels, to avoid

any gut tissue. Larvae were kept frozen on dry ice during the entire dissection process. The shavings were comprised of epidermal tissue, hemolymph, fat body and muscle, but no gut tissue. The remainder of the larva had gut tissue along with the other larval tissues. These two fractions of larval components (shavings & remainder) from each dissected larvae, were analyzed separately for IAPV with RT-PCR.

### **Statistical Analysis:**

*JMP*<sup>®</sup> 8.0.2 statistical package (SAS Institute Inc. 2009) was used to analyze the data. Virus prevalence was compared among ALCB populations using Pearson's Chi-square ( $\chi^2$ ) test. The larval mortality, cocoon formation and adult emergence data were compared between infected versus control bees using ANOVA. Adult mortality data were subjected to survivorship analysis using Parametric Survival Fit with a LogNormal distribution, comparing the failure probability/ probability of death between infected versus control males and females using the Likelihood Ratio Chi-square ( $\chi^2$ ) test.

## **Results**

### **Which RNA viruses infect ALCBs and alkali bees, and how prevalent are they in commercial field populations?**

Molecular diagnosis for seven different RNA viruses detected some viral species in both ALCBs and alkali bees (Table 1). KBV, ABPV and CBPV were not detected in any of the samples. Adult ALCBs tested positive for DWV, IAPV and BQCV, while DWV was the only virus detected in field-collected, surface-sterilized ALCB eggs (13%) and larvae (20%). In addition, DWV and IAPV were detected in ALCB pollen provisions collected from alfalfa fields. In our alkali bee survey samples, both the adult bees and larvae were detected positive for DWV and IAPV, while BQCV and SBV were detected only in adults.

In comparison to relatively low virus prevalence in these two solitary bee species, honey bee workers collected from four apiaries in the same area were heavily infected with RNA viruses (Table 1). Multiple viral co-infections were also more common in honey bees than the other two bees. More than 60% of the honey bees were infected with three or more viruses; whereas, very few ALCBs or alkali bees were detected with multiple viruses, and none of the solitary bees had more than two co-occurring virus species. Furthermore, the concurrent presence or absence of honey bees in the alfalfa fields along with ALCBs had varying impact on the prevalence of RNA viruses in ALCBs (Table 2). Except for IAPV, which was slightly higher in ALCB populations where honey bees were present (Pearson's  $\chi^2 = 4.12$ ;  $p = 0.0425$ ), no significant difference was observed in the prevalence of any other virus (DWV, Pearson's  $\chi^2 = 1.39$ ,  $p = 0.2392$ ; BQCV, Pearson's  $\chi^2 = 1.05$ ,  $p = 0.3049$ ).

#### **Does IAPV replicate actively in ALCBs?**

Active infections of IAPV were detected in virus-fed ALCBs. The negative-sense IAPV RNA was detected in ALCB larvae and adults (Figure 1) using a specific forward primer to reverse transcribe RNA to cDNA. In the no-RT controls (where the reverse transcription step was eliminated) no PCR product was observed, indicating that the product obtained with the negative strand RT-PCR reaction was derived from RNA. Sequences obtained from the RT-PCR products matched those of the IAPV structural polyprotein in the GenBank, confirming the identity of IAPV.

#### **Does IAPV have any impact on ALCBs when fed to larvae?**

All the virus-fed larval samples (5 larvae per replicate) collected 5 and 10 d post-treatment for virus analysis using RT-PCR tested positive for IAPV infections, while none of the control larvae had IAPV. Around 20% of the larvae also had DWV with no difference between the

treatments. None of the larvae had BQCV, KBV, SBV, ABPV or CBPV. Low levels of DWV and BQCV present in the virus solution, seems to be unable to cause any infection. IAPV infections in ALCB larvae had no impact on larval mortality (d.f. = 5; F = 0.30; p = 0.6108) (Figure 2). Further, the viral infections did not impact larval development as measured by successful cocoon formation (d.f. = 5; F = 0.67; p = 0.4596). However, a significantly greater percentage of virus-infected larvae ( $49.4 \pm 2.7\%$ ) pupated and then emerged as second-generation adults, as compared to uninfected control larvae ( $35.7 \pm 5.3\%$ ), most of which entered into diapause as prepupae (d.f. = 5; F = 15.65; p = 0.0167).

#### **Does the IAPV infection remain localized or does it become systemic in ALCB larvae?**

Dissection of infected ALCB larvae into two components with and without gut tissue (Figure 3) and subsequent molecular analysis suggested that IAPV infection remains restricted to larval gut tissue. No RT-PCR product was observed in larval shavings that lacked gut tissue (lanes 2-9, Figure 4); whereas, strong PCR reactions for IAPV were observed in remaining larval sample containing gut tissue (lanes 10-17). Actin was equally amplified in all the components, indicating the presence of good quality mRNA in all samples.

#### **What is the impact of IAPV on ALCBs when the virus was acquired as newly-emerged adults?**

When newly emerged (24-h post-emergence) ALCB adults were fed IAPV, both males and females had a significantly shorter life-span than the untreated bees (Fig. 5) (Treatment  $\chi^2 = 12.93$ , p = 0.0003; Sex  $\chi^2 = 79.96$ , p < 0.0001; Treatment x Sex  $\chi^2 = 0.17$ , p = 0.6828). RT-PCR analysis of samples collected 5 d post-treatment (3 bees per cage; total N= 42 bees per treatment) revealed 100% IAPV infection among virus-fed bees, while none of the control bees tested positive for IAPV. Around 25% bees also had DWV with no difference between the treatments.

50% mortality was observed around 25 d post-treatment in infected females, compared to more than 30 days in untreated control females. Viral infection similarly affected male survivorship; however, males (both infected and uninfected) lived for significantly less time than the females. Some degree of hair loss and integument blackening was observed in IAPV-infected bees after one week of infection.

## Discussion

We report the first molecular detection of single-stranded RNA viruses from ALCBs and alkali bees. Our study corroborates the microscopic- and serologically-based report of small isometric ssRNA like viruses from ALCBs in 1980; however, unlike Hackett [36], we did not detect any CBPV in our samples. Our results support the recent literature depicting a much broader host range of these RNA viruses than previously anticipated [28,30,37]. This study also provides evidence for active infections of RNA viruses in non-*Apis* bees. Like most other viruses associated with honey bees, IAPV has a positive-sense single-stranded RNA genome, with a complementary negative-sense RNA transcribed in the host during replication and used as a template for viral genome multiplication. A successful infection occurs when virus replicates, and the detection of the negative-strand RNA intermediates in an infected host serves as a marker for active virus multiplication [38]. We detected replication of IAPV in ALCBs. Previously, DWV and IAPV have been shown to replicate in honey bees and the parasitic mite (*V. destructor*) that vectors these viruses [35,39-42].

To our knowledge, this is the first experimental study looking at the consequences of IAPV infections in non-*Apis* bees. Larval and adult bioassays were performed on ALCBs to evaluate the virulence and effects of IAPV infection, one of the many factors associated with honey bee colony collapse disorder. IAPV infection disrupted ALCB diapause resulting in increased second-generation adult emergence, when virus was fed to ALCB larvae, and IAPV

also caused premature mortality in infected adults. The effect of IAPV on ALCBs diapause is very intriguing and may have significant impact on ALCB's pollination capability in the field. Typically the ALCB overwinters as a post-feeding, diapausing larva, called the prepupa, in a tough silken cocoon. The larvae typically pupate and emerge as adults in late spring or early summer. However, in US populations, up to 40% of the brood may avert diapause and emerge as adults in the same season in which they were produced, thus becoming bivoltine [43]. The precise mechanism behind this facultative bivoltinism is currently unknown; however, several factors such as long photoperiod, high temperatures, nutritional stress and maternal inheritance have been suggested as possible elicitors for bivoltinism [12]. Our results indicate that IAPV infections can disrupt diapause, in addition to these other factors. More research is needed to thoroughly understand the physiology behind the role of all viral infections detected in ALCB field populations in diapause disruption.

The second-generation females contribute little in terms of pollination and progeny rearing, since when they emerge the floral resources are scarce and their larvae cannot complete their development before weather conditions become too cold [12]. The impact of IAPV infections on ALCB is likely to exceed the approximate 14% reduction in numbers of bees entering diapause and the loss of overwintering bees. Second-generation adults can influence the epidemiology of chalkbrood disease by increasing disease transmission [12]. The ALCB is a linear-cavity nester; the emerging second-generation adults will chew through any chalkbrood-infected cadavers on their way to the nest entrance, disseminating the fungal spores to their brood and other bees. Normally such spore-filled cadavers would be removed during the fall/winter cleanup commonly-practiced by producers under the loose-cell, ALCB management system [44,45]. Since chalkbrood is highly virulent in the developing brood, any increase in disease adversely impacts the success of progeny rearing. Our data help to explain in part the poor

reproductive output of ALCBs in the United States, which currently requires farmers to import bees from Canada to restock their fields every year.

IAPV significantly reduced the survival of infected ALCB adults, having direct consequences on pollination services and reproductive success. ALCB females nest for about 6-8 weeks under field conditions, constructing 1-2 brood cells complete with eggs per day [46,47]. Shortening the adult life-span translates both into loss of reproduction and effective pollination workforce in the field; this means that more bees will be required to achieve the same levels of pollination as uninfected bees, making pollination economically more challenging. U.S. farmers are currently using 100,000-150,000 bees/ha in their fields. If IAPV prevalence increases, this number may need to be further increased to achieve the same seed set. Other health problems, such as chalkbrood, pesticide exposure, poor nutrition and adverse climate conditions may also interact with the viral infections in bees in the field, compounding the problem. Our results are similar to what has been found in honey bees, where IAPV caused adult mortality and had a debilitating impact on honey bee colony vigor and survival [48,49]. Infected ALCB adults exhibited some hair loss, but we did not observe any spasms or paralytic symptoms in infected ALCB adults, characteristics of an IAPV infection in honey bees [50].

In ALCB larvae, IAPV infection was restricted to the gut tissue, which may explain the lack of larval mortality. Diapause disruption by IAPV in our larval bioassays may be an indirect consequence of gut infection, perhaps impairing digestion and nutrient uptake. Nutrition is thought to affect diapause in other insects, and may even have played a role in the evolution of sociality in the Hymenoptera [51]. Further, virus infection in the larval gut may predispose larvae to other pathogens that invade via the gut. Little research has been done to determine how RNA viruses disseminate in different stages of bees. KBV, a close relative of IAPV, infects a variety of tissues in honey bee pupae, including the gut epithelium, musculature, hemocytes, oenocytes and tracheal epithelium [52]. Given the impact of IAPV on ALCB adult survivorship, it is highly



likely that IAPV infection disseminated throughout the adult body, unlike the infection in larvae. Our contention is supported by previous studies on other Picornaviruses including DWV, cricket paralysis virus, aphid lethal paralysis virus and *Solenopsis invicta* virus, where the virus was detected in various body tissues away from the gut [39,53-55]. In our research (Singh et al. unpublished data), we found IAPV virus to disseminate from the midgut to other tissues in bumble bee queens, including ovaries and eggs.

RNA viruses spread in honey bee colonies using both horizontal transmission routes (mediated by varroa mites, contaminated food and equipment) and vertical transmission routes (from queen and drones to offspring) [56]. However, unlike honey bees, these solitary bees do not share food between adults via stomodeal trophallaxis and are not parasitized by varroa mites. Detection of virus in field-collected pollen provisions of ALCBs suggests that the pollen may be a major route of virus transmission in alfalfa pollinators. This is supported by previous detection of RNA viruses in pollen loads of honey bees [57] and our own recent work [30] providing evidence for the role of pollen in the epidemiology of viruses in the pollinator community. Further, the detection of DWV in surface-sterilized eggs of ALCBs suggests that vertical transmission may also be important in these bees. The much lower prevalence of RNA viruses in ALCBs and alkali bees than in honey bees could be due to many factors, including differences in bee life cycles, bee susceptibilities, or how the viruses are transmitted. More research is needed to understand the importance of different transmission routes in the epidemiology of these viruses, and how this might affect the pollinator community, particularly for viruses with a broad host range, such as the DWV, IAPV and BQCV.

In conclusion, this study provides evidence for RNA virus infections in and their impact on non-*Apis* bees and may help explain in part the problem of local sustainability of ALCB populations in the United States. We also provide more insight into the pathogenicity and epidemiology of RNA viruses in bees.

## **Acknowledgements**

The help of Ellen Klinger, Craig Huntzinger and Miranda Trostle in Rosalind James's lab, Logan, UT is greatly appreciated in collecting field bee samples and in setting-up lab larval bioassays. We thank R. Rivas and G. Hallman from USDA-ARS Subtropical Agricultural Research Center at Weslaco, TX, USA, for  $\gamma$ -irradiation of bee pollen. Help of Amanda Mahoney, Penn State University, in designing bumble bee actin primers is also appreciated. We thank Douglas Walsh from Washington State University, for providing alkali bee samples. We also appreciate the cooperation of alfalfa seed growers and a honey beekeeper from Box Elder and Cache Counties, UT, for allowing us to collect bee samples from their fields and bee yards.

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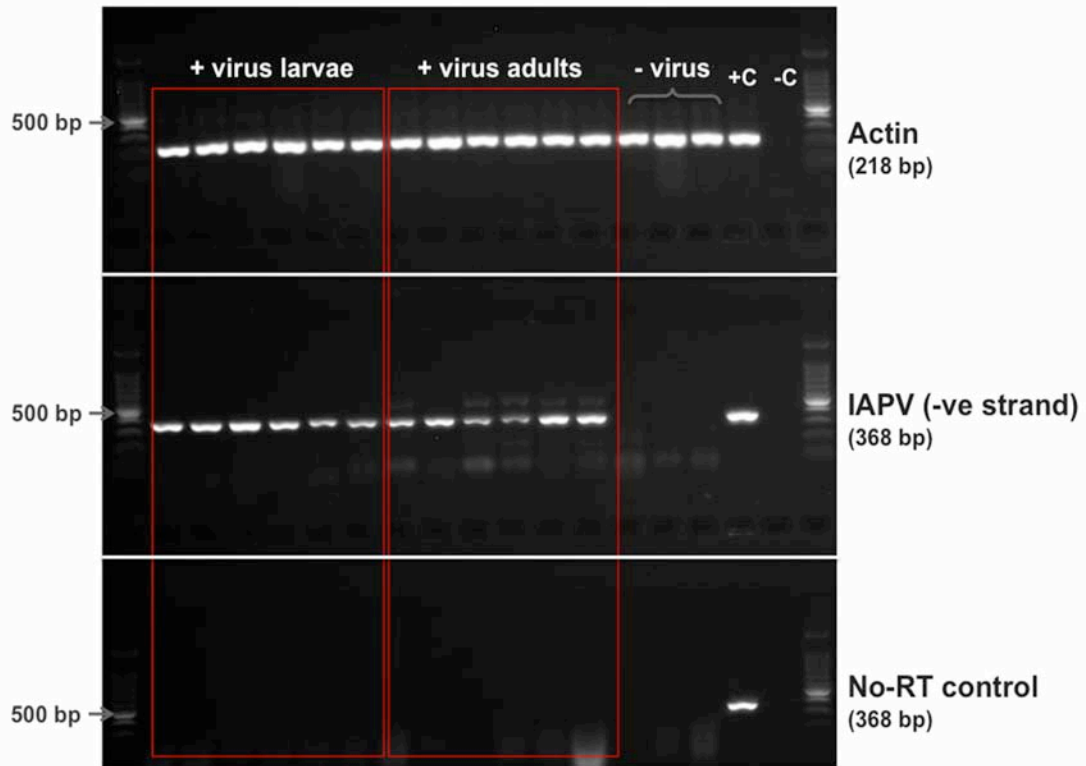
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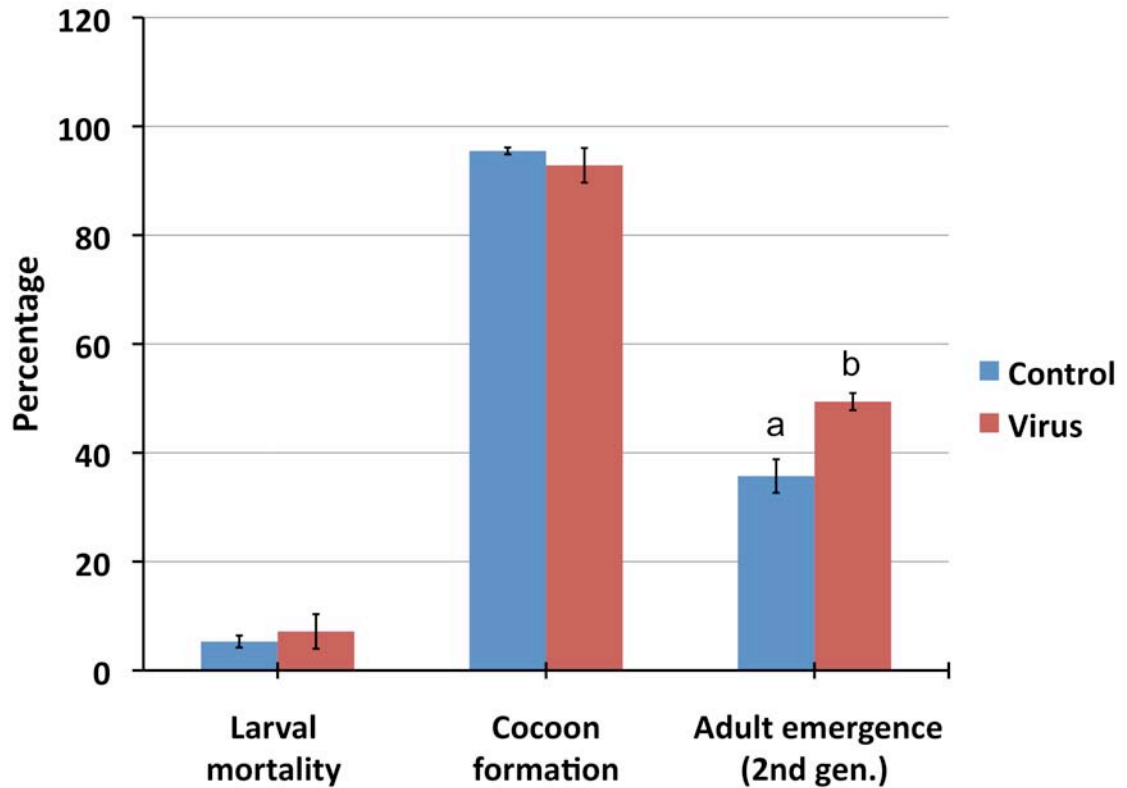
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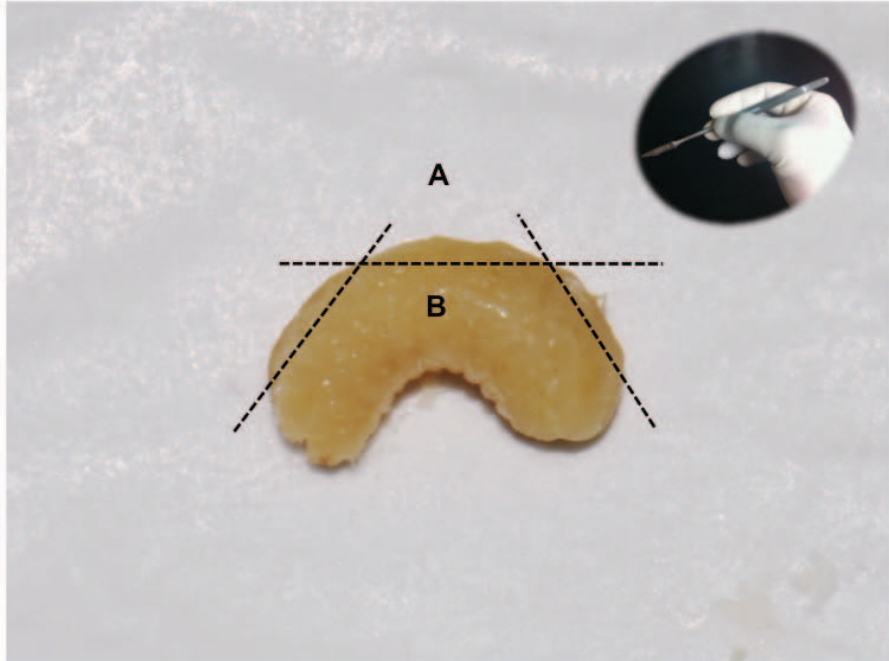




**Figure 1: Detection of replicative form or negative strand of Israeli acute paralysis virus (IAPV) in the alfalfa leafcutting bee, *Megachile rotundata*.** For each sample, cDNA was synthesized from negative-sense RNA by using an IAPV forward primer (IAPV short-F). PCR reactions were performed using IAPV short forward and reverse primers that amplify 368 bp of IAPV structural polyprotein gene, followed by gel electrophoresis. Actin was used as an internal control for the quality of RNA extraction in all the samples. +C = positive control (cloned IAPV cDNA); -C = negative control; **No-RT control** = control where the reverse transcription step was omitted and PCR performed directly on the mRNA sample; **+ virus larvae** = larvae from virus treatment collected 5 days post-treatment (lanes 2-4) and 10 days post-treatment (lanes 5-7); **+ virus adults** = adults from virus treatment collected 5 days post-treatment (lanes 8-13); **- virus** = larvae from control treatment (lanes 14-16).



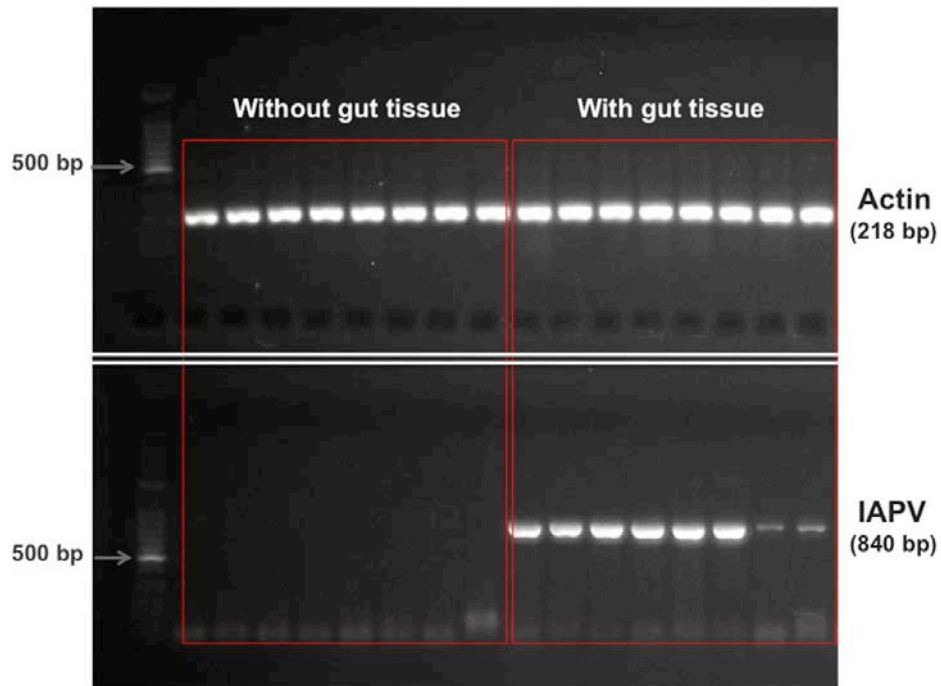
**Figure 2: Impact of Israeli acute paralysis virus (IAPV) on the alfalfa leafcutting bee, *Megachile rotundata*, when fed to larvae.** IAPV was fed to ALCB larvae in  $\gamma$ -irradiated pollen, in sterile 96-well tissue culture plates. Bees fed on irradiated pollen but no virus, served as a control. Larvae were incubated at 29°C and 70% RH. (ANOVA; larval mortality d.f. = 5, F = 0.30, p = 0.6108; cocoon formation d.f. = 5, F = 0.67, p = 0.4596; adult emergence d.f. = 5, F = 15.65 p = 0.0167).



**A= Shavings** (Epidermal tissue, fat body, hemolymph, muscles, but **no gut tissue**)

**B= Remainder** (All larval tissues including the gut)

**Figure 3: Pictorial description of the alfalfa leafcutting bee, *Megachile rotundata*, larval dissection.** Thin slices of larval tissue (indicated by dotted lines) were carefully shaved from frozen fifth-instar ALCB larvae on the dorsal surface using sterile surgical scalpels.



**Figure 4: Presence or absence of Israeli acute paralysis virus (IAPV) in the alfalfa leafcutting bee (*Megachile rotundata*) larval tissue, with and without gut.** Larval tissues with and without gut (from experimentally infected larvae) were analyzed for IAPV with RT-PCR using IAPV capsid1 primers to amplify 840 bp of IAPV structural polyprotein gene. Actin was used as an internal control to indicate the quality of RNA extraction in the samples.

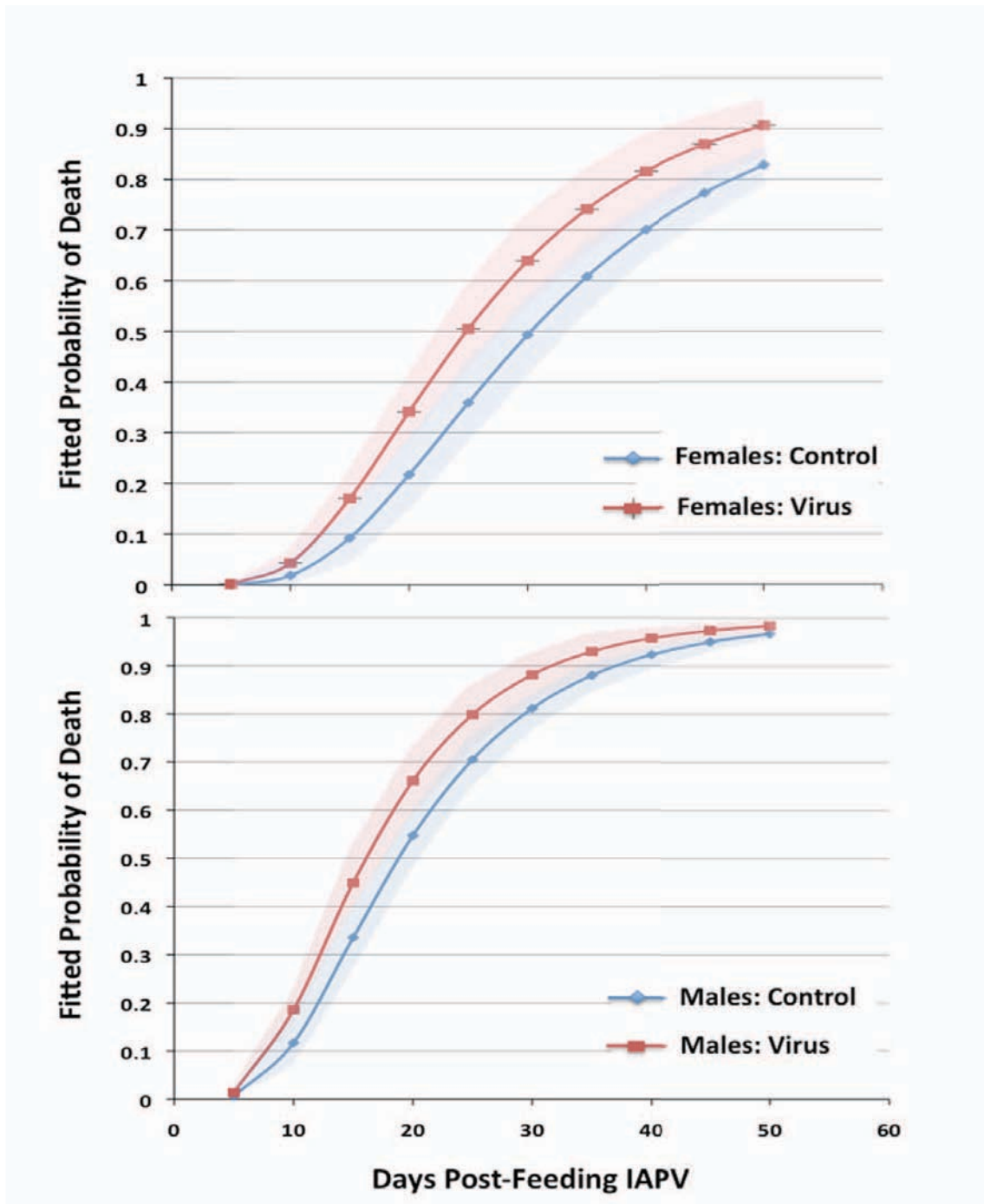


Figure 5: Impact of Israeli acute paralysis virus (IAPV) on the alfalfa leafcutting bee, *Megachile rotundata*, when the virus was acquired as newly-emerged adults. IAPV was fed to newly-emerged ALCB adult males and females, in 50% sucrose solution. Controls were fed only 50% sucrose. Bees were kept at 29°C, 70% RH and 12:12 h (L:D) photoperiod. Daily

mortality data were recorded and subjected to survivorship analysis using Parametric Survival Fit with a LogNormal distribution, comparing the failure probability between infected versus control males and females using the Likelihood Ratio Chi-square ( $\chi^2$ ) test (Treatment  $\chi^2 = 12.93$ ,  $p = 0.0003$ ; Sex  $\chi^2 = 79.96$ ,  $p < 0.0001$ ; Treatment x Sex  $\chi^2 = 0.17$ ,  $p = 0.6828$ ). In **Red**: probability of death,  $\pm$  95% confidence intervals in IAPV-fed treatment; in **Blue**; probability of death,  $\pm$  95% confidence intervals in control treatment.

**Table 1: Prevalence of RNA viruses in alfalfa (*Medicago sativa*) pollinators, collected from Utah and Washington State.**

Bee species	Virus Prevalence (% bees)				# Co-infections <sup>o</sup>		
	DWV	IAPV	BQCV	SBV	2	3	4
<b>ALCB</b>	17.5 ± 13.2*	12.5 ± 15.5	5.0 ± 7.1	0	7.5	0	0
<b>Alkali bee</b>	34.3**	17.1	2.9	2.9	5.7	0	0
<b>Honey bee</b>	87.1 ± 17.0*	62.9 ± 33.8	89.9 ± 10.9	20.0 ± 40.1	27.5	57.5	6.3

ALCB: alfalfa leafcutting bee; DWV: deformed wing virus; IAPV: Israeli acute paralysis virus.  
BQCV: black queen cell virus; SBV: sacbrood virus.

None of the samples were positive for Kashmir bee virus (KBV), acute bee paralysis virus (ABPV) and chronic bee paralysis virus (CBPV).

\* Mean ± SD; adults only; 20 ALCB adults per field from 4 separately owned farms; 20 honey bee workers per hive, 3 hives per apiary, and 4 apiaries.

\*\* Combined sample of adults & larvae (N=35).

<sup>o</sup> % bees infected with multiple viruses (2, 3 or 4 virus species).

**Table 2: Variation in the prevalence of RNA viruses in alfalfa leafcutting bee (*Megachile rotundata*) and honey bee (*Apis mellifera*) populations collected from four locales in Utah State.**

				Virus Prevalence (%)		
Farm	Location	Bee Species	Foraging Situation	DWV	IAPV	BQCV
A	Corinne, UT	ALCB	ALCB alone	10	10	0
B	Elwood, UT	ALCB	ALCB + HB	5	35	0
B	Elwood, UT	HB	ALCB + HB	85	55	95
C	Riverside, UT	ALCB	ALCB alone	35	0	15
D	Bear river city, UT	ALCB	ALCB + HB	20	5	5

DWV: deformed wing virus; IAPV: Israeli acute paralysis virus; BQCV: black queen cell virus.

ALCB: alfalfa leafcutting bee; HB: honey bee; UT: state of Utah in the United States.

ALCB + BH: alfalfa leafcutting bees and honey bees foraging together

None of the samples were positive for sacbrood virus (SBV), Kashmir bee virus (KBV), acute bee paralysis virus (ABPV) and chronic bee paralysis virus (CBPV).

N = 20 for ALCBs for each location, and N = 60 for HBs.



## Chapter 5

### Conclusion

Knowledge of the degree of host specificity is important to the understanding of pathogen transmission dynamics. This thesis provides strong evidence that the so-called ‘honey bee viruses’ are not restricted to the genus *Apis*. Detection of different RNA viruses in as many as nine non-*Apis* bee species and four species of wasps indicates broad occurrence of viruses in hymenopteran insects visiting the flowering plants. Notably, multiple virus species were found co-infecting non-*Apis* pollinators as well as honey bees in this study, which corroborate many reports of multiple viral co-infections in honey bees throughout the world [1-3]. This is particularly important since elevated levels of multiple viruses have been found associated with CCD colonies [4,5]. Importantly, IAPV was detected only in non-*Apis* pollinators collected near IAPV-infected apiaries in the initial survey, suggesting a possible spread of this virus from honey bees into other pollinators species. Alternatively, this virus may have spilled over from some other wild bee species that we did not sample. Phylogenetic studies could be used to improve our understanding of origin and the initial spread of the virus.

Our data also indicates a wide prevalence of RNA viruses in commercial bumble bee colonies. The prevalence and spread of RNA viruses, especially DWV and BQCV, in bumble bee colonies was similar to honey bees in the United States [3,6]. In comparison to honey bees and bumble bees, the virus prevalence was lower in ALCBs and the alkali bees, which could be due to several factors, including differences in bee life cycles, sociality, interspecies interactions, different virus transmission routes, and the kinds of flowers visited. Both honey bees and bumble bees are social insects that live in colonies that function as a single unit and are active for several months in a year. Close interactions among colony members provide favorable environment for the spread of infections. On the other hand, ALCBs and alkali bees are solitary and are active for

only couple of months. The potential for pathogen transmission between honey bees and bumble bees is very high due to the geographical proximity, close relatedness and frequent sharing of floral resources. Oral transfer of food between nest mates (trophallaxis), common in eusocial Apinae including honey bees and bumble bees [7], is another important variable that can impact virus prevalence in bee populations via high within colony transmission. Honey bees have more frequent trophallaxis in comparison to bumble bee [8]; whereas, ALCBs and alkali bees do not share food. In addition, unlike most other flowers, alfalfa flowers are usually only visited once, then they are tripped, do not replenish their nectaries, and quickly senesce, reducing the chances of pathogen spread. Since bumble bee colonies and to a limited extent alfalfa leaf-cutter bees are shipped across large geographical distances, our findings reflect a real threat for the spread of new or more virulent strains of these pathogens. More research is needed to understand the importance of different transmission routes in the epidemiology of these viruses and how this might affect the pollinator community.

I found that pollen serves as one of the routes of inter-taxa transmission of RNA viruses among hymenopteran pollinators. Molecular detection of DWV, BQCV and SBV in pollen loads of forager bees, and the subsequent phylogenetic analyses of these viruses support my theory that RNA viruses are disseminating freely among the pollinators via the pollen itself. Moreover, DWV and SBV were detected in the pollen loads of uninfected bees, directly implicating pollen as a route of transmission of viruses into healthy colonies. That honey bees can serve as a carrier for some pollen-borne plant viruses by moving pollen from plant to plant, has been previously demonstrated [9,10]. In addition, other recent studies also support my theory. One such study has shown higher concentration of DWV in foraging workers of *B. huntii* than in males, implying a possible association between the virus infections and foraging activities [11]. Another study, which tracked the pathogen load in honey bee colonies over the season, has also found peak infection of common honey bee viruses during the months when bees are actively

foraging [12].

Further, the pathogenicity of RNA viruses in pollen (bee bread) stored in the honey bee hives was experimentally demonstrated in this study. Despite an exposure to sun and desiccation, viruses in stored pollen remained infective when fed to virus-free honey bees, even after six months of storage under ambient conditions. Pollen-borne plant viruses transmitted by honey bees during pollination are also known to remain infective for several weeks after being stored in the bee bread in the hive [13]. More information on how long different RNA viruses can remain infective in stored pollen and honey would be highly valuable for beekeepers.

Although the pollen that tested positive for the virus was not associated with any particular plant species, the possibility of plants serving as the reservoirs of these viruses cannot be ignored at this point. The order Picornavirales contains viruses infecting plants and animals, including humans [14,15]. Moreover, a dicistrovirus that infects aphids can also become associated with and persist in plant phloem cells [16]. Having said that, based on the current information, the possibility of pollen playing a greater role than just as a physical carrier of these viruses is probably low. However, the dynamics of this viral transmission route via pollen needs to be further studied before rejecting or accepting this hypothesis.

The containment greenhouse experiments involving IAPV have further demonstrated that RNA viruses could be transmitted between bee species foraging on the same flowers. No directionality was involved in the virus movement, at least between honey bees and bumble bees. Importantly, bee-collected pollen can potentially lead to long distance spread of RNA viruses without the movement of pollinators themselves. Since more than 200 tons of honeybee-collected pollen, usually freshly-frozen, is used annually worldwide for rearing commercial bumble bees colonies [17], this route could significantly impact the virus epidemiology and viral movement between widely separated regions or countries. This may also explain, at least in part, the similar prevalence of RNA viruses between commercial bumble bee colonies and honey bees in this

study. No good alternative is available to replace honey bee-collected pollen for commercial bumble bee rearing, therefore, it is imperative that risk mitigation strategies such as decontamination of pollen be encouraged for rational use of pollen in bee rearing. This same concern may also extend over to honey bees, since many beekeepers purchase pollen to feed their bees; some of this pollen is imported into the United States from other countries for human consumption.

When fed to virus-free bumble bee queens, IAPV disseminated throughout the bee body, including the ovaries and eggs. Other picornaviruses, such as poliovirus, cricket paralysis virus and DWV have also been shown to infect a variety of host tissues, depending upon the host's condition [11,18,19]. IAPV infection, however, remained restricted in the gut of the ALCB larvae. Little information is available on how RNA viruses disseminate within different stages of bees.

It is possible that IAPV is transmitted in bumble bee populations both vertically and horizontally. Detection of IAPV in surface-sterilized eggs of initially virus-free queens after feeding virus indicates transovarial transmission. Vertical transmission can also occur through infection of young gynes (future queens) within the nest via virus shed in the bee feces or contamination of the hive food. IAPV can spread horizontally in natural bumble bee populations possibly through the shared use of flowers, much like the intestinal protozoan *Crithidia bombi*, which is heavily shed in the bee feces [20].

This thesis also provides comprehensive data on the impacts of IAPV in different species of managed bees. Like other insect-infecting RNA viruses [21], IAPV infections seem to be asymptomatic at low levels, but highly virulent when bees are exposed to high doses. IAPV infections negatively impacted the colony health and survival in bumble bees and honey bees. High adult mortality and poor colony build up was observed in infected honey bees. IAPV infection significantly reduced the ALCB adult survivorship when acquired as newly emerged

adults and disrupted prepupal diapause when young larvae were infected.

Typical paralytic symptoms [22] were observed in IAPV-infected honey bees, and bees with these symptoms predominantly left the colony to die or were removed from the colony by undertaker bees to be found dead on the greenhouse floor. In contrast, only a few bumble bees showed paralytic seizures and few were found dead on the floor. IAPV-infected ALCB did not exhibit any spasms or paralytic symptoms. This suggests the possible impact of host sociality on virus ecology. Honey bees are well known for their hygienic behavior [23,24]. There is even an indication that altruistic or sacrificial behavior is involved, where the infected or sick individuals in honey bees colonies leave the nest to die away from their nest mates [25,26]. Detection of IAPV in the bee nervous tissue or brain provides the initial clue to the underlying causes of the paralytic symptoms associated with the virus; however, more research is needed to understand the physiological and molecular basis of such behavior. The rapid adult mortality and associated behavior observed in IAPV-infected honey bees in this study is especially interesting considering the recent honey bee colony losses involving similar characteristics [27]. However, further studies are needed to understand the role of IAPV and related viruses in the recent declines in field populations of different pollinators.

This thesis establishes the role of pollen as one of the major routes of inter-taxa virus transmission in the pollinator community. The apparent capacity of pollen as a means of virus movement offers an alternative perspective on the co-occurrence of multiple viruses in bee populations and adds another dimension to RNA virus epidemiology. Our finding that RNA viruses have a broad host range and are freely circulating in the pollinator community has important implications on export/import and movement of managed bees and bee products that may bring in new or more virulent strains of pathogens. Our data on the health impacts, pathology and transmission dynamics of IAPV increases current understanding of RNA virus ecology and epidemiology and may help explain bee disease patterns and pollinator population declines in

general. The potential for inter-taxa disease transmission calls for a broader epidemiological approach and to study the disease dynamics of pollinator community as a whole instead of dealing on individual species basis. Community-based disease modeling approaches could be used to better understand disease spread and potentially predict disease epidemics in pollinators.

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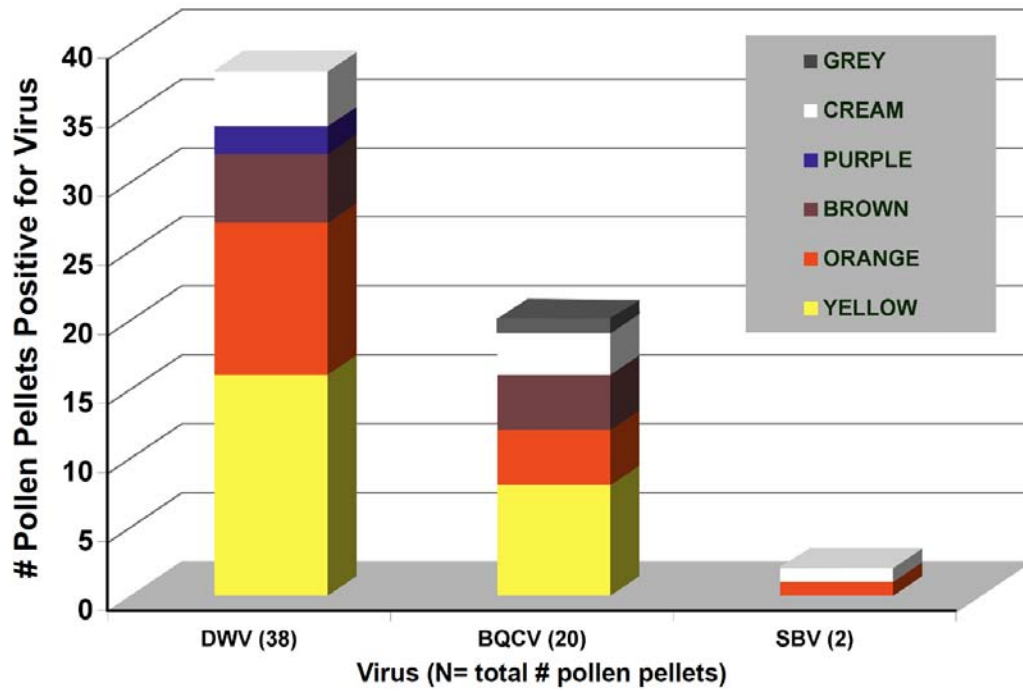
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## Appendix A

### Color and number of pollen pellets having detectable virus



DWV=Deformed wing virus; SBV= Sacbrood virus; BQCV=Black queen cell virus. N= total number of pellets with detectable virus.

## Appendix B

### Primer sequences for gene regions detected and sequenced in the chapter 2

Virus	Primer Name	Gene	Location (nt)	Product (bp)	Accession No.	Forward (5'-3') Primer	Reverse (5'-3') Primer	Created by	Purpose or use in this paper
IAPV	IAPV Capsid1	Capsid	7776-8616	840	NC009025	GGTCCAAACCTCGAAATCAA	TTGGTCCGGATGTTAATGGT	This paper	Diagnostic assay or phylogenetic analysis
DWV	DWV VP1a	Capsid	2624-3047	424	NC004830	CTCGTCATTTTGTCCCGACT	TGCAAAGATGCTGTCAAACC	This paper	Diagnostic assay or phylogenetic analysis
DWV	DWV VP1b	Capsid	2948-3475	528	NC004830	GGCGTGGTTCATTAGAATATAGG	AAGCAGATCCCCACCTAAAAA	This paper	Phylogenetic analysis
DWV	DWV VP1c	Capsid	3415-3860	446	NC004830	GCTGGAGTGTGGCATAGCTT	CGAGACTCCTCTCCTTCTGG	This paper	Phylogenetic analysis
KBV	KBV Capsid1	Capsid	7941-8565	625	NC004807	TGTTTGTGGCAATCCAGCTA	TACGTCTTCTGCCCATTTCC	This paper	Diagnostic assay
BQCV	BQCV 3'UTR	Capsid/ 3'UTR	7850-8550	700	NC003784	TGGTCAGCTCCCACTACCTTAAAC	GCAACAAGAAGAAACGTAAACCAC	[56]	Diagnostic assay or phylogenetic analysis
SBV	SBV VP1b	Capsid	1655-2347	693	AF092924	GCACGTTTAATTGGGGATCA	CAGGTTGTCCCTTACCTCCA	This paper	Diagnostic assay or phylogenetic analysis

## Appendix C

### Primer sequences and the gene regions detected for RNA viruses in pollinators in the chapter 3

Virus	Primer Name	Gene	Location (nt)	Product (bp)	Accession No.	Forward (5'-3') Primer	Reverse (5'-3') Primer	Reference
DWV	DWV vp1a	Capsid	2624-3047	424	NC004830	CTCGTCATTTTGTCCTGACT	TGCAAAGATGCTGTCAAACC	(1)
BQCV	BQCV 3'UTR	Capsid/3'UTR	7850-8550	700	NC003784	TGGTCAGCTCCCACTACCTTAAAC	GCAACAAGAAGAAACGTAAACCAC	(2)
IAPV	IAPV Capsid1	Capsid	7776-8616	840	NC009025	GGTCCAAACCTCGAAATCAA	TTGGTCCGGATGTTAATGGT	(1)
KBV	KBV Capsid1	Capsid	7941-8565	625	NC004807	TGTTGTGGCAATCCAGCTA	TACGTCTTCTGCCCATTTCC	(1)
SBV	SBV vp1b	Capsid	1655-2347	693	AF092924	GCACGTTTAATTGGGGATCA	CAGGTTGTCCCTTACCTCCA	(1)
ABPV	ABPV	RNA polymerase	8460-9360	900	AF150629	TTATGTGTCCAGAGACTGTATCCA	GCTCCTATTGCTCGGTTTTTCGGT	(2)
CBPV	CBPV	RNA polymerase	1-455	455	AY763287	AGTTGTCATGGTTAACAGGATACGAG	TCTAATCTTAGCACGAAAGCCGAG	(3)

(1) Singh, R., A. L. Levitt, et al. (2010). "RNA Viruses in Hymenopteran Pollinators: Evidence of Inter-Taxa Virus Transmission via Pollen and Potential Impact on Non-Apis Hymenopteran Species." *PLoS ONE* 5(12): 16.

(2) Benjeddou, M., N. Leat, et al. (2001). "Detection of acute bee paralysis virus and black queen cell virus from honeybees by reverse transcriptase PCR." *Applied and Environmental Microbiology* 67(5): 2384-2387.

(3) Ribiere, M., C. Triboulot, et al. (2002). "Molecular diagnosis of chronic bee paralysis virus infection." *Apidologie* 33(3): 339-351.

## Appendix D

### Primer sequences and the gene regions detected for RNA viruses in pollinators in the chapter 4

Virus	Primer Name	Gene	Location (nt)	Product (bp)	Accession No.	Forward (5'-3') Primer	Reverse (5'-3') Primer	Created by
DWV	DWV vp1a	Capsid	2624- 3047	424	NC004830	CTCGTCATTTTGTCCCGACT	TGCAAAGATGCTGTCAAACC	(1)
BQCV	BQCV 3'UTR	Capsid/ 3'UTR	7850- 8550	700	NC003784	TGGTCAGCTCCCACTACCTTAAAC	GCAACAAGAAGAAACGTAAACCAC	(2)
IAPV	IAPV Capsid1	Capsid	7776- 8616	840	NC009025	GGTCCAAACCTCGAAATCAA	TTGGTCCGGATGTTAATGGT	(1)
IAPV	IAPV short	Capsid	7856- 8224	368	EU224279	GGTCCAAACCTCGAAATCAA	ATGGAGCGGCATCTATGACT	This paper
KBV	KBV Capsid1	Capsid	7941- 8565	625	NC004807	TGTTTGTGGCAATCCAGCTA	TACGCTTTCTGCCCATTTCC	(1)
SBV	SBV vp1b	Capsid	1655- 2347	693	AF092924	GCACGTTTAATTGGGGATCA	CAGGTTGTCCCTTACCTCCA	(1)
ABPV	ABPV	RNA polymerase	8460- 9360	900	AF150629	TTATGTGTCCAGAGACTGTATCCA	GCTCCTATTGCTCGGTTTTTCGGT	(2)
CBPV	CBPV	RNA polymerase	1-455	455	AY763287	AGTTGTCATGGTTAACAGGATACGAG	TCTAATCTTAGCACGAAAGCCGAG	(3)

(1) Singh, R., A. L. Levitt, et al. (2010). "RNA Viruses in Hymenopteran Pollinators: Evidence of Inter-Taxa Virus Transmission via Pollen and Potential Impact on Non-Apis Hymenopteran Species." *PLoS ONE* 5(12): 16.

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(3) Ribiere, M., C. Triboulot, et al. (2002). "Molecular diagnosis of chronic bee paralysis virus infection." *Apidologie* 33(3): 339-351.

## VITA

### Rajwinder Singh

#### EDUCATION

- PhD, Entomology, 2011  
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Advisor: Dr G. S. Battu, Senior Insect Pathologist
- BS, Agriculture (Honors in Plant Protection), Punjab Agricultural University, Ludhiana, India, 2002

#### AWARDS AND HONORS

- ASPEE Agricultural Research & Development Foundation **Gold Medal** in BS (Honors in Plant Protection) in the Punjab Agricultural University, College of Agriculture, 2002
- Dr Avtar Singh Atwal **Gold Medal** for All Round Best Student in Master's program in the Punjab Agricultural University, 2004-05
- Dr Sardar Singh Medal in Master's program of the Punjab Agricultural University Entomology Department, 2005
- William Yendol Memorial Research Travel Award, Penn State Entomology department, 2008 & 2009

#### PUBLICATIONS

- Singh R, Levitt AL, Rajotte EG, et al. (2010) RNA Viruses in Hymenopteran Pollinators: Evidence of Inter-Taxa Virus Transmission via Pollen and Potential Impact on Non-*Apis* Hymenopteran Species. PLoS ONE 5(12)
- Singh R and Battu GS (2007) Laboratory evaluation of selected phagostimulants- adjuvants for tank-mix formulation of HearNPV (PAU-1 Strain) against *Helicoverpa armigera* (Hübner). J. Insect Sci. Ludhiana. 20(1): 58-64
- Singh R and Battu GS (2005) Laboratory evaluation of selected carbohydrate-based phagostimulants in effecting quantal sunflower leaf feeding response by *Helicoverpa armigera* (Hübner). Biopest. News 9(2): 3-4

#### PRESENTATIONS:

- 'Prevalence and Impact of RNA Viruses in two major Pollinators of Alfalfa Seed crop', at 82<sup>nd</sup> Annual meeting of Eastern branch Entomological Society of America, Harrisburg, PA, March 2011
- 'Ecology and Epidemiology of RNA Viruses in the Pollinator Community', Guest research talk at USDA-ARS Pollinating Insects-Biology, Management & Systematics Research Unit, Logan UT, July 2010
- 'Viral pathogens in the pollinator community: their distribution, inter-species transmission and impact on bee health'. 9<sup>th</sup> Annual NAPPC (North American Pollinator Protection Campaign) International conference, Crystal City, VA, Oct 2009