HONEY BEE HOST-VIRUS INTERACTIONS

AT THE INDIVIDUAL AND CELLULAR LEVEL

by

Alexander James McMenamin

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

August 2021

ACKNOWLEDGEMENTS

I would like to first thank my mentor, Dr. Michelle Flenniken. Michelle has an exacting standard for her students that made me a better scientist by demanding an attention to detail, a high quality of work, and commitment to what you set out to do. Above all, Michelle built a relationship with me that demonstrated that she cared about my personal and professional growth. I can only hope to take Michelle's discernment in science and interpersonal relationships forward into my career. As a testament to Michelle's uncanny ability to find qualified, personable employees, I cannot go without thanking some of the Flenniken lab members, past and present. Laura Brutscher, Katie Daughenbaugh, Cayley Faurot-Daniels, Brian Ross, Fenali Parekh and Naomi Kaku. You are all impressive scientists but also very generous friends. The demands of science would be a lot less tolerable without people like you. I would also like to thank my committee members Dr. Blake Wiedenheft, Dr. Doug Kominsky and Dr. Laura Burkle for their guidance and investment in my training. Lastly, I would like to thank Project Apis m and Costco for providing the funding that let me do some of this exciting work for the last two years. Hope to see you all in the next thing.

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

TABLE OF CONTENTS CONTINUED

TABLE OF CONTENTS CONTINUED

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

LIST OF FIGURES CONTINUED

Figure Page

ABSTRACT

Honey bees are important pollinators of the fruits, nuts and vegetable crops that feed our growing population. Unfortunately, honey bee colony losses have averaged 38% from 2008-2018. These losses are due to a variety of factors, including reduced quality forage, pesticide exposure in agricultural fields, parasites like the *Varroa destructor* mite, and pathogens. The most diverse group of pathogens effecting honey bees are small RNA viruses. Honey bees have evolved numerous strategies to restrict virus infection, including the RNA interference (RNAi) pathway. Bees infected with a model virus, Sindbis-GFP (SINV) have differential expression of hundreds of genes, including RNAi genes and several heat shock protein (HSP) encoding genes. Therefore, we hypothesized that heat shock proteins are antiviral in honey bees. To induce the heat shock response (HSR), SINV-infected bees were heat shocked at 42ºC for 4 hours. Heat shock resulted in a 74-90% reduction in SINV RNA copies as compared to bees maintained at 32ºC. Heat shocked and/or virus-infected bees had increased expression of several core HSR protein-encoding genes, but heat shock did not consistently result in the increased expression of RNAi genes (*argonaute-2* and *dcr-like*). This indicates that heat shock proteins are contributing to an antiviral response. SINV-infected bees also had higher expression of a recently identified antiviral gene – *bee antiviral protein-1* (*bap1*). Therefore, we further characterized *bap1* using computation approaches including phylogenetic analysis, which determined that this gene is taxonomically restricted to Hymenoptera and *Blatella germanica* (the German cockroach). Structural predication programs indicated that *bap1* is a highly disordered protein. Intriguingly, transcriptome and correlation analyses determined that *bap1* was coexpressed with several genes implicated in antiviral immunity (i.e., *ago2*, *tudor-sn* and *TEP7).* Although the precise antiviral function of *bap1* remains to be elucidated, we further developed experimental tools that will enable more incisive investigation of *bap1* and other antiviral genes. Primary cultures of larval hemocytes (immune cells) and mixed-cell pupal tissue cultures supported productive replication of sacbrood virus, deformed wing virus, and Flock House virus. Infected pupal cell cultures exhibited virus-specific transcriptional responses in *bap1*, *ago2,* and *dcr-like* expression. Together, these data further elucidate honey bee antiviral immunity and provide new tools for studying honey bee host-virus interactions.

CHAPTER ONE

OVERVIEW OF HONEY BEE HOST-PATHOGEN INTERACTIONS

Honey Bee Colony Health Is Impacted By Virus Infections

There are roughly 18,000 described bee species (Hymenoptera: Anthophila) across the globe, distributed on every continent except Antarctica [1,2]. Bees likely emerged in the mid-cretaceous and underwent a synchronized adaptive radiation with angiosperms (flowering plants) [2-5]. Angiosperms are the largest and most diverse group of vascularized plants, and frequently depend on animal-mediated pollination [4-8], especially insect-mediated. Bees, in particular, are prolific pollinators of flowering plants [1]. Society benefits from this ecosystem service as many of the fruits, nuts, and vegetables consumed by people are produced by flowering plants that depend on insect pollination [9-13]. In fact, about one third of the human diet in the US is attributable to insect pollinators [12]. The managed species *Apis mellifera* (the Western honey bee) is of particular importance to agriculture, because honey bees can be reared in portable moveable frame boxes (Langstroth hives) that facilitate husbandry. In fact, the value of crop pollination by the honey bee has been estimated at $150 million globally and $15 million in the US alone [10,11]. The agricultural need for pollinators has outpaced the growth of honey bee colonies, in part because of an average of 38% honey bee colony loses annually which are increasingly important agricultural insects in the face of pollinator declines [12,14-17].

Honey bees are cosmopolitan agricultural pollinators that are impacted by numerous stressors including agrochemicals, lack of quality forage and habitat, colony and habitat management, the *Varroa destructor* mite, and pathogens including bacteria, fungi,

trypanosomatids, and viruses [18-31]. The largest and most diverse group of honey bee pathogens are small positive-sense single-strand RNA viruses. While the list of honey bee viruses is ever growing due to virus discovery efforts, there are several honey bee-infecting viruses that have been more extensively studied [24,32-39] (reviewed in [21,22]). Virus infections in bees can cause a wide range of symptoms, including deformity, paralysis, impaired foraging and death (reviewed in [21,40]). However, individual bees can also harbor high viral loads (~ 100 billion copies per bee) and show no apparent symptoms [24,41-43]. While it is not clear why some bees experience obvious symptoms while others do not, apparently asymptomatic bees likely experience covert negative impacts of infection like energetic costs and dysregulation of homeostasis [44,45]. Differences in outcome of infection are likely attributable to various factors including nutritional status, virus tropism, host genetics, environmental factors and the immunocompetence of the individual [46,47].

Honey bee antiviral defense

Honey bees have a suite of sophisticated immune pathways and effectors including autophagy, endocytosis, apoptosis, eicosanoid signaling, melanization, and the Toll and Imd (Immune Deficiency), pathways involving NF-κB (Nuclear Factor κB) homologues Dorsal and Relish, JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription), JNK (c-Jun N-terminal kinase), MAPK (Mitogen-Activated Protein Kinases), and RNA interference (RNAi) pathways [48-56]. RNAi is a sequence-specific post-transcriptional gene silencing mechanism, which includes the microRNA (miRNA) and small interfering RNA (siRNA) pathways. The siRNA pathway is induced when a Dicer endoribonuclease recognizes long exogenous double

stranded RNA (dsRNA), a virus-associated molecular pattern (VAMP) and processes it into 21-22 nucleotide (nt) double stranded siRNAs [57,58]. Together, Dicer and a dsRNA-binding protein called R2D2 form the RNA induced silencing complex (RISC)-loading complex. The RISC loading complex facilitates the loading of the siRNA duplex into Argonaute-2 (Ago2), the core catalytic component of RISC [59,60]. After loading, the passenger strand is released while the guide strand is retained. The loaded Argonaute protein surveils the cell for cognate RNAs and, through Watson-Crick base pairing with the siRNA, binds to the complementary sequence, and then Ago2 degrades the target [61-63] (reviewed in [51,64-67]). Infected honey bees that are fed or injected with virus-specific dsRNA have a virus-limiting response, and RNAi-mediated reduced expression of *dcr-like* results in higher virus abundance compared to a control that received non-specific dsRNA (ns-dsRNA) and SINV [52,53,68-72]. These data strongly indicate that RNAi is a major antiviral response in honey bees, as it is in other insects like *Drosophila melanogaster* and mosquitoes. Interestingly, like mammals, honey bees and bumble bees have an induced antiviral response when treated with non-sequence specific dsRNA, although this response is less well characterized than the siRNA pathway [52,53,71,73-77].

Honey bees that were infected with a virus (i.e., Sindbis-GFP) and/or injected with dsRNA exhibited differential expression of hundreds of genes compared to mock-infected bees [52,53]. Among these differentially expressed genes are several heat stress response proteins. Heat shock proteins (HSPs) maintain cellular homeostasis and maintain proteostasis – including protein synthesis, folding, function, and regulation of degradation [78]. In *D. melanogaster* the heat shock response (HSR) antagonizes virus infection [79]. In fact, in general, there is a positive

association between HSPs and immunocompetence [79-83]. There is likely a myriad of roles in which HSPs contribute to an immune response, including HSPs serving as chaperones of antiviral effector proteins. For example, the ATP-dependent loading and assembly of RISC is mediated by heat shock proteins [60,84]. However, while honey bees have a conserved set of HSR genes, the role of HSPs honey bee antiviral immunity is still an active area of research. For example, the HSR and an effective humoral response are antagonistic to one another. Not only does wounding and bacterial infection decrease expression of heat shock factor (HSF) target genes, but heat shock (45 °C for 4 hours) reduced the expression of antimicrobial peptides *hymenoptaecin, defensin 1,* and *abaecin* [85]. On the contrary, virus infection resulted in higher expression of several genes associated with a heat stress response, including *heat shock protein 90, activator of hsp90, hsp60, heat shock protein 83-like, dnaj shv-like, protein lethal(2)essential for life-like,* and *hsp10* [53]. Heat *shock protein 70 cognate 3* expression was increased in bees naturally infected with Israeli acute paralysis virus (IAPV) in one study [48], but its expression was decreased in bees experimentally inoculated with IAPV in a second study [54]. Interestingly, however, heat-stress (temperatures >37 °C) reduced deformed wing virus (DWV) loads in naturally infected honey bees [86,87]. Though that study did not examine the expression of HSPs and observed higher mortality in heat stressed bees, making it unclear whether DWV was reduced due to an immune response or because of general proteostatic stress. Additional experiments are needed to elucidate the role of the HSR pathway and HSPs in honey bee antiviral defense.

Virus-infected bees that hadhigher expression of HSPs also had higher expression of a novel transcript, *bee antiviral protein-1* (*bap1*) (Genbank: MF116383). The antiviral role of *bap1* was confirmed *in vivo*, via RNAi-mediated knockdown in virus-infected bees. *Bap1* was originally annotated as a *putative cyclin dependent serine/threonine kinase* due to sequence similarity to an *Apis cerana* transcript (XM_017051141.1) and *in silico* analysis [53,88]. Serine/threonine kinases phosphorylate other proteins and thereby have a wide range of biological functions as mediators of the cell cycle and cell signaling in response to various stimuli (reviewed in [89]). The mammalian protein kinase R (PKR) is a very well-studied example of a serine/threonine kinase involved in antiviral defense. PKR is an interferon-inducible kinase that detects virus infection by binding dsRNA and subsequently arrests translation to suppress virus replication (reviewed in [90]). In honey bees, various kinases have been implicated in antiviral defense pathways (reviewed in [65]). For example, the Toll pathway, though primarily considered an antibacterial or antifungal response pathway in insects, is activated or expression of its components are upregulated during infection by some viruses via an unknown mechanism [49,52-55,91-94]. Toll receptor activation ultimately leads to activation of the protein kinase Pelle which phosphorylates Cactus, which is the inhibitor of Dorsal, an NFκB homologue (reviewed in [95]). The phosphorylation of Cactus leads to its degradation and subsequent activation of Dorsal which is translocated to the nucleus and results in upregulation of expression of various antimicrobial peptides (e.g., Defensins, Abaecin, and Apidaecin) [96,97] (reviewed in [56,65]). The exact contribution of the Toll pathway to antiviral defense is an ongoing area of research. However, the role of protein kinases in this response is clear.

Continued efforts will likely identify other protein kinases that are involved in antiviral defense. However, *in silico* protein-domain searches suggest bap1 does not contain a conserved kinase domain, and therefore is not likely a protein kinase. This work will be facilitated by the ongoing efforts to develop new techniques to study honey bee host-virus interactions, including the development of infectious virus clones and cell culture models [98-103].

Investigating honey bee host-virus interactions using primary honey bee cell cultures

Cell culture is a powerful tool to study biology at a mechanistic level. Scientists extensively rely on primary and immortalized cell cultures to elucidate antiviral responses [104-106]. Immortalized cell lines are a workhorse of a virology lab because they can be maintained in culture indefinitely, they are easily manipulated and they are virtually genetically invariable which results in experiments with high reproducibility (reviewed in [107]). Cell cultures are invaluable to studies of host-pathogen interaction because they can be manipulated to alter (increase or decrease) the expression of a candidate gene in infected cells to interrogate its antiviral capacity. The development of immortalized lines for non-model organisms, including honey bees, remains an ongoing effort. While approximately 1,000 cell lines have been derived from insect tissues, including 19 hymenopteran cell lines (the insect order that includes bees, ants, and wasps), only one immortalized honey bee cell line has been established from embryonic tissue, the fibroblast-like AmE-711 [100,108] (reviewed in [107]). Unfortunately, AME711 is slow-growing, difficult to maintain in the laboratory and it is persistently infected with deformed wing virus (DWV) [108]. Additionally, it should be considered that the immune response in a fibroblast-like cell line may not reflect the relevant immune response in other

tissues like the gut, epithelium or fat body. Therefore, it is important to also develop robust primary cell culture techniques in addition to immortalized cell lines. In fact, there are established protocols to establish primary cultures of various honey bee tissues such as neurons, gut cells and embryos [109-111]. Cultures from pluripotent embryonic tissues generally result in tissue differentiation into primarily fibroblast-type cultures, limiting their utility in immunological studies but providing the opportunity to generate transgenic cultures [100,111]. On the other hand, more complex culture types may more closely recapitulate an organismal immune response while lowering the sample-to-sample variability associated with individual level studies. One such example is cultures derived from pupal tissues, in which the pupa is dissected into head, thorax and abdomen and sterile needles are used to disrupt the tissues which can then be cultured for extended periods of time [109,110]. Depending on the question, a single-tissue culture may be desired. For example, one may be interested in the role of hemocytes in host-pathogen interactions since they are the primary insect immune cell, and they are involved in antiviral immunity in *Drosophila melanogaster* [112-114]. It is unclear whether hemocytes play a role in honey bee antiviral immunity, but there is some indirect evidence that they may be involved. Genes involved in migration, phagocytosis, and expression immunoglobulin domain-encoding, were expressed at greater levels in honey bees infected with SINV, relative to mock-infected bees [53]. Developing cell culture techniques to study antiviral immune responses in complex multi-tissue cultures and in single tissue cultures will be very important to further elucidate honey bee host-virus interactions.

Hypotheses and Project Summaries

Intellectual Merit

Honey bee (*Apis melifera*) colony health is threatened by viruses that produce a wide range of symptoms, including mortality (reviewed in [21,40]). The development of tools and techniques to study the basic biology of honey bee-infecting viruses, and how bees respond to infection will be crucial to developing strategies to mitigate colony deaths due to virus infection. It is paramount that, in addition to known pathways and effectors, new or novel ones are identified and explored as potential targets. For example, heat shock proteins and *bee antiviral protein-1* (*bap1*; GenBank:MF116383) are intriguing potential new and novel proteins that limit virus infection. The establishment of robust cell culture techniques for both immortalized and primary cell cultures will facilitate efforts to describe the functions of novel pathways and proteins, thanks to their manipulability.

Hypothesis Chapter 4

The heat shock response participates in antiviral immunity in honey bees.

Research aims Chapter 4

Aim 1. Determine the effect of heat shock (42°C for 4 hrs) on the outcome of virus infection. To determine whether the response to heat shock in honey bees resulted in an antiviral response, honey bees were (a) injected with buffer, (b) injected with buffer and heat shocked (c) injected with SINV or (d) injected with SINV and heat shocked. This experiment revealed that bees that received virus and heat shock showed 74-90% reduction in SINV RNA copies relative to SINV-infected bees maintained at a constant temperature.

Aim 2. Assess the transcriptional regulation of six heat shock proteins and three immune genes in response to virus infection and heat shock. Quantitative PCR was used to measure the expression of heat shock proteins *protein lethal(2)essential for life like*, *dnaj shv-like*, *hsc70-3*, *hsc70-4*, *hsp83-like*, and *hsp90* as well as immune genes *mf116383* (herein renames *bap1*), *ago2*, and *dcr-like*. Both heat shock proteins and immune genes had increased expression in bees that were infected, heat shocked, or both infected and heat shocked. Immune genes do not always have increased expression in bees that were infected and heat shocked suggesting their upregulation may not be necessary for the protective effect of heat shock. However, the expression of immune genes and heat shock proteins are positively correlated, potentially due to coregulation.

Hypothesis Chapter 5

Bee antiviral protein-1 (GenBank: *MF116383,* a *putative cyclin dependent serine/threonine kinase*) is an antiviral immune gene evolving under positive selection, and it is coexpressed with other immune genes.

Research aims Chapter 5

Aim 1. Confirm that *bap1* is an antiviral immune gene. When *bap1* was first annotated, Brutscher et al also confirmed *in vivo* that it limits virus infection [53]. The same approach was used to confirm that finding. First, *bap1* expression is induced by SINV and ns-dsRNA. Using an RNAi approach, the expression of *bap1* was reduced in bees that were infected with SINV, which resulted in higher SINV RNA copies than the ns-dsRNA control bees that were infected.

Aim2. Determine the phylogenetic distribution of *bap1* and whether it is evolving under positive selection. Searches against protein databases with Hidden Markov Models failed to find any homology to protein kinases and therefore this gene, formerly *MF116383,* is renamed here as *bee antiviral protein-1* (*bap1*). Bayesian phylogenetic inference suggests *bap1* is a rapidly evolving protein that is taxonomically restricted to Hymenoptera and *Blatella germanica* (the German cockroach). Additionally, the majority of Bap 1 amino acids are evolving under neutral selection.

Aim 3. Description of *bap1* and identification of highly coexpressed genes. Online tools reveal that Bap1 is a highly disordered protein that is trafficked to the endoplasmic reticulum, via a signal peptide. Additionally, *post hoc* analysis of a transcriptome dataset generated from bees infected with SINV and/or injected with dsRNA demonstrates that *bap1* is coexpressed with several immune genes including *TEP7*, *Tudor-SN,* and most notably, *ago2*. The coexpression of *bap1* and *ago2* is confirmed in an independent data set.

Hypothesis Chapter 6

Primary cell cultures derived from honey bee tissue will support replication of viruses known to infect honey bees and mount an immune response to infection.

Research aims Chapter 6

Aim1. Determine whether primary cell cultures derived from honey bee tissues support virus infection. Honey bee hemocytes or cells derived from purple eyes pupal cells were infected with sacbrood virus (SBV), DWV or Flock House virus (FHV). Quantitative PCR and negative strand-specific PCR demonstrate that these primary cultures support replication of SBV,

DWV, and FHV. Infected pupal cell cultures are shown to produce infectious virions by infecting naïve cells using material from previously infected tissues.

Aim2. Assay the expression of immune genes in response to infection. Quantitative PCR was used to measure the expression of *bap1*, *ago2*, and *dcr-like* in pupal cell cultures. These cultures had unique transcriptional responses of these three genes to SBV, DWV and FHV.

References Cited

1. Michener, C.D. The Bees of the World; The Johns Hopkins University Press: Boston, MA, USA, 2000.

2. Danforth, B.N.; Sipes, S.; Fang, J.; Brady, S.G. The history of early bee diversification based on five genes plus morphology. Proceedings of the National Academy of Sciences **2006**, 103, 15118, doi:10.1073/pnas.0604033103.

3. Peters, R.S.; Krogmann, L.; Mayer, C.; Donath, A.; Gunkel, S.; Meusemann, K.; Kozlov, A.; Podsiadlowski, L.; Petersen, M.; Lanfear, R.; et al. Evolutionary History of the Hymenoptera. Current Biology **2017**, 27, 1013-1018, doi:https://doi.org/10.1016/j.cub.2017.01.027.

4. Crane, P.R.; Friis, E.M.; Pedersen, K.R. The origin and early diversification of angiosperms. Nature **1995**, 374, 27-33, doi:10.1038/374027a0.

5. Soltis, P.S.; Soltis, D.E. The origin and diversification of angiosperms. American Journal of Botany **2004**, 91, 1614-1626, doi:https://doi.org/10.3732/ajb.91.10.1614.

6. Regal, P.J. Ecology and Evolution of Flowering Plant Dominance. Science **1977**, 196, 622, doi:10.1126/science.196.4290.622.

7. Burger, W.C. Why Are There So Many Kinds of Flowering Plants? BioScience **1981**, 31, 572-581, doi:10.2307/1308218.

8. Pellmyr, O. Evolution of insect pollination and angiosperm diversification. Trends in Ecology & Evolution **1992**, 7, 46-49, doi:https://doi.org/10.1016/0169-5347(92)90105-K.

9. Delplane, K.S.; Mayer, D.F. Crop Pollination by Bees; CABI Publishing: Wallingford, UK, 2000.

10. Gallai, N.; Salles, J.-M.; Settele, J.; Vaissière, B.E. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological Economics **2009**, 68, 810-821, doi:10.1016/j.ecolecon.2008.06.014.

11. Calderone, N.W. Insect pollinated crops, insect pollinators and US agriculture: Trend analysis of aggregate data for the period 1992-2009. PLoS ONE **2012**, 7, 24-28, doi:10.1371/journal.pone.0037235.

12. Eilers, E.J.; Kremen, C.; Smith Greenleaf, S.; Garber, A.K.; Klein, A.-M. Contribution of Pollinator-Mediated Crops to Nutrients in the Human Food Supply. PLOS ONE **2011**, 6, e21363, doi:10.1371/journal.pone.0021363.

13. Klein, A.-M.; Vaissière, B.E.; Cane, J.H.; Steffan-Dewenter, I.; Cunningham, S.a.; Kremen, C.; Tscharntke, T. Importance of pollinators in changing landscapes for world crops. Proceedings. Biological sciences / The Royal Society **2007**, 274, 303-313, doi:10.1098/rspb.2006.3721.

14. Aizen, M.a.; Harder, L.D. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Current biology : CB **2009**, 19, 915-918, doi:10.1016/j.cub.2009.03.071.

15. Aizen, M.A.; Garibaldi, L.A.; Cunningham, S.A.; Klein, A.M. Long-Term Global Trends in Crop Yield and Production Reveal No Current Pollination Shortage but Increasing Pollinator Dependency. Current Biology **2008**, 18, 1572-1575, doi:https://doi.org/10.1016/j.cub.2008.08.066.

16. Burkle, L.A.; Marlin, J.C.; Knight, T.M. Plant-Pollinator Interactions over 120 Years: Loss of Species, Co-Occurrence and Function. Science (New York, N.Y.) **2013**, 339, 1611-1616.

17. Goulson, D.; Hanley, M.E.; Darvill, B.; Ellis, J.S. Biotope associations and the decline of bumblebees (Bombus spp.). Journal of Insect Conservation **2006**, 10, 95-103, doi:10.1007/s10841-006-6286-3.

18. Goulson, D.; Nicholls, E.; Botias, C.; Rotheray, E.L. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science **2015**, 347, 1255957, doi:10.1126/science.1255957.

19. Cornman, R.S.; Tarpy, D.R.; Chen, Y.; Jeffreys, L.; Lopez, D.; Pettis, J.S.; VanEngelsdorp, D.; Evans, J.D. Pathogen webs in collapsing honey bee colonies. PLoS ONE **2012**, 7, e43562, doi:10.1371/journal.pone.0043562.

20. Brutscher, L.M.; McMenamin, A.J.; Flenniken, M.L. The buzz about honey bee viruses. PLoS Pathogens **2016**, 12, e1005757, doi:10.1371/journal.ppat.1005757.

21. Grozinger, C.M.; Flenniken, M.L. Bee Viruses: Ecology, Pathogenicity, and Impacts. Annual Review of Entomology **2019**, 64, 205-226, doi:10.1146/annurev-ento-011118-111942.

22. McMenamin, A.J.; Flenniken, M.L. Recently identified bee viruses and their impact on bee pollinators. Current Opinion in Insect Science **2018**, 26, 120-129, doi:https://doi.org/10.1016/j.cois.2018.02.009.

23. Runckel, C.; DeRisi, J.; Flenniken, M.L. A draft genome of the honey bee trypanosomatid parasite crithidia mellificae. PLoS ONE **2014**, 9, doi:10.1371/journal.pone.0095057.

24. Runckel, C.; Flenniken, M.L.; Engel, J.C.; Ruby, J.G.; Ganem, D.; Andino, R.; DeRisi, J.L. Temporal Analysis of the Honey Bee Microbiome Reveals Four Novel Viruses and Seasonal Prevalence of Known Viruses, Nosema, and Crithidia. PLoS ONE **2011**, 6, e20656, doi:10.1371/journal.pone.0020656.

25. Schwarz, R.S.; Bauchan, G.R.; Murphy, C.A.; Ravoet, J.; de Graaf, D.C.; Evans, J.D. Characterization of two species of trypanosomatidae from the honey bee apis mellifera: Crithidia mellificae langridge and mcghee, and lotmaria passim n. gen., n. sp. Journal of Eukaryotic Microbiology **2015**, 1-17, doi:10.1111/jeu.12209.

26. Ravoet, J.; Schwarz, R.S.; Descamps, T.; Yañez, O.; Tozkar, C.O.; Martin-Hernandez, R.; Bartolomé, C.; De Smet, L.; Higes, M.; Wenseleers, T.; et al. Differential diagnosis of the honey bee trypanosomatids Crithidia mellificae and Lotmaria passim. Journal of Invertebrate Pathology **2015**, 130, 21-27, doi:https://doi.org/10.1016/j.jip.2015.06.007.

27. Cornman, R.S.; Chen, Y.P.; Schatz, M.C.; Street, C.; Zhao, Y.; Desany, B.; Egholm, M.; Hutchison, S.; Pettis, J.S.; Lipkin, W.I.; et al. Genomic Analyses of the Microsporidian Nosema ceranae, an Emergent Pathogen of Honey Bees. PLOS Pathogens **2009**, 5, e1000466, doi:10.1371/journal.ppat.1000466.

28. Genersch, E. American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. Journal of Invertebrate Pathology **2010**, 103, S10-S19, doi:10.1016/j.jip.2009.06.015.

29. Genersch, E.; Von Der Ohe, W.; Kaatz, H.; Schroeder, A.; Otten, C.; Büchler, R.; Berg, S.; Ritter, W.; Mühlen, W.; Gisder, S.; et al. The German bee monitoring project: A long term study to understand periodically high winter losses of honey bee colonies. Apidologie **2010**, 41, 332-352, doi:10.1051/apido/2010014.

30. Forsgren, E. European foulbrood in honey bees. Journal of Invertebrate Pathology **2010**, 103, S5-S9, doi:10.1016/j.jip.2009.06.016.

31. Nazzi, F.; Le Conte, Y. Ecology of Varroa destructor, the Major Ectoparasite of the Western Honey Bee, Apis mellifera. Annual Review of Entomology **2016**, 61, 417-432, doi:10.1146/annurev-ento-010715-023731.

32. Daughenbaugh, K.F.; Kahnonitch, I.; Carey, C.C.; McMenamin, A.J.; Wiegand, T.; Erez, T.; Arkin, N.; Ross, B.; Wiedenheft, B.; Sadeh, A.; et al. Metatranscriptome Analysis of Sympatric Bee Species Identifies Bee Virus Variants and a New Virus, Andrena-Associated Bee Virus-1. Viruses **2021**, 13, doi:10.3390/v13020291.

33. Schoonvaere, K.; Smagghe, G.; Francis, F.; de Graaf, D.C. Study of the Metatranscriptome of Eight Social and Solitary Wild Bee Species Reveals Novel Viruses and Bee Parasites. Frontiers in Microbiology **2018**, 9, doi:10.3389/fmicb.2018.00177.

34. de Miranda, J.R.; Cornman, R.S.; Evans, J.D.; Semberg, E.; Haddad, N.; Neumann, P.; Gauthier, L. Genome Characterization, Prevalence and Distribution of a Macula-Like Virus from Apis mellifera and Varroa destructor. Viruses **2015**, 7, 3586-3602, doi:10.3390/v7072789.

35. Remnant, E.J.; Shi, M.; Buchmann, G.; Blacquiere, T.; Holmes, E.C.; Beekman, M.; Ashe, A. A Diverse Range of Novel RNA Viruses in Geographically Distinct Honey Bee Populations. J Virol **2017**, 91, doi:10.1128/JVI.00158-17.

36. Levin, S.; Galbraith, D.; Sela, N.; Erez, T.; Grozinger, C.; Chejanovsky, N. Identification of a novel Rhabdovirus in populations of pollinators and their parasites from two continents (in preparation). **2017**.

37. Levin, S.; Galbraith, D.; Sela, N.; Erez, T.; Grozinger, C.M.; Chejanovsky, N. Presence of Apis Rhabdovirus-1 in Populations of Pollinators and Their Parasites from Two Continents. Frontiers in Microbiology **2017**, 8, doi:10.3389/fmicb.2017.02482.

38. Galbraith, D.A.; Fuller, Z.L.; Ray, A.M.; Brockmann, A.; Frazier, M.; Gikungu, M.W.; Martinez, J.F.I.; Kapheim, K.M.; Kerby, J.T.; Kocher, S.D.; et al. Investigating the viral ecology of global bee communities with high-throughput metagenomics. Scientific Reports **2018**, 8, 8879, doi:10.1038/s41598-018-27164-z.

39. Deboutte, W.; Beller, L.; Yinda, C.K.; Shi, C.; Smets, L.; Vanmechelen, B.; Conceição-Neto, N.; Dallmeier, K.; Maes, P.; de Graaf, D.C.; et al. Hymenoptera associated eukaryotic virome lacks host specificity. bioRxiv **2020**, 2020.2009.2015.298042, doi:10.1101/2020.09.15.298042.

40. McMenamin, A.J.; Genersch, E. Honey bee colony losses and associated viruses. Current Opinion in Insect Science **2015**, 8, 121-129, doi:10.1016/j.cois.2015.01.015.

41. Daughenbaugh, K.F.; Martin, M.; Brutscher, L.M.; Cavigli, I.; Garcia, E.; Lavin, M.; Flenniken, M.L. Honey bee infecting Lake Sinai viruses. Viruses **2015**, 7, 3285-3309, doi:10.3390/v7062772.

42. Francis, R.M.; Nielsen, S.L.; Kryger, P. Patterns of viral infection in honey bee queens. Journal of General Virology **2013**, 94, 668-676, doi:https://doi.org/10.1099/vir.0.047019-0.

43. D'Alvise, P.; Seeburger, V.; Gihring, K.; Kieboom, M.; Hasselmann, M. Seasonal dynamics and co-occurrence patterns of honey bee pathogens revealed by high-throughput RT-qPCR analysis. Ecology and Evolution **2019**, 9, 10241-10252, doi:10.1002/ece3.5544.

44. Ravindran, M.S.; Bagchi, P.; Cunningham, C.N.; Tsai, B. Opportunistic intruders: how viruses orchestrate ER functions to infect cells. Nat Rev Microbiol **2016**, 14, 407-420, doi:10.1038/nrmicro.2016.60.

45. Fan, Y.; Sanyal, S.; Bruzzone, R. Breaking Bad: How Viruses Subvert the Cell Cycle. Front Cell Infect Microbiol **2018**, 8, 396-396, doi:10.3389/fcimb.2018.00396.

46. Bull, J.C.; Ryabov, E.V.; Prince, G.; Mead, A.; Zhang, C.; Baxter, L.A.; Pell, J.K.; Osborne, J.L.; Chandler, D. A Strong Immune Response in Young Adult Honeybees Masks Their Increased Susceptibility to Infection Compared to Older Bees. PLoS Pathogens **2012**, 8, 11-14, doi:10.1371/journal.ppat.1003083.

47. Chandra, S.; Chandra, R.K. Nutrition, immune response, and outcome. Prog Food Nutr Sci **1986**, 10, 1-65.

48. Chen, Y.P.; Pettis, J.S.; Corona, M.; Chen, W.P.; Li, C.J.; Spivak, M.; Visscher, P.K.; DeGrandi-Hoffman, G.; Boncristiani, H.; Zhao, Y.; et al. Israeli Acute Paralysis Virus: Epidemiology, Pathogenesis and Implications for Honey Bee Health. PLOS Pathogens **2014**, 10, e1004261, doi:10.1371/journal.ppat.1004261.

49. Nazzi, F.; Brown, S.P.; Annoscia, D.; Del Piccolo, F.; Di Prisco, G.; Varricchio, P.; Della Vedova, G.; Cattonaro, F.; Caprio, E.; Pennacchio, F. Synergistic Parasite-Pathogen Interactions Mediated by Host Immunity Can Drive the Collapse of Honeybee Colonies. PLOS Pathogens **2012**, 8, e1002735, doi:10.1371/journal.ppat.1002735.

50. Kingsolver, M.B.; Hardy, R.W. Making connections in insect innate immunity. Proceedings of the National Academy of Sciences of the United States of America **2012**, 109, 18639-18640, doi:10.1073/pnas.1216736109.

51. Kingsolver, M.B.; Huang, Z.; Hardy, R.W. Insect antiviral innate immunity: Pathways, effectors, and connections. Journal of Molecular Biology **2013**, 425, 4921-4936, doi:10.1016/j.jmb.2013.10.006.

52. Flenniken, M.L.; Andino, R. Non-Specific dsRNA-Mediated Antiviral Response in the Honey Bee. PLoS ONE **2013**, 8, 1-16, doi:10.1371/journal.pone.0077263.

53. Brutscher, L.M.; Daughenbaugh, K.F.; Flenniken, M.L. Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. Scientific Reports **2017**, 7, 6448, doi:10.1038/s41598-017-06623-z.

54. Galbraith, D.A.; Yang, X.; Nino, E.L.; Yi, S.; Grozinger, C. Parallel epigenomic and transcriptomic responses to viral infection in honey bees (Apis mellifera). PLoS Pathog **2015**, 11, e1004713, doi:10.1371/journal.ppat.1004713.

55. Ryabov, E.V.; Fannon, J.M.; Moore, J.D.; Wood, G.R.; Evans, D.J. The Iflaviruses Sacbrood virus and Deformed wing virus evoke different transcriptional responses in the honeybee which may facilitate their horizontal or vertical transmission. PeerJ **2016**, 4, e1591, doi:10.7717/peerj.1591.

56. Brutscher, L.M.; Daughenbaugh, K.F.; Flenniken, M.L. Antiviral defense mechanisms in honey bees. Current Opinion in Insect Science **2015**, 2, 1-12, doi:10.1016/j.cois.2015.04.016.

57. Deddouche, S.; Matt, N.; Budd, A.; Mueller, S.; Kemp, C.; Galiana-Arnoux, D.; Dostert, C.; Antoniewski, C.; Hoffmann, J.A.; Imler, J.-L. The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in drosophila. Nature Immunology **2008**, 9, 1425-1432, doi:10.1038/ni.1664.

58. Galiana-Arnoux, D.; Dostert, C.; Schneemann, A.; Hoffmann, J.A.; Imler, J.-L. Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. Nature Immunology **2006**, 7, 590-597, doi:10.1038/ni1335.

59. van Rij, R.P.; Saleh, M.-C.; Berry, B.; Foo, C.; Houk, A.; Antoniewski, C.; Andino, R. The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes & Development **2006**, 20, 2985-2995, doi:10.1101/gad.1482006.

60. Iwasaki, S.; Kobayashi, M.; Yoda, M.; Sakaguchi, Y.; Katsuma, S.; Suzuki, T.; Tomari, Y. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. Mol Cell **2010**, 39, 292-299, doi:10.1016/j.molcel.2010.05.015.

61. Wang, X.-H.; Aliyari, R.; Li, W.-X.; Li, H.-W.; Kim, K.; Carthew, R.; Atkinson, P.; Ding, S.-W. RNA Interference Directs Innate Immunity Against Viruses in Adult Drosophila. Science **2006**, 312, 452-454, doi:10.1126/science.1125694.

62. Zambon, R.A.; Vakharia, V.N.; Wu, L.P. RNAi is an antiviral immune response against a dsRNA virus in Drosophila melanogaster. Cellular Microbiology **2006**, 8, 880-889, doi:10.1111/j.1462-5822.2006.00688.x.

63. Myles, K.M.; Wiley, M.R.; Morazzani, E.M.; Adelman, Z.N. Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. Proceedings of the National Academy of Sciences **2008**, 105, 19938, doi:10.1073/pnas.0803408105.

64. Brutscher, L.M.; Flenniken, M.L. RNAi and antiviral defense in the honey bee. Journal of Immunology Research **2015**, 2015, doi:10.1155/2015/941897.

65. McMenamin, A.J.; Daughenbaugh, K.F.; Parekh, F.; Pizzorno, M.C.; Flenniken, M.L. Honey Bee and Bumble Bee Antiviral Defense. Viruses **2018**, 10, 395, doi:10.3390/v10080395.

66. Bronkhorst, A.W.; van Rij, R.P. The long and short of antiviral defense: small RNA-based immunity in insects. Current Opinion in Virology **2014**, 7, 19-28, doi:https://doi.org/10.1016/j.coviro.2014.03.010.

67. Gammon, D.B.; Mello, C.C. RNA interference-mediated antiviral defense in insects. Current Opinion in Insect Science **2015**, 8, 111-120, doi:10.1016/j.cois.2015.01.006.

68. Doublet, V.; Poeschl, Y.; Gogol-Döring, A.; Alaux, C.; Annoscia, D.; Aurori, C.; Barribeau, S.M.; Bedoya-Reina, O.C.; Brown, M.J.F.; Bull, J.C.; et al. Unity in defence: honeybee workers exhibit conserved molecular responses to diverse pathogens. BMC Genomics **2017**, 18, 207, doi:10.1186/s12864-017-3597-6.

69. Chejanovsky, N.; Ophir, R.; Schwager, M.S.; Slabezki, Y.; Grossman, S.; Cox-Foster, D. Characterization of viral siRNA populations in honey bee colony collapse disorder. Virology **2014**, 454-455, 176-183, doi:10.1016/j.virol.2014.02.012.

70. Desai, S.D.; Eu, Y.J.; Whyard, S.; Currie, R.W. Reduction in deformed wing virus infection in larval and adult honey bees (Apis mellifera L.) by double-stranded RNA ingestion. Insect Molecular Biology **2012**, 21, 446-455, doi:10.1111/j.1365-2583.2012.01150.x.

71. Niu, J.; Meeus, I.; Cappelle, K.; Piot, N.; Smagghe, G. The immune response of the small interfering RNA pathway in the defense against bee viruses. Current Opinion in Insect Science **2014**, 6, 22-27, doi:10.1016/j.cois.2014.09.014.

72. Maori, E.; Paldi, N.; Shafir, S.; Kalev, H.; Tsur, E.; Glick, E.; Sela, I. IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. Insect Molecular Biology **2009**, 18, 55-60.

73. McMenamin, A.J.; Daughenbaugh, K.F.; Flenniken, M.L. The Heat Shock Response in the Western Honey Bee (Apis mellifera) is Antiviral. Viruses **2020**, 12, 245, doi:10.3390/v12020245.

74. Piot, N.; Snoeck, S.; Vanlede, M.; Smagghe, G.; Meeus, I. The effect of oral administration of dsRNA on viral replication and mortality in Bombus terrestris. Viruses **2015**, 7, 3172-3185, doi:10.3390/v7062765.

75. Niu, J.; Smagghe, G.; De Coninck, D.I.; Van Nieuwerburgh, F.; Deforce, D.; Meeus, I. In vivo study of Dicer-2-mediated immune response of the small interfering RNA pathway upon systemic infections of virulent and avirulent viruses in Bombus terrestris. Insect Biochem Mol Biol **2016**, 70, 127-137, doi:10.1016/j.ibmb.2015.12.006.

76. Jarosch, A.; Moritz, R.F.A. RNA interference in honeybees: off-target effects caused by dsRNA. Apidologie **2012**, 43, 128-138, doi:10.1007/s13592-011-0092-y.

77. Nunes, F.M.F.; Aleixo, A.C.; Barchuk, A.R.; Bomtorin, A.D.; Grozinger, C.M.; Simões, Z.L.P. Non-Target Effects of Green Fluorescent Protein (GFP)-Derived Double-Stranded RNA (dsRNA-GFP) Used in Honey Bee RNA Interference (RNAi) Assays. Insects **2013**, 4, 90-103.

78. Taylor, R.C.; Berendzen, K.M.; Dillin, A. Systemic stress signalling: understanding the cell non-autonomous control of proteostasis. Nature Reviews Molecular Cell Biology **2014**, 15, 211-217, doi:10.1038/nrm3752.

79. Merkling, S.H.; Overheul, G.J.; Mierlo, J.T.V.; Arends, D.; Gilissen, C.; Rij, R.P.V. The heat shock response restricts virus infection in Drosophila. Nature Scientific Reports **2015**, 5, 1-15, doi:10.1038/srep12758.

80. Prithika, U.; Deepa, V.; Balamurugan, K. External induction of heat shock stimulates the immune response and longevity of Caenorhabditis elegans towards pathogen exposure. Innate Immunity **2016**, 22, 466-478, doi:10.1177/1753425916654557.

81. Singh, V.; Aballay, A. Heat-shock transcription factor (HSF)-1 pathway required for Caenorhabditis elegans immunity. Proceedings of the National Academy of Sciences **2006**, 103, 13092, doi:10.1073/pnas.0604050103.

82. Singh, V.; Aballay, A. Heat Shock and Genetic Activation of HSF-1 Enhance Immunity to Bacteria. Cell Cycle **2006**, 5, 2443-2446, doi:10.4161/cc.5.21.3434.

83. Chen, J.; Xie, C.; Tian, L.; Hong, L.; Wu, X.; Han, J. Participation of the p38 pathway in Drosophila host defense against pathogenic bacteria and fungi. Proceedings of the National Academy of Sciences **2010**, 107, 20774, doi:10.1073/pnas.1009223107.

84. Dorner, S.; Lum, L.; Kim, M.; Paro, R.; Beachy, P.A.; Green, R. A genomewide screen for components of the RNAi pathway in Drosophila cultured cells. Proceedings of the National Academy of Sciences **2006**, 103, 11880–11885.

85. McKinstry, M.; Chung, C.; Truong, H.; Johnston, B.A.; Snow, J.W. The heat shock response and humoral immune response are mutually antagonistic in honey bees. Scientific Reports **2017**, 7, 8850, doi:10.1038/s41598-017-09159-4.

86. Dalmon, A.; Peruzzi, M.; Le Conte, Y.; Alaux, C.; Pioz, M. Temperature-driven changes in viral loads in the honey bee Apis mellifera. Journal of Invertebrate Pathology **2018**, doi:https://doi.org/10.1016/j.jip.2018.12.005.

87. Bordier, C.; Dechatre, H.; Suchail, S.; Peruzzi, M.; Soubeyrand, S.; Pioz, M.; Pélissier, M.; Crauser, D.; Conte, Y.L.; Alaux, C. Colony adaptive response to simulated heat waves and consequences at the individual level in honeybees (Apis mellifera). Scientific Reports **2017**, 7, 3760, doi:10.1038/s41598-017-03944-x.

88. Elsik, C.G.; Worley, K.C.; Bennett, A.K.; Beye, M.; Camara, F.; Childers, C.P.; de Graaf, D.C.; Debyser, G.; Deng, J.; Devreese, B.; et al. Finding the missing honey bee genes: lessons learned from a genome upgrade. BMC Genomics **2014**, 15, 86, doi:10.1186/1471-2164-15-86.

89. Edelman, A.M.; Blumenthal, D.K.; Krebs, E.G. Protein serine/threonine kinases. Annu Rev Biochem **1987**, 56, 567-613, doi:10.1146/annurev.bi.56.070187.003031.

90. García, M.A.; Meurs, E.F.; Esteban, M. The dsRNA protein kinase PKR: Virus and cell control. Biochimie **2007**, 89, 799-811, doi:https://doi.org/10.1016/j.biochi.2007.03.001.

91. Zambon, R.A.; Nandakumar, M.; Vakharia, V.N.; Wu, L.P. The Toll pathway is important for an antiviral response in Drosophila. Proc Natl Acad Sci U S A **2005**, 102, 7257-7262, doi:10.1073/pnas.0409181102.

92. Xi, Z.; Ramirez, J.L.; Dimopoulos, G. The Aedes aegypti Toll Pathway Controls Dengue Virus Infection. PLOS Pathogens **2008**, 4, e1000098, doi:10.1371/journal.ppat.1000098.

93. Ferreira, Á.G.; Naylor, H.; Esteves, S.S.; Pais, I.S.; Martins, N.E.; Teixeira, L. The Toll-Dorsal Pathway Is Required for Resistance to Viral Oral Infection in Drosophila. PLOS Pathogens **2014**, 10, e1004507, doi:10.1371/journal.ppat.1004507.

94. Thoetkiattikul, H.; Beck, M.H.; Strand, M.R. Inhibitor κB-like proteins from a polydnavirus inhibit NF-κB activation and suppress the insect immune response. Proceedings of the National Academy of Sciences of the United States of America **2005**, 102, 11426, doi:10.1073/pnas.0505240102.

95. Drier, E.A.; Steward, R. The dorsoventral signal transduction pathway and the Rel-like transcription factors inDrosophila. Seminars in Cancer Biology **1997**, 8, 83-92, doi:https://doi.org/10.1006/scbi.1997.0059.

96. Meng, X.; Khanuja, B.S.; Ip, Y.T. Toll receptor-mediated Drosophila immune response requires Dif, an NF-kappaB factor. Genes Dev **1999**, 13, 792-797, doi:10.1101/gad.13.7.792.

97. Lourenço, A.P.; Florecki, M.M.; Simões, Z.L.P.; Evans, J.D. Silencing of Apis mellifera dorsal genes reveals their role in expression of the antimicrobial peptide defensin-1. Insect Molecular Biology **2018**, 27, 577-589, doi:https://doi.org/10.1111/imb.12498.

98. Lamp, B.; Url, A.; Seitz, K.; Eichhorn, J.; Riedel, C.; Sinn, L.J.; Indik, S.; Koglberger, H.; Rumenapf, T. Construction and Rescue of a Molecular Clone of Deformed Wing Virus (DWV). PLoS One **2016**, 11, e0164639, doi:10.1371/journal.pone.0164639.

99. Seitz, K.; Buczolich, K.; Dikunová, A.; Plevka, P.; Power, K.; Rümenapf, T.; Lamp, B. A molecular clone of Chronic Bee Paralysis Virus (CBPV) causes mortality in honey bee pupae (Apis mellifera). Scientific Reports **2019**, 9, 16274, doi:10.1038/s41598-019-52822-1.

100. Goblirsch, M.J.; Spivak, M.S.; Kurtti, T.J. A Cell Line Resource Derived from Honey Bee (Apis mellifera) Embryonic Tissues. PLoS ONE **2013**, 8, 1-13, doi:10.1371/journal.pone.0069831.

101. Gusachenko, O.N.; Woodford, L.; Balbirnie-Cumming, K.; Campbell, E.M.; Christie, C.R.; Bowman, A.S.; Evans, D.J. Green Bees: Reverse Genetic Analysis of Deformed Wing Virus Transmission, Replication, and Tropism. Viruses **2020**, 12, doi:10.3390/v12050532.

102. Ryabov, E.V.; Christmon, K.; Heerman, M.C.; Posada-Florez, F.; Harrison, R.L.; Chen, Y.; Evans, J.D. Development of a Honey Bee RNA Virus Vector Based on the Genome of a Deformed Wing Virus. Viruses **2020**, 12, doi:10.3390/v12040374.

103. Posada-Florez, F.; Lamas, Z.S.; Hawthorne, D.J.; Chen, Y.; Evans, J.D.; Ryabov, E.V. Pupal cannibalism by worker honey bees contributes to the spread of deformed wing virus. Scientific Reports **2021**, 11, 8989, doi:10.1038/s41598-021-88649-y.

104. van Cleef, K.W.R.; van Mierlo, J.T.; van den Beek, M.; van Rij, R.P. Identification of Viral Suppressors of RNAi by a Reporter Assay in Drosophila S2 Cell Culture. In Antiviral RNAi: Concepts, Methods, and Applications, van Rij, R.P., Ed.; Humana Press: Totowa, NJ, 2011; pp. 201-213.

105. Baum, B.; Cherbas, L. Drosophila Cell Lines as Model Systems and as an Experimental Tool. In Drosophila: Methods and Protocols, Dahmann, C., Ed.; Humana Press: Totowa, NJ, 2008; pp. 391-424.

106. Harschnitz, O.; Studer, L. Human stem cell models to study host–virus interactions in the central nervous system. Nature Reviews Immunology **2021**, doi:10.1038/s41577-020-00474-y.

107. Guo, Y.; Goodman, C.L.; Stanley, D.W.; Bonning, B.C. Cell Lines for Honey Bee Virus Research. Viruses **2020**, 12, 236, doi:10.3390/v12020236.

108. Carrillo-Tripp, J.; Dolezal, A.G.; Goblirsch, M.J.; Miller, W.A.; Toth, A.L.; Bonning, B.C. In vivo and in vitro infection dynamics of honey bee viruses. Scientific Reports **2016**, 6, 22265, doi:10.1038/srep22265.

109. Genersch, E.; Gisder, S.; Hedtke, K.; Hunter, W.B.; Möckel, N.; Müller, U. Standard methods for cell cultures in Apis mellifera research. Journal of Apicultural Research **2013**, 52, 1-8, doi:10.3896/IBRA.1.52.1.02.

110. Hunter, W.B. Medium for development of bee cell cultures (Apis mellifera: Hymenoptera: Apidae). In Vitro Cellular & Developmental Biology - Animal **2010**, 46, 83-86, doi:10.1007/s11626-009-9246-x.

111. Chan, M.M.; Choi, S.Y.; Chan, Q.W.; Li, P.; Guarna, M.M.; Foster, L.J. Proteome profile and lentiviral transduction of cultured honey bee (Apis mellifera L.) cells. Insect Mol Biol **2010**, 19, 653-658, doi:10.1111/j.1365-2583.2010.01022.x.

112. Tassetto, M.; Kunitomi, M.; Andino, R. Circulating Immune Cells Mediate a Systemic RNAi-Based Adaptive Antiviral Response in Drosophila. Cell **2017**, 169, 314-325.e313, doi:10.1016/j.cell.2017.03.033.

113. Saleh, M.-c.; Tassetto, M.; van Rij, R.P.; Goic, B.; Gausson, V.; Berry, B.; Jacquier, C.; Antoniewski, C.; Andino, R. Antiviral immunity in Drosophila requires systemic RNA interference spread. Nature **2009**, 458, 346-350, doi:10.1038/nature07712.

114. Lavine, M.D.; Strand, M.R. Insect hemocytes and their role in immunity. Insect Biochemistry and Molecular Biology **2002**, 32, 1295-1309, doi:https://doi.org/10.1016/S0965-1748(02)00092-9.

CHAPTER TWO

RECENTLY IDENTIFIED BEE VIRUSES AND THEIR IMPACT ON BEE POLLINATORS

Contributions of Authors and Co-authors

Manuscript in Chapter Two

Author: Alexander J. McMenamin

Contributions: Wrote and edited the review

Co-author: Michelle L. Flenniken

Contributions: Wrote and edited the review

Manuscript Information

Alexander J. McMenamin, Michelle L. Flenniken

Recently identified bee viruses and their impact on bee pollinators

Current Opinion in Insect Science

Status of Manuscript
___ Prepared for submission to a peer-reviewed journal
___ Officially submitted to a peer-reviewed journal
___ Accepted by a peer-reviewed journal
_X_ Published in a peer-reviewed journal

Feb 13; doi: 10.1016/j.cois.2018.02.009.

Recently identified bee viruses and their impact on bee pollinators

Alexander J. McMenamin[1,2,3] and Michelle L. Flenniken[1,2]

[1]Department of Plant Sciences and Plant Pathology, [2]Pollinator Health Center, [3]Department of Microbiology and Immunology, Montana State University, Bozeman, MT, USA

*corresponding author: michelle.flenniken@montana.edu

Abstract

Bees are agriculturally and ecologically important plant pollinators. Recent high annual losses of honey bee colonies, and reduced populations of native and wild bees in some geographic locations, may impact the availability of affordable food crops and the diversity and abundance of native and wild plant species. Multiple factors including viral infections affect pollinator health. The majority of well-characterized bee viruses are picorna-like RNA viruses, which may be maintained as covert infections or cause symptomatic infections or death. Next generation sequencing technologies have been utilized to identify the sequences of additional bee-infecting viruses including the Lake Sinai viruses, Rhabdoviruses, and Bunyaviruses. In addition, sequence data is instrumental for defining specific viral strains and characterizing associated

pathogenicity, such as the recent characterization of Deformed wing virus master variants (DWV-A, -B, and -C) and their impact on bee health.

Introduction

Bees are important pollinators of plant species in natural and agricultural ecosystems. Recent high annual losses of honey bee colonies and reduced populations of native and wild bees involve multiple factors including pathogens, agrochemical exposure, and inadequate habitat and nutritional resources [1–14]. Although no single abiotic or biotic factor is responsible for recent bee deaths, viruses have been associated with honey bee colony losses [12,15–21] and individual mortality and morbidity in native and wild bees [22–30] (reviewed in [31]). Bee-infecting viruses are primarily positive sense single-stranded RNA viruses (+ssRNA) in the order *Picornavirales* [32,33]. Common bee viruses include: the Dicistroviruses (Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), Acute bee paralysis virus (ABPV), and Black queen cell virus (BQCV)); the Iflaviruses (Deformed wing virus (DWV), Kakugo virus, Varroa destructor virus-1/DWV-B, Sacbrood virus (SBV), and Slow bee paralysis virus (SBPV)); and taxonomically unclassified viruses (Chronic bee paralysis virus (CBPV) and the Lake Sinai viruses (LSVs)) (reviewed in [32–34]). Recently identified +ssRNA viruses include Bee macula-like virus (BeeMLV) in the *Tymoviridae* family, Apis mellifera flavivirus and Apis mellifera nora virus 1 [35,36]. The first bee-infecting negative sense single-stranded RNA viruses (-ssRNA) were also

recently described, specifically Apis mellifera rhabdovirus-1 (ARV-1) and Apis mellifera rhabdovirus-2 (ARV-2) [36], also known as Bee rhabdovirus (BRV) [37]. To date, only a single honey bee-infecting double-stranded DNA virus, Apis mellifera filamentous virus (AmFV), has been sequenced and characterized [38–40].

Bee virus discovery has been facilitated and accelerated by next generation sequencing technologies and it is likely that additional bee-associated viruses will be discovered. Virus identification and characterization are important first steps toward understanding the role of viruses in bee health. Typically, bee viruses are defined by the organism from which they were first identified (e.g., honey bee viruses), though many bee viruses have wide host ranges and are transmitted between genera [23,25,30,41–49] (reviewed in [31]). Virus nomenclature varies; a virus may be named after the symptoms or diseases associated with infection, the host from which it was isolated, or for the geographic regions or features (e.g., mountains, rivers, etc.) near where the virus was first identified. Viruses are assigned to families based on the type and sequence of nucleic acid that makes up their genome. The International Committee on the Taxonomy of Viruses (ICTV; URL: https://talk.ictvonline.org/taxonomy/) is responsible for maintaining a taxonomic catalogue of viruses, though the most up to date resource for virus sequences is the National Center for Biotechnology Information (NCBI) non-redundant nucleic acid data base [50]. Importantly, recent identification of a virus does not necessarily make it an emerging virus, and identification of viruses in additional hosts is not indicative of spillover from a reservoir population into a novel host population. The directionality of virus transmission is

difficult to discern, but field-based studies indicate bee viruses are transmitted from both managed bees to wild bees (e.g., DWV [42]) and from wild bees to managed bees (e.g., ABPV [25,42]). *Varroa destructor*, an ectoparasitic mite that commonly infests honey bee colonies and feeds on developing and adult bees, is an active vector for numerous viruses, including DWV [51–57], KBV [58], IAPV [59], and CBPV [60], and may be a passive vector or host of many more (e.g., ABPV (reviewed in [61]), BeeMLV [35], LSV [62],VDV-2 and -3 [63], Moku Virus [64], BQCV [65,66], and SBV [65–67]). Furthermore, mite-mediated DWV transmission has been shown to bottleneck virus populations [68] and result in enhanced replication of recombinant DWV-1/VDV-1 (DWV-B) viruses in honey bee hosts [69,70].

Several viruses discovered in honey bees replicate in other bee species, as evidenced by negative strand detection or amplification over the course of infection including ABPV [22], BQCV [49,71], DWV [24,25,43,49,72], IAPV [29,43,44,73,74], KBV [29], LSVs [45,75], and SBPV [74], though the pathogeneses of these viruses in native bees is underexplored. Virus transmission between sympatric pollinator species is mediated by shared floral resources, including pollen [44,72], and is evidenced by a lack of host-species-specific clustering of virus sequences in phylogenetic analyses [25,43,44]. While it is clear that bee viruses replicate within and are transmitted between several bee species, their pathogeneses may be host or virus strain dependent (reviewed in [31]) and are likely influenced by additional factors including nutritional status [76], host genetic makeup [77], host sex [78], and bee age [79]. In wild bee species viral infection may result in deformity [23], systemic infection [24], reduced reproductive success

[29], and/or mortality [22,25]. Second to honey bees, the consequences of bee virus infections have been most investigated in bumble bees. DWV replicates in multiple bumble bee species including *Bombus huntii* [24]*, B. impatiens* [24,43]*, B. lapidarius* [25]*, B. lucorum* [25]*, B. monticola* [25]*, and B. vagans* [43]), though symptomatic infection has only been described in *B. terrestris* [23–25] and *B. pascorum* [23] (see [31] for a comprehensive review). *Bombus terrestris* exhibits DWV-associated wing deformities and mortality [23,25]. Similarly, negative consequences of virus infection of *B. terrestris* include reduced fecundity and colony founding associated with KBV infection [29], reduced fecundity due to IAPV infection [29,73], and mortality due to ABPV infection [22]. However, the signs, symptoms, and severity of virus infection may differ across bee species. For example, one study determined that exposure to a mixed inoculum of viruses isolated from honey bees (i.e., IAPV, SBV, and DWV) resulted in lower mortality in two native bee species, *Megachile rotunda* and *Colletes inaequalis*, as compared to honey bees (*Apis mellifera)* [80]. This result may indicate specific virus-host adaptation that may result in enhanced, unchanged, or diminished virulence, depending on the specific virus-host combination and directionality of virus transmission. Additional investigation of bee virus pathogenesis and intra- and inter-species transmission is required to better understand the role of viruses on bee health and their impact on bee losses [25,42] (reviewed in [31]).

Next generation sequencing and bee virus discovery

Pioneering historic research on bee viruses by Bailey, Ball, and others relied on conventional tools including studies documenting virus-associated disease transmission using filterable agents, electron microscopy, and antibody-mediated virus detection [32,81,82]. Virus genome discovery in bees has been greatly accelerated by next generation sequencing and new assembly tools (Table 1) [35,36,63,64,83]. Bee virus discovery efforts have primarily focused on Western honey bee (*Apis mellifera*) and bumble bee (*B. pascorum* and *B. lapidarius*) samples [45], although there have been a few studies in other honey bee-associated species including identification of Moku virus (MV) from *Vespula pensylvanica* wasps which prey on honey bees in Hawaii [64], and Varroa destructor virus 2 (VDV2) and VDV3 from *Varroa destructor* [63]. Furthermore, recovery of Aphid lethal paralysis virus (ALPV) and Big Sioux River virus (BSRV) genome sequence in aphids [84] and DWV in Argentine ants in New Zealand [85] indicates that “bee viruses”, like other insect viruses, likely infect a broad range of insects and arachnids.

Table 1. Recently described bee-associated viruses.

Virus/Group	Location(s)	Genome Type	Approximate Genome Size	Family	Associated Species	Ref(s)
Aphid lethal paralysis virus	US, Spain	+ssRNA	4.1 kb	*Dicistroviridae*	*Apis mellifera*	[30,83,87, 90]
Apis mellifera filamentous virus	Switzerland, US, Belgium	dsDNA	498.5 kb	unclassified	*Apis mellifera* *Osmia cornuta* *Osmia bicornis* *Andrena vaga* *Andrena ventralis*	[30,38-40]
Apis mellifera bunya virus-1	South Africa	-ssRNA	6 kb	*Bunyaviridae*	*Apis mellifera*	[36]
Apis mellifera bunya virus-2	South Africa	-ssRNA	6.5 kb	*Bunyaviridae*	*Apis mellifera*	[36]
Apis dicistrovirus	Netherlands	+ssRNA	9.1 kb	*Dicistroviridae*	*Apis mellifera*	[36]
Apis mellifera flavivirus	South Africa	+ssRNA	20.4 kb	*Flaviviridae*	*Apis mellifera*	[36]
Apid mellifera nora virus-1	South Africa	+ssRNA	10 kb (partial)	Picorna-like	*Apis mellifera*	[36]
Apis mellifera rhabdovirus-1 / bee rhabdovirus-1	US, Israel Tonga Netherlands South Africa	-ssRNA	14.6 kb	*Rhabdoviridae*	*Apis mellifera* *Bombus impatiens* *Varroa destructor*	[36,37]
Apis mellifera rhabdovirus-2	US, Israel Tonga Netherlands South Africa	-ssRNA	14 kb	*Rhabdoviridae*	*Apis mellifera* *Varroa destructor*	[36,37]
Big Sioux River virus	United States	+ssRNA	9.6 kb	Dicistroviridae	*Apis mellifera*	[83]
Halictus scabiosae Adlikon virus	Switzerland	+ssRNA	5.2 kb	unclassified	*Halictus scabiosae*	[88]
Lake Sinai viruses	United States Europe Australia China	+ssRNA	5.9 kb	unclassified	*Apis mellifera* *Andrena vaga* *Andrena ventralis* *Osmia bicornis* *Osmia cornuta* *Bombus spp.* *Messor spp.*	[30,45, 83,87-91]
Moku virus	United States	+ssRNA	10 kb	*Iflaviridae*	*V. pensylvanica* *Apis mellifera* *Varroa destructor*	[64]
Varroa destructor macula-like virus	United States Belgium	+ssRNA	6.5 kb	*Tymoviridae*	*Apis mellifera* *Varroa destructor* *Osmia cornuta* *Bombus spp.*	[30,35, 45,91]
Varroa destructor virus 2	Israel	+ssRNA	9.6 kb	*Iflaviridae*	*Varroa destructor*	[63]
Varroa destructor virus 3	Israel	+ssRNA	4.2 kb	unclassified	*Varroa destructor*	[63]
Varroa tymo-like virus	United States	+ssRNA	6.2 kb	*Tymoviridae*	*Varroa destructor*	[35]

Table 1. Recently described bee-associated viruses.
Recent development of next generation sequencing technologies has led to a rapid expansion in the number of viruses in bees and associated taxa (e.g., *Varroa* and *Vespula pensylvanica).* This table is a list of bee-associated virus genomes that have been published in the last ten years; this list does not include new variants of previously described viruses like Deformed wing virus [111]. Viruses of note include the first negative-sense single-stranded RNA viruses associated with bees (i.e., ABV-1 and -2, ARV-1 and -2) and the expanded host range of Apis mellifera filamentous virus, which includes several solitary bee species.

The first metagenomic study of honey bees examined the entire RNA profile of honey bee samples obtained from Colony Collapse Disorder-affected and healthy colonies [17]. Initially, high prevalence of IAPV and KBV were associated with CCD-affected colonies, but subsequent analyses and additional studies indicate that no single virus is universally associated with CCD or colony losses [15,16,20,32,82]. One of the first studies to utilize next generation (or ultra-high throughput) sequencing for virus discovery in honey bee samples identified four new honey bee associated viruses including two Dicistroviruses (i.e., Aphid-lethal paralysis virus-like virus (ALPV-like 1) and BSRV) and the unique Lake Sinai virus group, including Lake Sinai virus 1 (LSV1) and Lake Sinai virus 2 (LSV2) [83]. Recently, the LSV group has been expanded and phylogenetically resolved into four clades [50,62,83,84,87–89]. The abundance of LSV1 and LSV2 RNA, which respectively peaked at approximately 7.06 $x10^{10}$ and 7.16 x 10^{11} genome copies per bee, and detection of the replicative form of the viral genome using strand-specific PCR, indicated that these viruses replicated in honey bees [83]. Subsequent studies in Belgium, Spain, and the US, and re-evaluation of sequencing data from CCD- and non-CCD affected samples, identified several additional LSVs and indicated that these viruses are globally distributed, abundant, and sometimes associated with poor colony health [16,36,62,75,90,91]. A

recent study identified new viruses from Halictid bees and defined a new virus genus, *Halictivirus*, which is phylogenetically similar to LSVs in the *Sinaivirus* genus [84]. ALPV-like virus was also detected in metagenomic sequencing data obtained from honey bee samples from Spain [90]. The negative replicative intermediate form of the APLV-like genome and of Varroa destructor macula-like virus (VdMLV), which was renamed Bee macula-like virus (BeeMLV), were detected using a multiplex-ligation probe dependent amplification based method (i.e. BeeDoctor®) on RNA isolated from honey bees in Belgium [35,91]. Metagenomic sequencing of honey bee samples from Spain also identified two plant viruses (i.e., Turnip ringspot virus and Turnip yellow mosaic virus), which were likely passively associated with honey bees [90]. In contrast, detection of the negative strand of another plant virus, Tomato ringspot virus, indicated that this virus may replicate in honey bees [92].

Recently, short read high-throughput and chain termination sequencing methods were used to assemble the Bee macula-like virus (BeeMLV) genome from poly-A augmented RNA samples obtained from *Varroa destructor* mites and honey bees [35]. BeeMLV is a polyadenylated +ssRNA virus approximately 6,500 nucleotides in length in the *Tymoviridae* family. BeeMLVs form a new species complex independent of other related viruses (i.e., Tymovirus, Marifivirus, and Maculavirus) [35]. The US and European strains of BeeMLV are > 70% identical at the nucleotide level and distinct from the related Varroa tymo-like virus (VTLV), which was discovered in *Varroa* samples [35]. In addition to the US and France, BeeMLV has been detected in bee and mite samples from Belgium, but was not detected in samples obtained from

Sweden, Norway, or the French territory Isle d'Ouessant [35]. Peak BeeMLV prevalence in French apiaries occurred in autumn and coincided with peak mite abundance, although levels of virus abundance in bees and mites were not correlated [35]. Greater relative abundance of BeeMLV subgenomic RNA relative to genomic RNA in honey bee samples is indicative of active viral infection. However, since the ratio of subgenomic to genomic RNA in mites was equivalent to bees in the same colony, BeeMLV in mite samples may be due to virus uptake during mite-feeding, rather than virus replication [35] .

Sequences from seven new honey bee-associated viruses were identified in a recent study by Remnant et al., that utilized RNA-sequencing to examine viral diversity in honey bees obtained from colonies that were either bred for or naturally evolved the trait of mite resistance [36]. Sequencing libraries generated from ribosomal RNA depleted honey bee RNA samples from the Netherlands, South Africa, and Tonga resulted in the identification of the first -ssRNA viruses in both bees and mites. These new viruses are in the family *Rhabdoviridae,* which comprises enveloped -ssRNA viruses that infect a broad range of species, including many arthropods [93]. Sequences derived from Apis mellifera rhabdovirus-1 (ARV-1), and Apis mellifera rhabdovirus-2 (ARV-2), which are phylogenetically closest to Farmington virus based on 30% aa identity of the RNA-dependent RNA polymerase (RdRp), were detected in all geographic locations. Subsequently, Bee rhabdovirus (BRV) sequences, which shared over 99% homology to ARV-1, were identified in honey bee and bumble bee (i.e., *B. impatiens*) samples from the US, as well as

honey bee and *Varroa* samples from Israel [37]. Due to the expanded host range of this virus, Levin et al. proposed to rename ARV-1 to BRV-1 [37].

Additional -ssRNA virus the Bunyavirus family (i.e., Apis mellifera bunyavirus-1 (ABV-1) and Apis mellifera bunyavirus-2 (ABV-2)) were discovered in bee samples obtained from South Africa, but only the largest genome segment, which includes the RdRp, was sequenced [36]. These viruses may actively infect bees or the bee-infecting trypanosomatid species, *Crithidia mellificae* and/or *Lotmaria passim* [94,95], since ABV-1 is most similar to Leishbunyavirus (LBV1), which was isolated from the insect trypanosomatid parasite (i.e. *Leptomonas moramango*) [96].

Three additional +ssRNA viruses were identified including the first bee-associated flavivirus, Apis mellifera flavivirus (AFV), from a sample obtained from one colony located in South Africa. AFV has a 20,414 nucleotide +ssRNA genome that contains a single open reading frame (ORF) of 6,615 amino acids [36]. The sequence of the first nora virus, Apis mellifera nora virus 1 (ANV-1), was also identified in a bee sample obtained from South Africa. To obtain the full genome of ANV-1, the RNASeq-derived contigs were aligned with the Drosophila pseudoobscura nora virus. Chain termination sequencing and RT-PCR were utilized to obtain the partial 10,091 nucleotide sequence, including the entire replicase-encoding gene, but not the first ORF [36]. Finally, Apis mellifera dicistrovirus (ADV), isolated from a honey bee sample from the Netherlands, adds to the growing list of identified honey bee dicistroviruses [36].

Honey bee antiviral defense mechanisms include RNA interference (RNAi) [97–104]. Therefore several recent sequencing efforts have assessed the small inhibitory RNA (siRNA) profiles of naturally and experimentally virus-infected bees and identified the signature 21- 22 nucleotide siRNAs produced by Dicer cleavage [36,70,99,105]. Likewise, the siRNA profile of ARV-1 and ARV-2 infected honey bees determined that ARV-1 and ARV-2 siRNAs had characteristics of Dicer processed small RNAs (e.g., 2-nucleotide overhang, 21-22 nucleotides long, and phased from ends of the genome [36]). Detection of Dicer-processed siRNAs indicates ARV-1 and ARV-2 actively infect honey bees and implicates the involvement of RNAi in honey bee antiviral defense against ARV infections [36].

Together, these studies illustrate that data from short-read libraries may be used to identify new virus sequences and indicate that many more await discovery, particularly since most studies used similar methods and focused on RNA viruses. Verification of sequence data using longer read methods (e.g., PacBio sequencing) and more accurate chain-termination sequencing is typically carried out to ensure that complete viral genomes are properly assembled and annotated [83,106]. In addition, detection of the replicative intermediate forms of the virus genome by strand-specific amplification (e.g., negative-strand specific tagged PCR), *in situ* hybridization, and/or northern blot analysis provides additional evidence that a recently identified virus is infectious to the host from which it was obtained [45,83,107,108]. Complete genome sequences facilitate phylogenetic analyses of these viruses, but more commonly such analyses are

performed using nucleotide and amino acid sequencing of key viral proteins, such as the RdRp and capsid proteins. The outcome of phylogenetic reconstruction may differ depending on the genome regions utilized in the analysis and with the availability of related sequences in the databases (e.g., Lake Sinai virus [36,45,62,75] and Bee Macula-like virus [35]).

Virus quasispecies

RNA viruses encode and rely on error-prone RdRp for genome replication, resulting in a mutation rate nearly a million-fold higher than eukaryotic host polymerases [109]. This generates a population of related viruses of high variation around one or more 'master genotypes'– known as a quasi-species swarm (reviewed in [109,110]). The degree of nucleotide identity that defines a new viral variant or strain varies by virus, and is not clearly defined for many honey bee viruses. For example, Lake Sinai virus sequences in the NCBI database range in sequence identity from 69 – 99% at the nucleotide level [62], whereas proposed DWV master variants range from 79-84% identity at the nucleotide level, with up to 98.2% identity among sequences identified as DWV-A [111]. A master variant or master type is the genotype with maximal fitness around which the quasispecies explores sequence space (reviewed in [110]). Recent phylogenetic analysis has suggested the possible existence of three DWV master variants [111].

The Deformed wing virus cluster

Deformed wing virus (DWV) is a picorna-like virus with an approximately 10 kb +ssRNA genome encapsidated by a 30 nm diameter icosahedral capsid (reviewed in [34,112]). DWV negatively impacts honey bee health and is a major correlate to colony failure, particularly in association with *Varroa destructor* [18,52,54–56,86,113]. *Varroa*-mediated transmission of DWV and mite infestation of DWV-infected honey bee colonies augments DWV abundance and DWV-associated deformities and death [52,55–57,66,68,114]. Mite-mediated transmission of DWV may also exert a selective bottleneck on DWV at the individual [69,70] and landscape levels [68], perhaps partially explaining the association between *Varroa*-vectored DWV and poor colony health and colony loss [19,68,115]. The observation that Australia, which lacks DWV and *Varroa*, has not reported elevated colony losses supports the hypothesis that the synergistic effect of DWV and *Varroa* drive colony loss [52,55,116]. However, several studies indicated that the association of DWV with overwintering colony mortality is sometimes independent of *Varroa* levels [18,19,113,115].

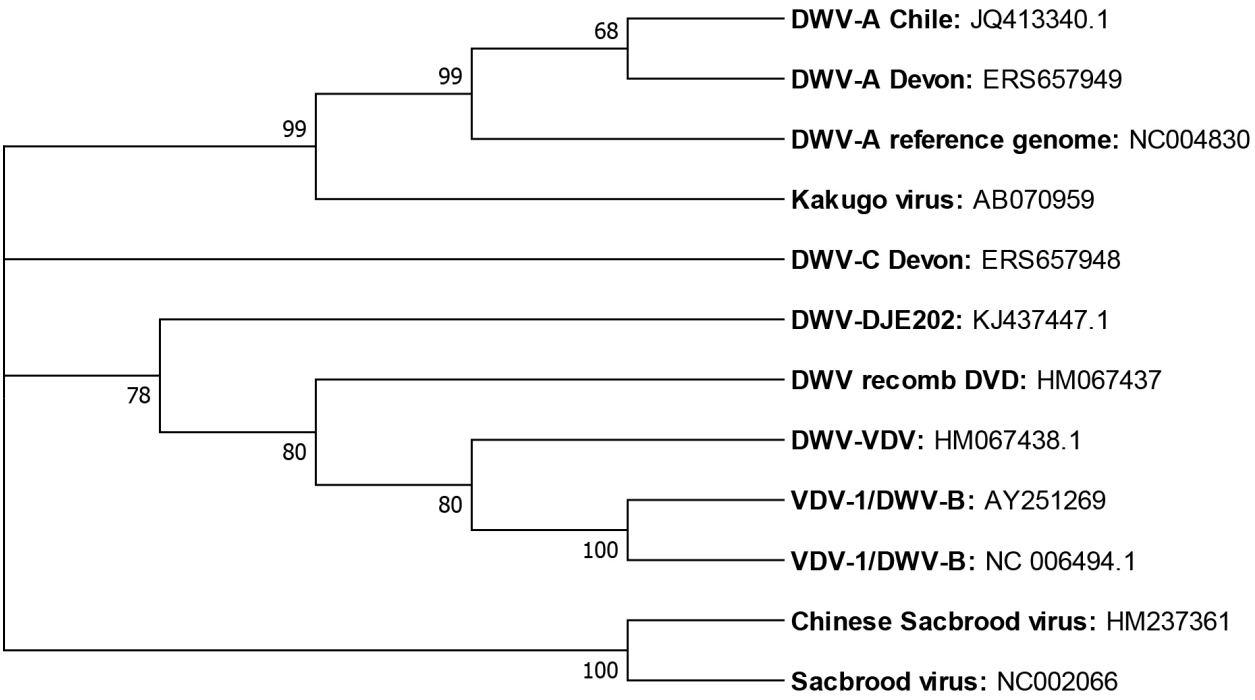


Figure 1. Deformed wing virus phylogenetic relationship inferred from whole genome nucleotide sequence.
Maximum Likelihood bootstrap supported consensus tree derived from Maximum Likelihood analysis in Mega v7.0.26 [120] using a whole genome nucleic acid MUSCLE alignment generated in Mega using a neighbor joining clustering method (max iterations=30, gap open penalty= -400, gap extension penalty= 0) [121]. There were a total of 11,045 positions (nucleotides and gaps) in the final dataset. A mixed-model approach implemented in Mega identified a General Time Reversible model with a discrete Gamma distribution of evolutionary rates among sites (5 categories (+*G*, parameter = 1.1861)) to be the best fit for these data [119,120]. The initial tree was obtained using Maximum Parsimony with Subtree-Pruning-Regrafting (SPR level 5) and a heuristic search involving 1000 random addition replicates to determine the optimal tree topology. Maximum Likelihood bootstrap support values (1000 replicates) are reported next to the branches [122]. The bootstrap consensus tree (topology of more than 50% of trees) was visualized and partially edited using Mega v7.0.26 [120]. Sacbrood virus was selected as the out-group since it is a closely related member of the *Iflaviridae* family. Genbank Accession numbers for whole genome sequences are as follows: DWV-A Chile (JQ413340.1), DWV-A Devon (ERS657949), DWV-A reference genome (NC_004830), Kakugo virus (AB070959), DW-C Devon (ERS657948), DWV-DJE202 (KJ437447.1), DWV recombinant DVD (HM067437), DWV-VDV recombinant (HM067438.1), VDV-1/DWV-B (AY251269 and NC_006494.1), Chinese Sacbrood virus (HM237361), Sacbrood virus (NC_002066).

In 2004, Varroa destructor virus 1 (VDV1), which shares 84% nucleotide identity with DWV, was isolated from *Varroa* mites [53]. Subsequently, DWV-VDV recombinants were identified as the predominant viral strains in *Varroa*-infested honey bees in the UK [69,70]. These recombinant viruses have since been detected in geographically widespread regions [117,118]. It has been proposed to designate VDV-1 as DWV 'master variant' B (VDV-1 Accession: AY251269) [68,111] and the reference sequence as DWV-A (Accession: NC_004830). In concurrence with this, Mordecai and colleagues recently described DWV-C (Accession: ERS657948) as a third 'master variant' of DWV [111]. DWV-A and DWV-B are 84.4% identical [53], and DWV-C shares 79.8% and 79.5% nucleotide identity with DWV-A and DWV-B, respectively (Figure 1) [111,119–122]. A quantitative Polymerase Chain Reaction assay that distinguishes DWV variants will facilitate future investigation of their relative impacts on bee health [123].

There are some mixed data in the literature concerning the relative virulence of DWV-A and DWV-B. The 2007 introduction and subsequent spread of *Varroa* into honey bee populations on the Hawaiian archipelago increased DWV prevalence and genome copy number by one million fold, and reduced DWV diversity, as indicated by fewer unique RdRp sequences [68]. More recently, Mordecai et al. reported that bees resistant to *Varroa* were predominantly infected by DWV-B and exhibited low prevalence and abundance of DWV-A [124]. Since these DWV-B infected colonies were qualitatively assessed to be resistant to *Varroa*, the authors suggest that

DWV-B is avirulent and that low DWV-A prevalence was due to superinfection exclusion by DWV-B [124]. However, observed resistance in this study population could also be due to selective breeding for resistance traits in the honey bee [124], while the discrepancies in DWV variant predominance between the Hawaiian and the UK study populations may be due to differences in genetic background of host, pathogen, or parasite. Alternatively, resistance to DWV-A infection could be due to the fact that the populations examined in the latter study had more extensive histories of association with *Varroa* [68,124].

At the colony level, both DWV-B and DWV-A are associated with significant overwintering colony mortality [18,19,113,115,125,126]. In a recent UK-based study, DWV-B genome equivalents and prevalence over time positively correlated with colony mortality, whereas DWV-A was not detected [115]. However, the explanatory power of their statistical models suggest the association between DWV-B and *Varroa* may be more critical for honey bee health than either alone [18,52,55,56,113,114,125,126]. This is important because it restates the difficulty of disentangling the close association of DWV with *Varroa* and their contribution to individual bee and colony mortality [19,52,55,113,127]. Additionally, while injection of adult bees with DWV-A resulted in greater mortality than that observed in the mock-infected control group, injection with DWV-B or a mixture of the two resulted in higher genome copies and greater mortality as compared to bees infected with DWV-A alone [26]. Therefore, laboratory data suggest DWV-B may be more virulent than DWV-A. However, these experiments need to be replicated in bees with different genetic backgrounds from distinct geographical locations to test the

generalizability of this conclusion. The development of infectious molecular clones of these viruses will greatly facilitate the study of their relative virulence and fitness. Indeed, an infectious clone of a DWV-A isolate was recently constructed and produced clinical infection when $5x10^6$ genome equivalents were injected into pupae [128]. This infectious molecular clone will facilitate competition assays [26] and tagged infectious clone experiments required to assess the relative fitness and pathogeneses of these variants [110]. Further development of additional molecular clones will rapidly expand our repertoire of tools that can be used to understand bee-infecting viruses.

Conclusion

Viruses contribute to bee deaths, although their relative role is often difficult to discern among several confounding variables [26,127,129]. Continued and invigorated efforts to quantitatively track known viruses and discover new virus genomes using next generation sequencing will further our understanding of the role of viruses on bee health and may facilitate our response to emerging and/or recently identified pathogens [130].

Acknowledgements

The Flenniken Laboratory is supported by the National Sciences Foundation CAREER Program, the United States Department of Agriculture National Institute of Food and Agriculture, Agriculture and Food Research Initiative (USDA-NIFA-AFRI) Program, Montana Department of Agriculture Specialty Crop Block Grant Program, the National Institutes of Health IDeA Program COBRE grant GM110732, National Science Foundation EPSCoR NSF-IIA-1443108, Hatch Multistate Funding (NC-1173), Project *Apis m*., the Montana State Beekeepers Association, Montana State University, and the Montana State University Agricultural Experiment Station. We would like to thank members of the Flenniken laboratory (Dr. Laura Brutscher and Dr. Katie Daughenbaugh) and Dr. Marie Pizzorno (Bucknell University) for reviewing this manuscript prior to publication.

References

1. Arbetman MP, Gleiser G, Morales CL, Williams P, Aizen MA: **Global decline of bumblebees is phylogenetically structured and inversely related to species range size and pathogen incidence**. *Proc. R. Soc. B* 2017, **284**:20170204.
2. Biesmeijer JC, Roberts SP., Reemer M, Ohlemüller R, Edwards M, Peeters T, Schaffers AP, Potts SG, Kleukers R, Thomas CD, et al.: **Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands**. *Science* 2006, **313**:351–354.
3. Burkle LA, Marlin JC, Knight TM: **Plant-pollinator interactions over 120 Years: Loss of species, co-occurrence and function**. *Science* 2013, **339**:1611–1616.
4. Cameron SA, Lozier JD, Strange JP, Koch JB, Cordes N, Solter LF, Griswold TL: **Patterns of widespread decline in North American bumble bees.** *Proc. Natl. Acad. Sci.* 2011, **108**:662–667.
5. Graystock P, Yates K, Darvill B, Goulson D, Hughes WOH: **Emerging dangers: Deadly effects of an emergent parasite in a new pollinator host**. *J. Invertebr. Pathol.* 2013, **114**:114–119.
6. Graystock P, Goulson D, Hughes WOH: **The relationship between managed bees and the prevalence of parasites in bumblebees**. *Peer J* 2014, **2**:e522.
7. Goulson D, Hanley ME, Darvill B, Ellis JS: **Biotope associations and the decline of bumblebees (*Bombus spp.*)**. *J. Insect Conserv.* 2006, **10**:95–103.
8. Goulson D, Lye GC, Darvill B: **Decline and conservation of bumble bees.** *Annu. Rev. Entomol.* 2008, **53**:191–208.
9. Goulson D, Nicholls E, Botías C, Rotheray EL: **Bee declines driven by combined stress from parasites, pesticides, and lack of flowers**. *Science* 2015, **347**:1255957.

   * This is a concise, pointed review of the factors contributing to honey bee colony losses and wild pollinator declines globally, which includes recommendations to mitigate these threats.

10. Potts SG, Biesmeijer K, Bommarco R, Breeze TD, Carvalheiro L, Franzén M, González-Varo JP, Holzschuh A, Kleijn D, Klein A, et al.: *Status and trends of European pollinators. key findings of the STEP project*. Pensoft Publishers; 2015.
11. Woodcock BA, Bullock JM, Shore RF, Heard MS, Pereira MG, Redhead J, Ridding L, Dean H, Sleep D, Henrys P, et al.: **Country-specific effects of neonicotinoid pesticides on honey bees and wild bees**. *Science* 2017, **356**:1393–1395.

   * In this study the authors use a field study with a randomized block design to test the effect of field-realistic applications of common pesticides in oil seed rape on introduced honey bees, bumble bees, and solitary bees. Their results show that while neonicotinoid pesticides may negatively effect pollinator populations by reducing reproductive output

(Hungary and the UK), the extent of their actual impact is context dependent.

12. Evans JD, Schwarz RS: **Bees brought to their knees: Microbes affecting honey bee health**. *Trends Microbiol.* 2011, **19**:614–620.
13. McMenamin AJ, Brutscher LM, Glenny W, Flenniken ML: **Abiotic and biotic factors affecting the replication and pathogenicity of bee viruses**. *Curr. Opin. Insect Sci.* 2016, **16**:14–21.
14. McMenamin AJ, Genersch E: **Honey bee colony losses and associated viruses**. *Curr. Opin. Insect Sci.* 2015, **8**:121–129.
15. Barron AB: **Death of the bee hive: Understanding the failure of an insect society**. *Curr. Opin. Insect Sci.* 2015, **10**:45–50.
16. Cornman RS, Tarpy DR, Chen Y, Jeffreys L, Lopez D, Pettis JS, vanEngelsdorp D, Evans JD: **Pathogen webs in collapsing honey bee colonies**. *PLoS One* 2012, **7**:e43562.
17. Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, Quan P, Briese T, Hornig M, Geiser DM, et al.: **A metagenomic survey of microbes in honey bee colony collapse disorder**. *Science* 2007, **318**:283–287.
18. Genersch E, von der Ohe W, Kaatz H, Schroeder A, Otten C, Büchler R, Berg S, Ritter W, Mühlen W, Gisder S, et al.: **The German bee monitoring project: A long term study to understand periodically high winter losses of honey bee colonies**. *Apidologie* 2010, **41**:332–352.
19. Highfield AC, Nagar A El, Mackinder LCM, Noël LMJ, Hall MJ, Martin SJ, Schroeder DC: **Deformed wing virus implicated in overwintering honeybee colony losses**. *Appl. Environ. Microbiol.* 2009, **75**:7212–7220.
20. Hou C, Rivkin H, Slabezki Y, Chejanovsky N: **Dynamics of the presence of Israeli acute paralysis virus in honey bee colonies with colony collapse disorder**. *Viruses* 2014, **6**:2012–2027.
21. vanEngelsdorp D, Evans JD, Saegerman C, Mullin C, Haubruge E, Nguyen BK, Frazier M, Frazier J, Cox-Foster D, Chen Y, et al.: **Colony collapse disorder: a descriptive study.** *PLoS One* 2009, **4**:e6481.
22. Bailey L, Gibbs AJ: **Acute infection of bees with paralysis virus**. *J. Insect Pathol.* 1964, **6**:395–407.
23. Genersch E, Yue C, Fries I, de Miranda JR: **Detection of Deformed wing virus, a honey bee viral pathogen, in bumble bees (*Bombus terrestris* and *Bombus pascuorum*) with wing deformities**. *J. Invertebr. Pathol.* 2006, **91**:61–63.
24. Li J, Peng W, Wu J, Strange JP, Boncristiani H, Chen Y: **Cross-species infection of Deformed wing virus poses a new threat to pollinator conservation.** *J. Econ. Entomol.* 2011, **104**:732–739.
25. Fürst MA, McMahon DP, Osborne JL, Paxton RJ, Brown MJF: **Disease associations between honeybees and bumblebees as a threat to wild pollinators**. *Nature* 2014, **506**:364–366.
26. McMahon DP, Natsopoulou ME, Doublet V, Fürst MA, Weging S, Brown MJF, Gogol-Döring A, Paxton RJ: **Elevated virulence of an emerging viral genotype as a driver of**

honeybee loss. *Proc. R. Soc. B* 2016, **283**:20160811.

** Injection of DWV-A, DWV-B, or a mixed inoculum into adult bees shows that DWV-B is more virulent in adult bees from UK colonies under laboratory conditions. Extensive field surveys show that DWV-B is at a higher prevalence than DWV-A in the UK but also that coinfections are not uncommon and both are widespread. Computer simulations using laboratory data suggest that DWV-B would result in the death of colonies one year earlier than DWV-A alone.

27. Graystock P, Meeus I, Smagghe G, Goulson D, Hughes WOH: **The effects of single and mixed infections of *Apicystis bombi* and Deformed wing virus in *Bombus terrestris***. *Parasitology* 2016, **143**:358–365.

* This study finds that experimental infection of *Bombus terrestris* with *Apicystis bombi* and Deformed wing virus results in 22% and 50% mortality, respectively, by 15 days after exposure. However, coinfection resulted in 86% mortality by 15 days after exposure. This study is one of the first to experimentally test the effect of coinfection of two widely spread parasites on bumble bee physiology and mortality.

28. Manley R, Boots M, Wilfert L: **Condition-dependent virulence of Slow bee paralysis virus in *Bombus terrestris*: are the impacts of honeybee viruses in wild pollinators underestimated ?** *Oecologia* 2017, **184**:305–315.

* In this paper, the authors show that under starvation conditions Slow bee paralysis virus infected bumble bees die faster than uninfected bees as compared to infected bees that are satiated. These results suggests that laboratory assays which provide food *ad libitum* may underestimate the impact of viral infection on wild pollinators since field conditions can often present nutritional stress.

29. Meeus I, de Miranda JR, de Graaf DC, Wäckers F, Smagghe G: **Effect of oral infection with Kashmir bee virus and Israeli acute paralysis virus on bumblebee (*Bombus terrestris*) reproductive success**. *J. Invertebr. Pathol.* 2014, **121**:64–69.
30. Ravoet J, De Smet L, Meeus I, Smagghe G, Wenseleers T, de Graaf DC: **Widespread occurrence of honey bee pathogens in solitary bees**. *J. Invertebr. Pathol.* 2014, **122**:55–58.
31. Tehel A, Brown MJF, Paxton RJ: **Impact of managed honey bee viruses on wild bees**. *Curr. Opin. Virol.* 2016, **19**:16–22.

** This is a comprehensive review of the impact of viruses, which were discovered in honey bees, on wild bee species. This review includes an extensive table summarizing bee virus infection.

32. Chen YP, Siede R: **Honey bee viruses.** *Adv. Virus Res.* 2007, **70**:33–80.
33. Brutscher LM, McMenamin AJ, Flenniken ML: **The buzz about honey bee viruses**. *PLoS Pathog.* 2016, **12**:e1005757.
34. de Miranda JR: **Classical bee viruses (in preparation)**. *Curr. Opin. Insect Sci.* 2017, [no volume].
35. de Miranda JR, Cornman SR, Evans JD, Semberg E, Haddad NJ, Neumann P, Gauthier L: **Genome characterization, prevalence and distribution of a Macula-like virus from *Apis mellifera* and *Varroa destructor***. *Viruses* 2015, **7**:3586–3602.
36. Remnant EJ, Shi M, Buchmann G, Blacquière T, Holmes EC, Beekman M, Ashe A: **A diverse range of novel RNA viruses in geographically distinct honey bee populations**. *J. Virol.* 2017, **91**:e00158-17.

    ** Next generation sequencing was utilized to identify seven novel viruses, including the first negative-sense single-stranded RNA viruses in honey bees, obtained from managed colonies located in the Netherlands, South Africa, and the Kingdom of Tonga that were infested by *Varroa* but showed no negative consequences of infestation.

37. Levin S, Galbraith D, Sela N, Erez T, Grozinger C, Chejanovsky N: **Presence of Apis rhabdovirus-1 in populations of pollinators and their parasites from two continents**. *Front. Microbiol.* 2017, **8**:2482.
38. Gauthier L, Cornman S, Hartmann U, Cousserans F, Evans JD, de Miranda JR, Neumann P: **The Apis mellifera filamentous virus genome**. *Viruses* 2015, **7**:3798–3815.
39. Hou C, Li B, Deng S, Chu Y, Diao Q: **Diagnosis and distribution of the Apis mellifera filamentous virus (AmFV) in honey bees (*Apis mellifera*) in China**. *Insectes Soc.* 2017, **64**:597–603.
40. Hartmann U, Forsgren E, Charrière J-D, Neumann P, Gauthier L: **Dynamics of Apis mellifera filamentous virus (AmFV) infections in honey bees and relationships with other parasites**. *Viruses* 2015, **7**:2654–2667.
41. Graystock P, Yates K, Evison SE, Darvill B, Goulson D, Hughes WOH: **The Trojan hives: Pollinator pathogens, imported and distributed in bumblebee colonies**. *J. Appl. Ecol.* 2013, **50**:1207–1215.
42. McMahon DP, Fürst MA, Caspar J, Theodorou P, Brown MJF, Paxton RJ: **A sting in the spit: widespread cross-infection of multiple RNA viruses across wild and managed bees**. *J. Anim. Ecol.* 2015, **84**:615–624.

    * This is an extensive survey of known bee-infecting viruses in bumble bee species across the UK. Mixed models of their prevalence data for several viruses indicates that while DWV and BQCV are more associated with honey bees while ABPV and SBPV are more associated with bumble bee species. These data highlight the complex ecology of bee-infecting viruses.

43. Levitt AL, Singh R, Cox-Foster DL, Rajotte E, Hoover K, Ostiguy N, Holmes EC: **Cross-**

species transmission of honey bee viruses in associated arthropods. *Virus Res.* 2013, **176**:232–240.

44. Singh R, Levitt AL, Rajotte EG, Holmes EC, Ostiguy N, vanEngelsdorp D, Lipkin WI, DePamphilis CW, Toth AL, Cox-Foster DL: **RNA viruses in hymenopteran pollinators: evidence of inter-taxa virus transmission via pollen and potential impact on non-Apis hymenopteran species.** *PLoS One* 2010, **5**:e14357.
45. Parmentier L, Smagghe G, de Graaf DC, Meeus I: **Varroa destructor Macula-like virus, Lake Sinai virus and other new RNA viruses in wild bumblebee hosts (*Bombus pascuorum*, *Bombus lapidarius* and *Bombus pratorum*)**. *J. Invertebr. Pathol.* 2016, **134**:6–11.

* This is the first report of VdMLV and LSVs in European bumble bees and the first evidence by detection of the negative strand that LSVs establish infections in bumble bee species. This paper adds another dimension to the ecology and evolution for LSVs, a globally distributed virus group.

46. Guzman-Novoa E, Hamiduzzaman M, Anguiano-Baez R, Correa-Benítez A, Castañeda-Cervantes E, Arnold NI: **First detection of honey bee viruses in stingless bees in North America**. *J. Apic. Res.* 2016, **54**:93–95.
47. Ueira-Veira C, Almeida LO, de Almeida FC, Amaral IMR, Brandeburgo MAM, Bonetti AM: **Scientific note on the first molecular detection of the acute bee paralysis virus in Brazilian stingless bees**. *Apidologie* 2015, **46**:628–630.
48. Lucia M, Reynaldi FJ, Sguazza GH, Abrahamovich AH: **First detection of deformed wing virus in *Xylocopa augusti* larvae (Hymenoptera: Apidae) in Argentina**. *J. Apic. Res.* 2014, **53**:466–468.
49. Radzevičiūtė R, Theodorou P, Husemann M, Japoshvili G, Kirkitadze G, Zhusupbaeva A, Paxton RJ: **Replication of honey bee-associated RNA viruses across multiple bee species in apple orchards of Georgia, Germany and Kyrgyzstan**. *J. Invertebr. Pathol.* 2017, **146**:14–23.
50. NCBI Research Coordinators: **Database resources of the National Center for Biotechnology Information.** *Nucleic Acids Res.* 2016, **44**:D7–D19.
51. Bowen-Walker P, Martin S, Gunn A: **The transmission of Deformed wing virus between honeybees (*Apis mellifera L.*) by the ectoparasitic mite *Varroa jacobsoni Oud***. *J. Invertebr. Pathol.* 1999, **73**:101–106.
52. Nazzi F, Brown SP, Annoscia D, Del Piccolo F, Di Prisco G, Varricchio P, Vedova G Della, Cattonaro F, Caprio E, Pennacchio F: **Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies**. *PLoS Pathog.* 2012, **8**:e1002735.
53. Ongus JR, Peters D, Bonmatin J, Bengsch E, Vlak JM, van Oers MM: **Complete sequence of a picorna-like virus of the genus *Iflavirus* replicating in the mite *Varroa destructor***. *J. Gen. Virol.* 2004, **85**:3747–3755.
54. Wilfert L, Long G, Legget HC, Schmid-Hempel P, Butlin R, Martin SJ, Boots M:

Deformed wing virus is a recent global epidemic in honeybees driven by *Varroa* mites. *Science* 2016, **351**:594–597.

* This paper presents a thorough phylogenetic analysis of global DWV isolates and shows the ecology of this virus is primarily shaped by honey bees and the parasitic mite Varroa destructor.

55. Di Prisco G, Annoscia D, Margiotta M, Ferrara R, Varricchio P, Zanni V, Caprio E, Nazzi F, Pennacchio F: **A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health**. *Proc. Natl. Acad. Sci.* 2016, **113**:3203–3208.

* Using several measures of immune stimulation this paper demonstrates that higher DWV copy numbers are associated with reduced immunocompetence in 5th instar honey bee larvae and increased fitness of feeding *Varroa* mites. These data suggest a mutualism between DWV and Varroa.

56. Gisder S, Aumeier P, Genersch E: **Deformed wing virus: Replication and viral load in mites (*Varroa destructor*)**. *J. Gen. Virol.* 2009, **90**:463–467.
57. Yue C, Genersch E: **RT-PCR analysis of Deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*)**. *J Gen Virol* 2005, **86**:3419–3424.
58. Chen Y, Pettis JS, Evans JD, Kramer M, Feldlaufer M: **Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor***. *Apidologie* 2004, **35**:441–448.
59. Di Prisco G, Pennacchio F, Caprio E, Boncristiani HF, Evans JD, Chen Y: ***Varroa destructor* is an effective vector of Israeli acute paralysis virus in the honeybee, *Apis mellifera***. *J. Gen. Virol.* 2011, **92**:151–155.
60. Celle O, Blanchard P, Olivier V, Schurr F, Cougoule N, Faucon JP, Ribière M: **Detection of Chronic bee paralysis virus (CBPV) genome and its replicative RNA form in various hosts and possible ways of spread**. *Virus Res.* 2008, **133**:280–284.
61. de Miranda JR, Cordoni G, Budge G: **The Acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex**. *J. Invertebr. Pathol.* 2010, **103**:S30–S47.
62. Daughenbaugh KF, Martin M, Brutscher LM, Cavigli I, Garcia E, Lavin M, Flenniken ML: **Honey bee infecting Lake Sinai viruses**. *Viruses* 2015, **7**:3285–3309.
63. Levin S, Sela N, Chejanovsky N: **Two novel viruses associated with the *Apis mellifera* pathogenic mite *Varroa destructor***. *Sci. Rep.* 2016, **6**:37710.

** Using next generation sequencing of total RNA and virus-enriched RNA from honey bees and *Varroa* mites identified two novel viruses isolated from *Varroa*. To our knowledge this is the first use of next generation sequencing to identify novel viruses associated with this parasitic mite of honey bees.

64. Mordecai GJ, Brettell LE, Pachori P, Villalobos EM, Martin SJ, Jones IM, Schroeder DC:

Moku virus; a new *Iflavirus* found in wasps, honey bees and *Varroa*. *Sci. Rep.* 2016, **6**:34983.

* Next generation sequencing of poly-A enriched RNA pools was used to identify Moku virus, a novel virus found in *Vespula pensylvanica* a social wasp and predator of honey bees. Partial genome sequence was recovered from honey bees and *Varroa*. This is the first potential evidence for a wasp-associated virus that may also infect bees, though this remains to be definitively shown.

65. Chantawannakul P, Ward L, Boonham N, Brown M: **A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in *Varroa* mites collected from a Thai honeybee (*Apis mellifera*) apiary**. *J. Invertebr. Pathol.* 2006, **91**:69–73.
66. Mondet F, de Miranda JR, Kretzschmar A, Le Conte Y, Mercer AR: **On the front line: Quantitative virus dynamics in honeybee (*Apis mellifera L.*) colonies along a new expansion front of the parasite *Varroa destructor***. *PLoS Pathog.* 2014, **10**:e1004323.
67. Shen M, Cui L, Ostiguy N, Cox-Foster D: **Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and Sacbrood virus) with the honeybee host and the parasitic *Varroa* mite**. *J. Gen. Virol.* 2005, **86**:2281–2289.
68. Martin SJ, Highfield AC, Brettell L, Villalobos EM, Budge GE, Powell M, Nikaido S, Schroeder DC: **Global honey bee viral landscape altered by a parasitic mite**. *Science* 2012, **336**:1304–1306.
69. Moore J, Jironkin A, Chandler D, Burroughs N, Evans DJ, Ryabov E V.: **Recombinants between Deformed wing virus and Varroa destructor virus-1 may prevail in *Varroa destructor*-infested honeybee colonies.** *J. Gen. Virol.* 2011, **92**:156–161.
70. Ryabov E V., Wood GR, Fannon JM, Moore JD, Bull JC, Chandler D, Mead A, Burroughs N, Evans DJ: **A virulent strain of Deformed wing virus (DWV) of honeybees (*Apis mellifera*) prevails after *Varroa destructor*-mediated, or *in vitro*, transmission**. *PLoS Pathog.* 2014, **10**:e1004230.
71. Peng W, Li J, Boncristiani H, Strange JP, Hamilton M, Chen Y: **Host range expansion of honey bee Black Queen Cell Virus in the bumble bee, *Bombus huntii***. *Apidologie* 2011, **42**:650–658.
72. Mazzei M, Carrozza ML, Luisi E, Forzan M, Giusti M, Sagona S, Tolari F, Felicioli A: **Infectivity of DWV associated to flower pollen: experimental evidence of a horizontal transmission route**. *PLoS One* 2014, **9**:e113448.
73. Piot N, Snoeck S, Vanlede M, Smagghe G, Meeus I: **The effect of oral administration of dsRNA on viral replication and mortality in *Bombus terrestris***. *Viruses* 2015, **7**:3172–3185.
74. Niu J, Smagghe G, De Coninck DIM, Van Nieuwerburgh F, Deforce D, Meeus I: **In vivo study of Dicer-2-mediated immune response of the small interfering RNA pathway upon systemic infections of virulent and avirulent viruses in *Bombus terrestris***. *Insect Biochem. Mol. Biol.* 2016, **70**:127–137.

75. Ravoet J, De Smet L, Wenseleers T, de Graaf DC: **Genome sequence heterogeneity of Lake Sinai virus found in honey bees and Orf1/RdRP-based polymorphisms in a single host**. *Virus Res.* 2015, **201**:67–72.
76. DeGrandi-Hoffman G, Chen Y, Huang E, Huang MH: **The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (*Apis mellifera L.*)**. *J. Insect Physiol.* 2010, **56**:1184–1191.
77. Delaplane KS, Pietravalle S, Brown MA, Budge GE: **Honey bee colonies headed by hyperpolyandrous queens have improved brood rearing efficiency and lower infestation rates of parasitic *Varroa* mites**. *PLoS One* 2015, **10**:e0142985.
78. Retschnig G, Williams GR, Mehmann MM, Yañez O, de Miranda JR, Neumann P: **Sex-specific differences in pathogen susceptibility in honey bees (*Apis mellifera*)**. *PLoS One* 2014, **9**:e85261.
79. Bull JC, Ryabov E V., Prince G, Mead A, Zhang C, Baxter LA, Pell JK, Osborne JL, Chandler D: **A strong immune response in young adult honeybees masks their increased susceptibility to infection compared to older bees**. *PLoS Pathog.* 2012, **8**:11–14.
80. Dolezal AG, Hendrix SD, Scavo NA, Carrillo-tripp J, Harris A, Wheelock MJ, Neal MEO, Toth AL: **Honey bee viruses in wild bees: Viral prevalence, loads, and experimental inoculation**. *PLoS One* 2016, **11**:e0166190.

** Experimental exposure of solitary bee species (i.e., *Megachile rotunda* and *Colletes inaequalis*) to viruses isolated from honey bees demonstrated less mortality in solitary bees, as compared to honey bees. This paper highlights the importance of understanding the effect these viruses have on particular host species.

81. Bailey L: **Viruses attacking the honey bee**. *Adv. Virus Res.* 1976, **20**:271–304.
82. Bailey L, Ball B V.: *Honey Bee Pathology*. Elsevier Ltd; 1991.
83. Runckel C, Flenniken ML, Engel JC, Ruby JG, Ganem D, Andino R, DeRisi JL: **Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, *Nosema*, and *Crithidia***. *PLoS One* 2011, **6**:e20656.
84. Feng Y, Krueger EN, Liu S, Dorman K, Bonning BC, Miller WA: **Discovery of known and novel viral genomes in soybean aphid by deep sequencing**. *Phytobiomes* 2017, **1**:36–45.
85. Sébastien A, Lester PJ, Hall RJ, Wang J, Moore NE, Gruber MAM: **Invasive ants carry novel viruses in their new range and form reservoirs for a honeybee pathogen**. *Biol. Lett.* 2015, **11**:20150610.
86. Johnson RM, Evans JD, Robinson GE, Berenbaum MR: **Changes in transcript abundance relating to colony collapse disorder in honey bees (*Apis mellifera*).** *Proc. Natl. Acad. Sci.* 2009, **106**:14790–14795.
87. Cepero A, Ravoet J, Gómez-Moracho T, Bernal J, Del Nozal MJ, Bartolomé C, Maside X, Meana A, González-Porto A V, de Graaf DC, et al.: **Holistic screening of collapsing**

honey bee colonies in Spain: A case study. *BMC Res. Notes* 2014, **7**:649.

88. Bigot D, Dalmon A, Roy B, Hou C, Germain M, Romary M, Deng S, Diao Q, Weinert LA, Cook JM, et al.: **The discovery *Halictivirus* resolves the *Sinaivirus* phylogeny**. *J. Gen. Virol.* 2017, **98**:2864–2875.

**This paper uses next generation sequencing to identify the novel virus Halictus scabiosae Adlikon virus, belonging to the new virus genus *Halictivirus* in sweat bee samples. Additionally, they find the first evidence of LSVs in ant species (*Messor spp.*) and, using HsAV as an outgroup, resolve the LSV phylogeny into four clades with no apparent geographical pattern.

89. Malfroy SF, Roberts JMK, Perrone S, Maynard G, Chapman N: **A pest and disease survey of the isolated Norfolk Island honey bee (*Apis mellifera*) population**. *J. Apic. Res.* 2016, **55**:202–211.
90. Granberg F, Vicente-Rubiano M, Rubio-Guerri C, Karlsson OE, Kukielka D, Belák S, Sánchez-Vizcaíno JM: **Metagenomic detection of viral pathogens in Spanish honeybees: Co-infection by Aphid lethal paralysis, Israeli acute paralysis and Lake Sinai viruses**. *PLoS One* 2013, **8**:e57459.
91. Ravoet J, Maharramov J, Meeus I, De Smet L, Wenseleers T, Smagghe G, de Graaf DC: **Comprehensive bee pathogen screening in Belgium reveals *Crithidia mellificae* as a new contributory factor to winter mortality**. *PLoS One* 2013, **8**:e72443.
92. Li JL, Scott Cornman R, Evans JD, Pettis JS, Zhao Y, Murphy C, Peng WJ, Wu J, Hamilton M, Boncristiani HF, et al.: **Systemic spread and propagation of a plant-pathogenic virus in European honeybees, *Apis mellifera***. *MBio* 2014, **5**:e00898-13.
93. Longdon B, Murray GGR, Palmer WJ, Day JP, Parker DJ, Welch JJ, Obbard DJ, Jiggins FM: **The evolution, diversity, and host associations of rhabdoviruses**. *Virus Evol.* 2015, **1**:vev014.
94. Schwarz RS, Bauchan GR, Murphy CA, Ravoet J, de Graaf DC, Evans JD: **Characterization of two species of Trypanosomatidae from the honey bee *Apis mellifera*: *Crithidia mellificae* Langridge and McGhee, and *Lotmaria passim* n. gen., n. sp.** *J. Eukaryot. Microbiol.* 2015, **62**:567–583.
95. Runckel C, DeRisi J, Flenniken ML: **A draft genome of the honey bee trypanosomatid parasite *Crithidia mellificae***. *PLoS One* 2014, **9**:e95057.
96. Akopyants NS, Lye L, Dobson DE, Lukeš J, Beverley S: **A novel Bunyavirus-like virus of trypanosomatid protist parasites**. *Genome Announc.* 2016, **4**:e00715-16.
97. Brutscher LM, Daughenbaugh KF, Flenniken ML: **Antiviral defense mechanisms in honey bees**. *Curr. Opin. Insect Sci.* 2015, **10**:71–82.
98. Brutscher LM, Flenniken ML: **RNAi and antiviral defense in the honey bee**. *J. Immunol. Res.* 2015, **2015**:941897.
99. Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E, Sela I: **IAPV, a bee-affecting virus associated with colony collapse disorder can be silenced by dsRNA ingestion**. *Insect Mol. Biol.* 2009, **18**:55–60.

100. Desai SD, Eu YJ, Whyard S, Currie RW: **Reduction in Deformed wing virus infection in larval and adult honey bees (*Apis mellifera L.*) by double-stranded RNA ingestion**. *Insect Mol. Biol.* 2012, **21**:446–455.
101. Brutscher LM, Daughenbaugh KF, Flenniken ML: **Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense**. *Sci. Rep.* 2017, **7**:6448.
102. Hunter W, Ellis J, vanEngelsdorp D, Hayes J, Westervelt D, Glick E, Williams M, Sela I, Maori E, Pettis J, et al.: **Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (Apis mellifera, Hymenoptera: Apidae)**. *PLoS Pathog.* 2010, **6**:e1001160.
103. Liu X, Zhang Y, Yan X, Han R: **Prevention of Chinese Sacbrood virus infection in *Apis cerana* using RNA interference**. *Curr. Microbiol.* 2010, **61**:422–428.
104. Flenniken ML, Andino R: **Non-specific dsRNA-mediated antiviral response in the honey bee**. *PLoS One* 2013, **8**:e77263.
105. Chejanovsky N, Ophir R, Schwager MS, Slabezki Y, Grossman S, Cox-Foster D: **Characterization of viral siRNA populations in honey bee colony collapse disorder**. *Virology* 2014, **454**–**455**:176–183.
106. Goodwin S, McPherson JD, McCombie WR: **Coming of age: Ten years of next-generation sequencing technologies**. *Nat. Rev. Genet.* 2016, **17**:333–351.
107. Craggs JK, Ball JK, Thomson BJ, Irving WL, Grabowska AM: **Development of a strand-specic RT-PCR based assay to detect the replicative form of hepatitis C virus RNA**. *J. Virol. Methods* 2001, **94**:111–120.
108. Plaskon NE, Adelman ZN, Myles KM: **Accurate strand-specific quantification of viral RNA**. *PLoS One* 2009, **4**:e7468.
109. Andino R, Domingo E: **Viral quasispecies**. *Virology* 2015, **479**–**480**:46–51.
110. Lauring AS, Andino R: **Quasispecies theory and the behavior of RNA viruses**. *PLoS Pathog.* 2010, **6**:e1001005.
111. Mordecai GJ, Wilfert L, Martin SJ, Jones IM, Schroeder DC: **Diversity in a honey bee pathogen: first report of a third master variant of the Deformed wing virus quasispecies**. *ISME J.* 2016, **10**:1264–1273.

    * This paper defines three DWV master variants and provides the first data on the existence of DWV-C

112. de Miranda JR, Genersch E: **Deformed wing virus**. *J. Invertebr. Pathol.* 2010, **103**:S48–S61.
113. Dainat B, Evans JD, Chen YP, Gauthier L, Neumann P: **Dead or alive: Deformed wing virus and *Varroa destructor* reduce the life span of winter honeybees**. *Appl. Environ. Microbiol.* 2012, **78**:981–987.
114. Yang X, Cox-Foster DL: **Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification.** *Proc. Natl. Acad. Sci.* 2005, **102**:7470–7475.

115. Natsopoulou ME, McMahon DP, Doublet V, Frey E, Rosenkranz P, Paxton RJ: **The virulent, emerging genotype B of Deformed wing virus is closely linked to overwinter honeybee worker loss**. *Sci. Rep.* 2017, **7**:5242.
116. Roberts JMK, Anderson DL, Durr PA: **Absence of Deformed wing virus and *Varroa destructor* in Australia provides unique perspectives on honeybee viral landscapes and colony losses**. *Sci. Rep.* 2017, **7**:6925.
117. Cornman RS: **Relative abundance of Deformed wing virus, Varroa destructor virus 1, and their recombinants in honey bees (*Apis mellifera*) assessed by kmer analysis of public RNA-Seq data**. *J. Invertebr. Pathol.* 2017, **149**:44–50.
118. Dalmon A, Desbiez C, Coulon M, Thomasson M, Conte Y Le, Alaux C, Vallon J, Moury B: **Evidence for positive selection and recombination hotspots in Deformed wing virus (DWV)**. *Sci. Rep.* 2017, **7**:41045.
119. Nei M, Kumar S: *Molecular Evolution and Phylogenetics*. Oxford University Press; 2000.
120. Kumar S, Stecher G, Tamura K: **MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets**. *Mol. Biol. Evol.* 2016, **33**:1870–1874.
121. Edgar RC: **MUSCLE: Multiple sequence alignment with high accuracy and high throughput**. *Nucleic Acids Res.* 2004, **32**:1792–1797.
122. Felsenstein J: **Confidence limits on phylogenies: An approach using the bootstrap**. *Evolution (N. Y).* 1985, **39**:783–791.
123. Kevill JL, Highfield A, Mordecai GJ, Martin SJ, Schroeder DC: **ABC assay : Method development and application to quantify the role of three DWV master variants in overwinter colony losses of European honey Bees**. *Viruses* 2017, **9**:314.
124. Mordecai GJ, Brettell LE, Martin SJ, Dixon D, Jones IM, Schroeder DC: **Superinfection exclusion and the long-term survival of honey bees in *Varroa*-infested colonies.** *ISME J.* 2016, **10**:1182–1191.
125. Dainat B, Evans JD, Chen YP, Gauthier L, Neumann P: **Predictive markers of honey bee colony collapse.** *PLoS One* 2012, **7**:e32151.
126. Dainat B, Neumann P: **Clinical signs of Deformed wing virus infection are predictive markers for honey bee colony losses.** *J. Invertebr. Pathol.* 2013, **112**:278–80.
127. Nazzi F, Pennacchio F: **Disentangling multiple interactions in the hive ecosystem**. *Trends Parasitol.* 2014, **30**:556–561.
128. Lamp B, Url A, Seitz K, Eichhorn J, Riedel C, Sinn LJ, Indik S, Köglberger H, Rümenapf T: **Construction and rescue of a molecular clone of Deformed wing virus (DWV)**. *PLoS One* 2016, **11**:e0164639.

** This work presents the first published infectious molecular clone of a honey bee-infecting virus. The development of infectious molecular clones will greatly improve our ability to dissect the biology of these viruses and understand how they contribute to poor pollinator health.

129. Manley R, Boots M, Wilfert L: **Emerging viral disease risk to pollinating insects: Ecological, evolutionary and anthropogenic factors.** *J. Appl. Ecol.* 2015, **52**:331–340.

130. Marston HD, Folkers GK, Morens DM, Fauci AS: **Emerging viral diseases: Confronting threats with new technologies.** *Sci. Transl. Med.* 2014, **6**:253ps10.

CHAPTER THREE

HONEY BEE AND BUMBLE BEE ANTIVIRAL DEFENSE

Contributions of Authors and Co-authors

Manuscript in Chapter Three

Author: Alexander J. McMenamin

Contributions: Wrote and edited the review

Co-author: Katie F. Daughenbaugh

Contributions: Co-wrote and edited the review

Co-author: Fenali Parekh

Contributions: Co-wrote and edited the review

Co-author: Marie C. Pizzorno

Contributions: Co-wrote and edited the review

Co-author: Michelle L. Flenniken

Contributions: Co-wrote and edited the review

Manuscript Information

Alexander J. McMenamin, Katie F. Daughenbaugh, Fenali Parekh, Marie C. Pizzorno,

Michelle L. Flenniken

Honey bee and bumble bee antiviral defense

Viruses

Status of Manuscript
___ Prepared for submission to a peer-reviewed journal
___ Officially submitted to a peer-reviewed journal
___ Accepted by a peer-reviewed journal
_X_ Published in a peer-reviewed journal

2018 Jul 27;10(8):395. doi: 10.3390/v10080395.

Honey Bee and Bumble Bee Antiviral Defense

Alexander J. McMenamin[1,4], Katie F. Daughenbaugh[2,4], Fenali Parekh[1,4], Marie Pizzorno[3], and Michelle L. Flenniken[2,4]

[1]Department of Microbiology and Immunology, [2]Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717

[3]Biology Department, Bucknell University, Lewisburg, PA, 17837

[4]Center for Pollinator Health, Montana State University, Bozeman, MT 59717

ABSTRACT

Bees are important plant pollinators in both natural and agricultural ecosystems. Managed and wild bees have experienced high average annual colony losses, population declines, and local extinctions in many geographic regions. Multiple factors, including virus infections, impact bee health and longevity. The majority of bee-infecting viruses are positive-sense single stranded RNA viruses. Bee-infecting viruses often cause asymptomatic infections but may also cause paralysis, deformity or death. The severity of infection is governed by bee host immune responses and influenced by additional biotic and abiotic factors. Herein, we highlight studies that have contributed to the current understanding of antiviral defense in bees, including the

Western honey bee (*Apis mellifera),* Eastern honey bee (*Apis cerana*), and bumble bee (*Bombus spp.*). Bee antiviral defense mechanisms include RNA interference (RNAi), endocytosis, melanization, encapsulation, autophagy, and conserved immune pathways including Jak/STAT (Janus kinase/signal transducer and activator of transcription), JNK (c-Jun N-terminal kinase), MAPK (mitogen-activated protein kinases), and the NF-κB mediated Toll and Imd (immune deficiency) pathways. Studies in Dipteran insects, including the model organism *Drosophila melanogaster* and pathogen-transmitting mosquitos, provide the framework for understanding bee antiviral defense. However, there are notable differences such as the more prominent role of a non-sequence specific, dsRNA-triggered, virus limiting response in honey bees and bumble bees. This virus-limiting response in bees is akin to pathways in a range of organisms including other invertebrates (i.e., oysters, shrimp, and sand flies), as well as the mammalian interferon response. Current and future research aimed at elucidating bee antiviral defense mechanisms may lead to development of strategies that mitigate bee losses, while expanding our understanding of insect antiviral defense and the potential evolutionary relationship between sociality and immune function.

INTRODUCTION

Bees – Hymenopteran insects that play an important ecological role as plant pollinators

There are over 4,000 bee species in the order Hymenoptera, including those that are social or solitary, native or introduced, managed or wild [1]. Bees are important pollinators of plant species, including agricultural crops (e.g., almonds, apples, cherries, squash, tomatoes) and ecologically important plants. In the United States honey bee pollination is valued at 14.6 billion annually [2], and insect pollination worldwide is valued at $175 billion annually [3]. Due to their abundance and economic importance, most of the research on bee host – virus interactions has focused on honey bees.

Western honey bees (*Apis mellifera*) are eusocial, cavity nesting bees that are native to Europe, Africa, and the Middle East; they were introduced into North America in the late 1600s [4-6]. The eastern honey bee (*Apis cerana*) is a related, but distinct species endemic to Asia, and detected in Australia in 2007 [7, 8]. Honey bee colonies consist of approximately 35,000 individual bees, including sterile female workers, a few hundred male bees (called drones), and a single reproductive female queen bee [9]. Honey bee colonies typically survive multiple years, while the longevity of individual worker bees depends on their cast (i.e., from six weeks to four months for worker bees, approximately eight weeks for drones, and several years for queen bees [9]). The majority of the approximately 2.5 million honey bee colonies in the United States (US) are managed by professional beekeepers and are involved in pollinating the almond crop, which

is the largest pollination event in the world [10, 11]. Since 2006, US beekeeping operations have suffered approximately 33% annual losses, which is an increase from historic levels of approximately 12-15% [10, 12-17]. Many biotic and abiotic factors contribute to these losses, including pathogenic infections, mite infestation levels, agrochemical-exposure, management, and lack of quality forage and habitat (reviewed in [18-24]). Viruses, including Deformed wing virus (DWV), are one of the factors that contribute to individual bee and colony deaths.

Bumble bees, including *Bombus terrestris* and *Bombus impatiens,* are also important agricultural pollinators of crops such as tomatoes and peppers, as well as blueberries and other ecologically important plant species [25]. Bumble bees are ground nesting bees that live in small annual colonies with distinct solitary and social life cycle phases [26]. Unlike honey bees, bumble bees (*B. terrestris)* rear one generation per year [27]. This means that the queen survives one year, and her reproductive daughters (gynes) start new colonies in the spring after an overwinter period of torpor (called diapause) [28, 29]. There are numerous species of bumble bees, some of which have suffered high losses and local extinctions that are partially attributed to habitat destruction and fragmentation, chemical-exposure, pathogens, and climate change, [26, 30-37]. The majority of bumble bee host pathogen research has focused on microsporidia (i.e., *Nosema bombi*) and trypanosomatid (i.e., *Crithidia bombi*) infections [38-42], though there is a growing body of virus literature, which is featured herein [43-48]. Recent metagenomic sequencing analysis of bumble bees (i.e., *Bombus terrestris*, *Bombus cryptarum*, *Bombus pascuorum*) obtained from several locations in Belgium identified several known bee infecting viruses (e.g., BQCV, VDV-

1/DWV-B, DWV), including potentially different strains, as well as numerous new bee-associated viruses including (+)ssRNA, (-)ssRNA, and dsDNA viruses [49]. Future studies aimed at characterizing the full genome sequences, virion structure, potential pathogenicity, host-specific antiviral responses, and inter-tax transmission of these viruses will greatly expand our understanding of bee virology [49]. Virus infections of social bees, including honey bees and bumble bees, may impact bee health at the superorganism (i.e., entire colony) and/or individual bee levels. Typically, colony population size is used as a proxy for colony health, whereas pathogen burden, life span, glandular protein content, and queen bee fecundity are used as proxies of individual bee health [12, 14, 16, 50-55].

Solitary bees including alfalfa leaf cutter bees (*Megachile rotundata*), blue orchard or mason bees (*Osmia lignaria*), and many other native and wild bee species are important plant pollinators. Some are generalist pollinators, whereas others are specialist pollinators that primarily interact with one or just a few plants (reviewed in [56]). Interestingly, numerous studies indicate that agricultural systems that include both managed and native and wild bee species have improved crop yield [57-60]. Less is known about the health of these bees, but in general habitat destruction, pathogenic infections, lack of quality forage, and agrochemical exposure are detrimental to bee health and population size (reviewed in [33]). Therefore, strategies that promote bee health including planting and/or maintaining pollinator forage, maintaining nesting sites (including bare earth for ground nesting bees), and reduced use of chemicals, particularly insecticides, will benefit all bee species. Though more research on the

impact of viruses on solitary bees is needed, many studies have shown that these bee species are infected by viruses originally discovered in honey bees [61], as well as viruses and other parasites that may be unique to particular hosts [49]. For example, high throughput sequencing of the metatranscriptomes of eight wild bee species including five solitary bee species (i.e., *Andrena cineraria*, *Osmia bicornis*, *Osmia cornuta*, *Andrena fulva*, and *Andrena haemorrhoa*) in Belgium resulted in strong support for Bee macula-like 2 virus infection of *A. haemorrhoa* and detection of several new partial virus genomes including a nege-like virus and a toti-like virus in *A. haemorrhoa* and *O. cornuta*, respectively [49].

A current focus of bee virus research is investigating intra- and inter- genera transmission of viruses. Though difficult to investigate, phylogenetic analyses of virus genome sequences obtained from co-foraging bee hosts have indicated that viruses are bidirectionally transmitted between managed and wild bee species [62-65] (reviewed in [66]). Additional studies are required to determine the extent of virus replication, as opposed to virus prevalence, and pathogenesis across bee taxa. Inter-genera virus transmission is likely influenced by virus prevalence and abundance in bee populations, as well as the dynamic composition of bee and forb species in specific geographic regions. In addition, plant-pollinator networks, and in turn pathogen transmission between co-foraging bees, are influenced by habitat loss and will likely be influenced by climate change [31, 37]. Investigating the co-evolutionary history of specific virus-host pairs, host antiviral immune responses, and viral counter measures in numerous bee species will greatly enhance our understanding of bee virus ecology.

Bee viruses

In this review we will use the term ‘bee virus’, though insect viruses generally have a broad host range and ‘bee viruses’ can infect a variety of bee hosts, as well as ants and mites [67-70] (reviewed in [66]). Because of their role in agriculture, honey bees (*Apis mellifera*) are the most investigated bee species, and thus the majority of bee-infecting viruses were discovered in honey bee samples. Most bee viruses are positive-sense single-stranded RNA viruses with approximately 30 nm diameter icosahedral capsids. These include Dicistroviruses (black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV)), Iflaviruses (deformed wing virus (DWV), sacbrood virus (SBV), slow bee paralysis virus (SBPV)), and yet-to-be taxonomically classified viruses including chronic bee paralysis virus (CBPV) and the Lake Sinai virus (LSV) group (reviewed in [68, 70, 71]. Recent sequencing efforts have discovered new bee viruses from additional families (reviewed in [72]) including viruses with negative-sense RNA genomes and enveloped virions [73]. To date, only one bee-infecting DNA virus, Apis mellifera filamentous virus (AmFv), has been described [74]. For a more thorough review of bee virology, see Grozinger and Flenniken [75] and Chen and Siede [70].

Bee viruses are transmitted vertically within species and horizontally, both within species and between different bee genera [62-64, 70]. Horizontal transmission is facilitated by food transfer (i.e., trophallaxis in social bee) between individual bees within a colony, and between colonies and bee species via the sharing of floral resources (i.e., nectar and pollen) [9, 62, 76]. Honey bee

viruses are also transmitted within and between honey bee colonies by the ectoparasitic mite *Varroa destructor* (i.e., DWV, IAPV, KBV) [77-85]. Several studies suggest that DWV replication in mites and/or mite-mediated virus transmission impacts the diversity of viral genomes at both a geographic scale (i.e., mite induced bottleneck of DWV strains in the Hawaiian Islands [86]), and at the individual bee level [87, 88]. Poor honey bee colony health is associated with high mite infestation coupled with deformed wing virus (DWV) infection [83, 85, 89-93] and the seasonal dynamics of mite infestation and DWV abundance are strongly correlated [12, 17, 54, 85, 90, 93-95]. The potential role of parasite-mediated virus transmission is under-explored for other bee species.

Virus infections in bees are primarily asymptomatic or they may result in deformity, paralysis, and/or death (reviewed in [70, 89, 96, 97]) [98, 99 , 100]. The extent of viral pathogenesis is influenced by biotic and abiotic stressors, including the synergistic negative effects of co-infection with multiple pathogens and/or agrochemical exposure, and governed by co-evolved host-virus interactions [37, 101] (reviewed in [75]). The mechanisms of bee antiviral defense, which are described in greater detail below, include conserved immune pathways (i.e., Jak/STAT (Janus kinase/signal transducer and activator of transcription), JNK (c-Jun N-terminal kinase), MAPK (mitogen-activated protein kinases), NF-κB (i.e., Dorsal / Relish) mediated Toll and Imd pathways, RNA-trigged responses (i.e., RNA interference (RNAi) and a non-sequence-specific dsRNA mediated mechanism), autophagy, endocytosis, and melanization (reviewed in [102, 103]) (Figure 1).

Bee Virology

The epidemiology of bee viruses has not been thoroughly investigated, though there have been several insightful studies including the German Bee Monitoring Project [90], apiary level surveillance programs carried out by the US Bee Informed Partnership [12] and the Ministry of Agriculture in Spain [104], honey bee colony health and virus prevalence and abundance studies [12-17, 53-55, 97, 105-110], and the ongoing Canadian National Honey Bee Health Survey [111]. These and other longitudinal monitoring studies have been instrumental in beginning to define how honey bee health relates to the prevalence and abundance of viruses, which varies by season, sampling date, and geographical location [54, 105]. Several studies indicate that at particular times of the year weak colonies are associated with higher pathogen levels, including IAPV, LSV2, DWV, *Nosema ceranae*, and mites [12-16, 53-55, 90, 95, 97, 106, 109, 110, 112-114], though additional monitoring efforts are required to determine impacts of virus infection at the colony level.

To examine the impact of viruses on individual bees, Bailey, Ball, and others used semi-purified viruses from infected bees/pupae to catalogue the symptoms associated with particular viruses [70, 115, 116]. Symptomatic virus infections in honey bees include a “hairless” or “greasy” phenotype associated with CBPV, wing deformity and shortened or bloated abdomens caused by DWV, paralysis associated with IAPV and ABPV [117] and complications with larval

development due to SBV and BQCV infections [70]. In addition, asymptomatic or covert infections may cause more subtle symptoms, such as the precocious foraging behavior and reduced lifespan associated with DWV-infections [118]. To date, only two infectious bee virus clones, BQCV [119] and DWV [120] have been described, and only the DWV clone is currently available. Bee cell culture includes the use of primary cells obtained from embryos, larvae, and adults for short-term studies and a single immortalized cell line [121], which can be difficult to maintain [122]. Therefore, most bee virus research is carried out using viruses isolated from both naturally and experimentally infected bees and/or bee pupae [115]. Isolating viruses from naturally infected bees typically includes several co-purified viruses [115, 122, 123], though nearly pure virus isolates have been obtained (e.g., LSV2 [112]). Likewise, propagation of DWV and IAPV in pupae has resulted in relatively pure virus preparations [97, 124-126]. The lack of infectious clones and robust cell culture models has made investigating bee viruses challenging, but the use of model viruses, including Sindbis-GFP [127, 128] and Flock house virus (FHV) [129], and semi-purified virus inocula in individual bee and cultured cell experiments has provided insight into the mechanisms of antiviral defense [61, 122, 127-130].

The genomes of several bee species including *Apis mellifera* [131], *Apis cerana* [7], *Bombus terrestris*, *Bombus impatiens* [132], and *Megachile rotundata* have been sequenced and partially annotated [133]. Genomic information facilitates the use of molecular techniques (e.g., high through-put sequencing, qRT-PCR, cloning, and RNA interference-mediated gene knock down)

to investigate and understand bee host – virus interactions at both the colony and individual levels.

BEE ANTIVIRAL DEFENSE

Bee antiviral defense mechanisms include dsRNA-triggered responses, hemocyte-mediated mechanisms (e.g., endocytosis, melanization, and encapsulation), and conserved pathways including JAK/STAT, JNK, MAPK, and the NF-κB mediated Toll and Imd pathways (reviewed in [102, 103]) (Figure 1).

Signal transduction cascades regulate the expression of genes, including those that are likely involved in limiting honey bee virus infections (reviewed in [102, 131]), though very few studies have characterized the role of potential antiviral effector proteins in bees [85, 127, 129]. Gene function in bees is largely based on the roles of orthologous genes in model organisms, including *Drosophila melanogaster* [134-140] and mosquitos [141, 142]. For example, *Drosophila melanogaster* encodes three NF-κB transcription factors (i.e., *relish*, *dif*, and *dorsal*) which are involved in promoting the expression of specific antimicrobial peptides (AMPs) [137, 143]. Honey bees have two major NF-κB like transcription factors (i.e., *dorsal* and *relish*) involved in the Toll and Imd pathways, respectively. Honey bees lack *dif*, but encode two dorsal homologues

(i.e., *dorsal-1* and *dorsal-2*) and there are two isoforms of *dorsal-1* (i.e., 1A and 1B) [144]. Toll and Imd pathway activation increases expression of *defensin-1*, a Hymenoptera-specific AMP *hymenoptaecin*, and both pathways govern expression of *abaecin* [144-146]. The roles of AMPs in host defense are best characterized in the context of bacterial and fungal infections where these cationic peptides act by disrupting pathogen cell membranes/walls [143, 145, 147]. The function of AMPs in virus infection is not yet known, though they serve as hallmarks of immune pathway activation [145, 148, 149].

There are several reports on the transcriptional level perturbations associated with virus infection and/or mite infestation of honey bees [87, 93, 97, 126-128, 150-153]. Even though these studies vary in terms of virus, infection route, inoculated versus field-acquired infections, bee age, sample date, and whether the presence of co-occurring infections were or were not examined, there were some general trends observed including increased expression of several AMPs (i.e., *apidaecin*, *hymenoptaecin*, *abaecin*, *lysozyme*, and *defensins*), immune genes (e.g., *peptidoglycan recognition protein (PGRP)-S2*, *TEPs, prophenoloxidase, eater*), transcription factors, components of the RNAi machinery (i.e., *dicer* and *argonaute-2*), and differential expression of genes involved in metabolic pathways and cellular differentiation [87, 93, 97, 126-128, 150, 152, 153]. Several studies determined that *protein lethal*(*2*)*essential for life-like*, which encodes a protein in the small heat shock protein (Hsp20) family was differentially expressed in virus-infected bees [127]. The differential expression of this and other Hsp family proteins in

virus-infected bees is intriguing, since the heat shock response is an important antiviral response in *Drosophila melanogaster* [154, 155] and *Anopheles gambiae* [156].

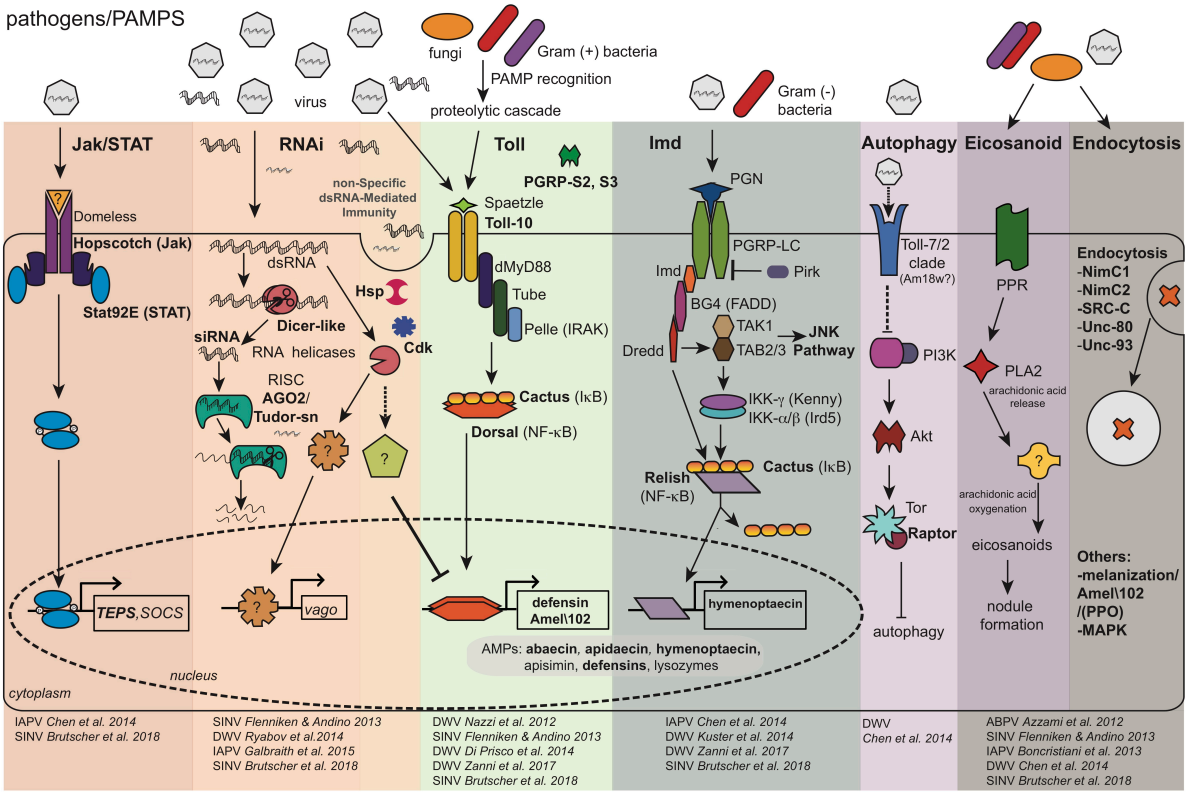


Figure 1. Honey Bee Immune Pathways - Highlighting Genes Implicated in Antiviral Immune Responses.
The honey bee genome encodes major members of insect immune pathways including: Jak/STAT (Janus kinase/Signal Transducer and Activator of Transcription); RNAi (RNA interference); Toll via NF-□B (Nuclear Factor □B/Dorsal); Imd (Immune deficiency) via NF-□B /Relish; JNK (c-Jun N-terminal kinase); and MAPK (Mitogen-Activated Protein Kinases), as well as orthologs of genes involved in the heat shock response (Hsp), autophagy, eicosanoid biosynthesis, endocytosis, melanization, and prophenoloxidase (PPO) response. Bold text indicates genes and proteins differentially expressed in virus-infected honey bees and/or bumble

bees. The first step in immune activation is host recognition of pathogen-associated molecular patterns (PAMPs) including viral dsRNA, bacterial peptidoglycans, and fungal □-glucans. In general, the Toll pathway is involved in defense against Gram(+) bacteria and fungi and the Imd pathway is activated by Gram(-) bacteria, but specific host-pathogen interactions are unique. This is particularly true for host – virus interactions since data from fruit-flies, mosquitoes, and honey bees indicate differential activation of immune genes and pathways. The **Jak/STAT pathway** is activated via ligand binding to the Domeless receptor; while *Drosophila melanogaster (Dm)* express Domeless ligands (*unpaired*, *upd*, *upd2*, and *upd3*), a honey bee *upd* ortholog has not been identified. Following Domeless-ligand binding, Hopscotch Janus kinases are transphosphorylated, leading to phosphorylation and dimerization of STAT92E-like proteins. Activated STATs transcriptionally regulate antimicrobial effectors TEP7 (Thioester-containing protein 7), TEPA, TEPB, and the Jak/STAT inhibitor SOCS (Suppressor of Cytokine Signaling). The honey bee genome also encodes for D-PIAS (Protein Inhibitor of Activated STAT), another inhibitor of the Jak/STAT pathway. The **RNAi-pathway** is initiated by *Dm*Dicer-2 cleavage of viral dsRNA into 21-22 bp siRNAs; *Am*Dicer-like shares ~30% aa identity with *Dm*Dicer-2. The siRNAs are then loaded into Ago2 (Argonaute-2*),* the catalytic component of the RISC (RNA Induced Silencing Complex). A single strand of the siRNA is retained in the RISC and used to specifically target cognate viral genome sequences for cleavage. In addition, *Dm*Dicer-2 serves as a **dsRNA sensor** that mediates a signal transduction cascade resulting in increased expression of *Dm*Vago, which suppresses viral replication. *Am*Dicer-like may serve as a **dsRNA sensor**, as honey bees have a *vago* ortholog which is up-regulated in DWV-infected honey bees, but not Sindbis-GFP-infected honey bees. In *B. terrestris,* Vago limits viral infection in fat bodies in a Dicer-dependent manner. Though the mechanism(s) of non-specific dsRNA-mediated antiviral responses in bees require additional characterization, a putative serine/threonine cyclin-dependent kinase (MF 116383) is involved in this virus-limiting response in honey bees. Additionally, several members of the heat shock protein family exhibit increased expression in Sindbis-GFP infected honey bees (i.e., *hsp90*, *activator of hsp90, 60 kda hsp*, *10 kda hsp*, *hsp83-like*, *hsc70-4* and *hsf5*), while dsRNA alone resulted in increased expression of *hsp90*. The **Toll pathway** is activated by a family of pathogen recognition receptors (PRRs) (e.g., peptidoglycan receptor proteins (PGRPs) and Gram(-) binding proteins) that bind fungal and bacterial PAMPs. The Toll pathway is activated in some insect host-virus combinations, although the activation mechanism is unknown. Following PAMP binding, a serine protease cascade results in cleavage of pro-Spaetzle into mature Spaetzle. The honey bee genome encodes two putative *spaetzle* orthologs, which bind the membrane-anchored Toll receptor. Toll dimerization results in the recruitment of dMyD88, Tube, and Pelle. Pelle is likely involved in degradation of NF-□B inhibitors (e.g., Cactus-1, Cactus-2, Cactus-3), resulting in the release of transcription factors Dorsal-1A and Dorsal-1B. Nuclear translocation of Dorsal results in increased expression of antimicrobial peptides (AMPs) and *Amel\102*. The **Imd pathway** is activated by Peptidoglycan recognition protein LC (PGRP-LC) binding to diaminopimelic-containing peptidoglycan of Gram(-) bacteria, followed by activation of the adaptor protein Immune deficiency (Imd), Relish phosphorylation by the IKK complex (I_kB kinase), and cleavage of Relish by the caspase Dredd (Death-related ced-3/Nedd2-like). Relish transcriptionally regulates expression of AMPs and

other genes involved in antimicrobial defense. The **JNK pathway** is also activated by TAK (Transforming growth factor-activated kinase 1) and TAB2/3 (TAK binding protein 2 and 3), resulting in AMP expression and/or apoptosis. In *Drosophila,* binding of vesicular stomatitis virus to the Toll-7 receptor promotes **autophagy**, likely by inhibiting the PI3/Akt/Tor (phosphatidylinositol 3-kinase / Protein kinase B / Target of rapamycin) pathway which suppresses autophagy. The honey bee genome encodes for one gene in the Toll-7/2 clade, *18-wheeler* (*am18w*), which shares ~49% aa identity with *Dm*Toll-7 and ~45% aa identity with *Dm*Toll-2. The role of the Am18w protein in antiviral defense and autophagy in honey bees is unknown. In insects, **Eicosanoid biosynthesis** begins with the induction of PLA2 (Phospholipase 2) from signal cascades downstream of viral, fungal, or bacterial PAMP recognition. Activated PLA2 hydrolyzes arachidonic acid (AA) from cellular phospholipids. Eicosonoid production likely occurs via oxidation of AA by an unidentified enzyme. Eicosanoids are critical for nodulation and aid in phagocytosis, micro-aggregation, adhesion, and release of prophenoloxidase (PPO) from hemocytes. Experimental evidence also suggests **endocytosis, melanization**, **and MAPK** pathways are involved in honey bee antiviral defense. Adapted with permission from Brutscher, et al. Current Opinion in Insect Science, 2015 [102].

Deformed wing virus infection of honey bees (*Apis mellifera*) is the most thoroughly examined virus-bee host pair [85, 89]. Deformed wing virus infects both honey bee larvae and adults, and high DWV infection levels of larvae results in wing deformity in adulthood [85, 87, 118, 157, 158]. Investigation of DWV pathogenesis is complicated by the synergistic negative effects of both *Varroa destructor* mite parasitization and mite-mediated virus transmission, as well as impairment of the antiviral NF-κB/Dorsal-1A pathway as a result of exposure to neonicotinoid insecticides (i.e., clothianidin and imidacloprid) [101]. The complex interactions between host, virus, vector, neonicotinoid exposure, and nutritional status, at both the individual bee and colony levels, were reviewed in this issue of *Viruses* by Nazzi and Pennacchio [159]. Together, several studies have demonstrated the key role of the NF-κB/Dorsal-1A pathway in limiting virus infection, including studies that determined that reduced expression of *dorsal-1A*, both experimentally and naturally, favored virus proliferation [93]. Furthermore, the NF-κB /Dorsal-

1A pathway regulates the expression of *Amel\102*, which is an immune gene likely involved in melanization and encapsulation; *Amel\102* expression is reduced in DWV-infected bees [85, 151]. Mite infestation and DWV levels are often correlated, though the immune response varies [87, 93, 151, 160]. For example, when mite infested bees have low DWV levels the expression of genes in the Toll pathway are increased, whereas high DWV levels are associated with differential expression of genes in the JNK pathway [151]. In addition, RNAi is involved in controlling DWV infection, as evidenced by the production of viral siRNAs in DWV-infected bees [87, 161] and increased cytopathology and DWV abundance in a persistently infected cell line after treatment with a potent RNAi inhibitor (CrPV-1A protein) [122]. Furthermore, and as described below, experimental introduction of DWV-targeting dsRNA reduced DWV abundance and DWV-associated mortality of both larvae and adult bees [87, 162].

Nutritional status and metabolism also influence the outcome of virus infections in honey bees. Several transcriptome studies of virus-infected bees identified differential expression of genes involved in metabolic processes and bees fed higher quality diets had lower virus loads [97, 126, 127, 151]. In addition, recent studies demonstrated that K_{ATP} channels, which respond to metabolic changes in the cell (e.g., the relative levels of ATP and ADP) play a role in limiting viral infection [129]. Specifically, bees fed a K_{ATP} agonist prior to challenge with FHV survived longer and had reduced virus levels compared to untreated bees or bees fed a K_{ATP} antagonist prior to virus challenge [129]. At least one study in *Drosophila melanogaster* links metabolic function to antiviral immunity by suggesting that K_{ATP} channels control FHV infection in an

RNAi-dependent manner [163]. Future studies will determine if changes in metabolic function are a result of the hosts' antiviral response or an energetic consequence of virus infections, but these results provide a clear link between honey bee metabolism and antiviral defense. This is striking, given that naturally infected honey bees, even asymptomatic bees, routinely harbor over one billion virus genome equivalents per individual [105], covert DWV infections reduce longevity [118], and honey bee immune gene expression, which is associated with metabolism, varies with season [153].

Viral dsRNA-triggered antiviral responses in honey bees and bumble bees, including sequence-specific RNAi and non-sequence-specific pathways.

During viral replication, most viruses produce long segments of dsRNA (e.g., replication intermediates of ssRNA viruses and RNA secondary structures in long transcripts of all viruses) that are recognized as pathogen-associated molecular patterns (PAMPs) by host dsRNA-binding proteins. Host recognition of viral dsRNA may result in activation of sequence-specific RNA interference (RNAi) and/or other antiviral immune pathways (e.g., Jak/STAT, Imd, and JNK) (reviewed in [102, 135, 136, 142, 164-166]).

Honey bee antiviral RNA interference

RNAi is a post-transcriptional sequence-specific gene silencing mechanism that is involved in regulating gene expression in most organisms [167, 168]. There are three distinct RNAi pathways including the microRNA (miRNA), piwi-interacting RNA (piRNA), and short-interfering RNA (siRNA) pathways (reviewed in [167, 169]). The siRNA pathway is an important antiviral defense mechanism in plants, fungi, nematodes, and arthropods [141, 167, 169-177]. In brief, cytosolic virally-produced dsRNA is recognized and cleaved by an endonuclease enzyme Dicer (Dcr) into 21-nucleotide small interfering RNAs (siRNAs). The siRNAs are then loaded into the RNA-induced silencing complex (RISC) which contains the protein Argonaute-2 (Ago2). Once loaded into RISC, one of the siRNA strands remains associated with Ago2 (i.e., the guide strand), while the passenger strand is degraded by Ago2 and the endonuclease C3PO (component 3 promoter of RISC) [178]. The guide-strand binds to a complementary target single-stranded RNA, leading to Ago2-mediated cleavage of the target RNA (reviewed in [167]). The antiviral role of RNAi was first reported in solitary insects including *Drosophila melanogaster* infected with Flock house virus (FHV) which is a positive-sense single stranded RNA (ssRNA) virus [179]. Later, flies deficient in *ago-2* were shown to be hypersensitive to viral infection [180], further implicating particular members of the RNAi pathway in antiviral defense. Interestingly, the systemic spread of a sequence-specific RNAi response to uninfected cells, mediated by a dsRNA uptake pathway, was shown to be necessary for an effective antiviral immune response [181, 182]. Recent *Drosophila* studies have revealed

that hemocytes and transposon-encoded reverse transcriptases are involved in amplification and systemic spread of siRNA-mediated antiviral defense [183-185].

The role of RNAi in honey bee (*Apis mellifera*) antiviral defense was demonstrated in laboratory-based experiments in which bees and/or larvae that were fed virus-specific dsRNA harbored reduced levels of virus, as compared to controls [128, 162, 186, 187]. Adult bees fed IAPV-specific dsRNAs had less mortality [187] and lower virus loads [97] post IAPV-infection as compared to bees fed non-sequence specific dsRNA. Likewise, larvae that were fed DWV-specific dsRNA prior to inoculation with DWV had a reduced percentage of wing-deformity and lower DWV virus load than larvae fed non-sequence specific dsRNA (i.e. dsRNA-GFP), although survival was not impacted [162]. The same study determined that adult bees fed dsRNA-DWV prior to DWV inoculation had reduced viral loads and increased longevity, compared to DWV-infected bees [162]. Intriguingly, particularly in the context of later studies described below, adult bees fed dsRNA-GFP prior to DWV inoculation also had increased longevity compared to DWV-infected bees, but the relative abundance of DWV RNA equivalents was similar to DWV-infected bees that were not fed dsRNA [162]. Together, these laboratory-based studies indicated that siRNAs and/or dsRNAs may be useful antiviral treatments, though other studies indicated dsRNA had additional biological impacts on bees [44, 47, 127, 188-191]. A field study in which honey bee colonies were fed both IAPV-specific dsRNA (Remebee-IAPV®) and IAPV determined that dsRNA-treated colonies in one of two locations had larger adult bee populations and produced a greater amount of honey, than colonies

that were only fed IAPV, though virus abundance was not assessed [192]. A more recent, colony level study characterized the siRNAs (21 - 22 nt) produced in bees obtained from naturally and experimentally IAPV-infected colonies [161]. Interestingly, the majority of sequenced IAPV-siRNAs corresponded to the negative sense-strand, whereas the viral siRNAs corresponding to DWV matched both strands [161]. Bee samples from both Colony Collapse Disorder (CCD)-affected and unaffected colonies contained viral siRNAs, indicating that even in the context of CCD, honey bees mounted an RNAi-mediated antiviral response [161].

Further support that RNAi plays an important antiviral role in honey bees is that the expression of *dicer-like* and *ago-2* is higher in virus-infected bees (i.e., IAPV or a model virus Sindbis-GFP), as compared to mock-infected bees [126, 127]. However, increased transcriptional regulation of the RNAi machinery was not observed in DWV-infected honey bees [87]. In addition, RNAi-mediated reduction of honey bee gene expression has become a useful tool to investigate gene function at different honey bee developmental stages including embryos [193], larvae [194, 195], pupae [144, 196], and adult bees [101, 127, 197-200], and provides further evidence that honey bee RNAi machinery is functional.

Honey bee non-sequence-specific dsRNA-mediated antiviral response

While sequence-specific RNAi is an important honey bee antiviral defense mechanism and experimental gene silencing tool, dsRNA also triggers a general non-sequence-specific antiviral

response (reviewed in [127, 173]). Initial observations that dsRNA treatment altered honey bee gene expression in a sequence independent manner were made in the context of targeted gene knock-down studies [188, 191]. As described previously, dsRNA is a viral PAMP that is likely recognized by dsRNA recognition proteins, which in turn activate signal transduction cascades that result in an antiviral transcriptional profile [127, 128]. The first study that demonstrated a non-specific dsRNA-mediated virus-limiting response in honey bees came from laboratory-based experiments in which adult honey bees were co-injected with Sindbis-GFP and virus-sequence specific dsRNA, non-sequence specific dsRNA, or poly(I:C) (a synthetic analogue of dsRNA), all of which reduced viral abundance at 72 hours post infection [128]. Perturbations in honey bee gene expression in response to either virus or dsRNA treatment at 72 hours post-inoculation were identified via microarray analyses compared to mock-infected control [128]. A more comprehensive examination of dsRNA-triggered honey bee antiviral defense mechanisms utilized transcriptome sequencing (RNASeq) to identify differentially expressed genes (DEGs) at 24, 48, and 72 hours post virus-infection and/or dsRNA administration [127]. This study determined that the number of DEGs increased over the course of virus infection and that many genes had unique temporal dynamics. Honey bee genes that were differentially expressed in virus-infected and/or dsRNA treated bees included *hopscotch* in the Jak-STAT pathway, *toll-10* and *tube* in the Toll pathway, *pirk* and *jra* in the Imd pathway, antimicrobial peptides (i.e., *apidaecin1*, *hymenoptaecin*, *abaecin*, and *defensin*), numerous heat shock response genes, *dicer* and *ago-2*, and *scavenger receptor class c*, which is involved in dsRNA uptake in *Drosophila*; most DEGs were functionally uncharacterized [127]. The virus-limiting functions of two of the

identified honey bee antiviral genes (i.e., *dicer*, NCBI GeneID 552127) and a *probable cyclin-dependent serine/threonine kinase (*NCBI GenBank MF116383) was confirmed in individual bee gene knock-down experiments. Reduced expression of either *dicer* or *MF116383* resulted in greater virus abundance [127]. In *Drosophila melanogaster* Dicer is involved in both specific and non-specific dsRNA responses including activating expression of the antiviral gene *vago* [201], though the mechanism of Vago-mediated virus reduction in *Drosophila* is not yet known. In mosquitos, Vago is a secreted protein that limits virus infection by linking the siRNA and Jak/STAT pathways through an unknown receptor [136, 202, 203]. *Vago* expression is increased in DWV-infected honey bees [87], though the neither the signal transduction pathway or transcription factor(s) involved have been identified. Furthermore, vago is not universally up-regulated in response to virus infection in bees (e.g., SINV-GFP, IAPV) [126, 127].

Recognition of non-self virally-produced dsRNA is the first step in activation of an antiviral state in many organisms. Cytosolically-located dsRNA recognition proteins include DExD (Asp-Glu-x-Asp) box helicases, such as Dicer, Retinoic acid-inducible gene I (RIG-I), and Melanoma differentiation-associated protein-5 (MDA-5), Protein kinase R, and endosomal Toll-like receptor 3 in diverse organisms including invertebrates and mammals [44, 47, 201-207]. Though identification of the honey bee proteins that detect and respond to dsRNA is ongoing, we hypothesize that further investigation of this non-sequence specific dsRNA virus limiting response in honey bees will result in the identification of genes that will have conserved antiviral roles in other invertebrate and vertebrate organisms.

Bumble bee RNA interference

Bumble bee and honey bee lineages diverged approximately 90 million years ago [208]. Similar to honey bees, the bumble bee (*B. terrestris*) RNAi machinery (i.e., *ago-2*, *dicer-2*) exhibits slightly increased expression in the context of virus infection (i.e., IAPV and SBPV) [44, 190]. Increased expression of key RNAi genes likely explains the observed enhancement of RNAi-mediated host gene knock-down in IAPV-infected *B. terrestris* [190]. The RNAi response is not very effective at reducing IAPV-abundance, as levels in experimentally inoculated bumble bees increased by over 1,000-fold in spite of activation of RNAi [190]. This result could be partially attributed to tissue specific variation of RNAi efficacy [44, 209, 210]. However, even experimental reduction of *dicer-2* expression in *B. terrestris* did not result in increased abundance of IAPV or SBPV [44]. IAPV, which is transmitted orally among bumble bees, was injected in these studies and injection of IAPV results in a more virulent infection than oral inoculation [44, 190], therefore additional studies are required to determine if the route of virus-inoculation impacts host-pathogen interactions and the efficacy of systemic RNAi. To further examine the virus-limiting role of RNAi, bumble bees were infected with Cricket paralysis virus (CrPV), which is a model virus that encodes a viral suppressor of RNAi (VSR) [211], and as expected, the RNAi response was reduced [190]. This study did not find evidence of IAPV-mediated suppression of RNAi, and the putative IAPV-1A-like VSR protein [97] was not detected by mass spectrometry analysis of infected bee samples [190].

Bumble bee non-sequence-specific dsRNA-mediated antiviral response

Similar to honey bees, viral dsRNA triggers both sequence-specific RNAi and non-sequence specific virus limiting responses in bumble bees, though the non-sequence specific pathway may be even more important for bumble bees [44, 47, 190]. This was demonstrated by a study in which bumble bees (*Bombus terrestris*) were fed IAPV only, or IAPV and either virus-sequence specific dsRNA or non-sequence-specific dsRNA (ns-dsRNA-GFP, 455 bp) for six consecutive days, beginning three days prior to virus-inoculation [47]. Survival was monitored for 22-days post-infection and revealed that bumble bees treated with non-sequence specific dsRNA better survived IAPV infection (60%) than bumble bees treated with virus-specific dsRNA (10%). However, in parallel, independent experiments that examined the potential effect of IAPV-dsRNA length (293 bp, 443 bp, and 586-bp) in addition to sequence specificity, virus abundance in bumble bee heads was reduced in all dsRNA-treated and virus-infected bees, as compared to bees that were virus-infected and not treated with dsRNA [47].

In addition to dsRNA-mediated antiviral responses, there are other immune pathways that likely play important roles in antiviral defense in bumble bees. Targeted analysis of canonical insect immune gene expression in virus-infected bumble bees unexpectedly indicated that *BtSVC-vago* (which is also referred to as Single von Willebrand factor C-domain protein (SVC) vago) expression was reduced in IAPV-infected bees and not affected in SBPV-infected bees, unlike

the observed up-regulation of this important antiviral gene in *Drosophila melanogaster* [44]. Experimental reduction of *BtSVC-vago* expression did not impact IAPV or SBPV abundance in the abdomen [47], however additional tissue specific analyses determined that *BtSVC-vago* reduction resulted in greater IAPV abundance in the fat body [212]. Similar to regulation of *Dmvago* by *Dm*Dicer-*2*, *BtSVC-vago* expression is governed by *Bt*Dicer-2 [212]. Reduction of *BtSVC-vago* expression did not impact *Btdicer-2 or Bthop* levels in fat bodies, and thus to date a connection between *Bt*SVC-vago expression and JAK/STAT activation has not been established [212]. However, reduced expression of *Bthop* resulted in higher SBPV load 48 hours post-infection, implicating the Jak/STAT pathway in bumble bee antiviral defense [44] (reviewed in [102]) (Figure 1). To date, the mechanism by which down-regulation of *BtSVC-vago* regulates expression of four AMPs (*BtAbaecin, BtApidaecin, BtDefensin and BtHymenoptaecin*) remains to be elucidated [212].

CONCLUSION

RNA virus infection results in the production of dsRNA molecules (e.g., virus replicative intermediates, RNA secondary structure, dsRNA genomes) in host cells. This viral pathogen associated molecular pattern (PAMP) is recognized as non-self by pathogen recognition receptors (PRRs) across diverse taxa, including plants, invertebrates (e.g., oysters, shrimp,

nematodes, ticks, fruit flies, sand flies, mosquitos, wasps, and bees), and mammals [177, 213-221]. Recognition of dsRNA by host PRRs induces different antiviral responses across different hosts. These include virus-specific RNAi in plants, nematodes, and arthropods, and non-sequence specific dsRNA-mediated induction of pathways that result in an 'antiviral state' that limits virus replication (e.g., mammalian interferon response, *C. gigas* oyster type I interferon-like response, *A. mellifera* and *B. terrestris* virus-limiting responses*)*. The extent to which these pathways are involved varies for each co-evolved virus – host pair. Overall the role of RNAi is greater in plants, fruit-flies, and mosquitos, while the role of other immune pathways including Jak/STAT, JNK, Toll, and non-sequence specific dsRNA-triggered pathways are more important in bees, oysters, and mammals. Importantly, these generalities are only true for the specific species that have been studied including *Arabidopsis thaliana*, *Xanthomonas oryzae*, *D. melanogaster*, *A. gambiae, A. mellifera*, *B. terrestris*, *C. gigas*, *Mus musculus*, *Homo sapiens*, and more.

The antiviral response(s) that are most important for any particular host-virus pair cannot be assumed based on broad organismal classification (e.g., insects, invertebrates, mammals) – each must be empirically determined. Since all host-virus interactions are inexorably complicated by the history of conflict and evolution shared (or not shared in the case of model viruses) by the host and a virus. These unique histories are reflected in the differential transcriptional responses and extent of parallel activation and/or cross-talk between host immune pathways, as well as

identification of the virus-evolved counter defense mechanisms, including suppressors of RNAi and other immune pathways [141, 222].

The parallels that exist between the antiviral responses, including the general, non-sequence specific dsRNA triggered induction of an antiviral state, in organisms separated by large evolutionary distances including honey bees, bumble bees, sand flies, shrimp, oysters and mammals, are very intriguing. Furthermore, it is interesting to hypothesize that in social bees this response may have evolved to rapidly respond to viruses and limit their transmission in the crowded hive environment and in the context of behaviors (e.g., trophallaxis) that promote virus transmission between individuals within the super-organism. Investigation of antiviral defense in some of the thousands of under-explored bee species, will further our understanding of the general and specific mechanisms that bees have evolved to combat specific viruses. This short review highlights studies that have contributed to our current understanding of bee antiviral defense mechanisms. Under-explored, burgeoning research areas include elucidation of the roles of alternative splicing [128, 223], epigenetic regulation [126, 224], and transgenerational immune priming in bee antiviral defense [225, 226]. Further examination of antiviral RNAi, including immune memory as a consequence of RNA virus integration into the bee genome [227], and potential transposon mediated amplification of virus-targeting secondary RNAs (as has been described in *Drosophila melanogaster* [183-185]) is also important. Lessons learned from evolutionary distant organisms, including those described in this special issue of *Viruses* focused on “Antiviral Defense in Invertebrates”, may help guide these studies.

Acknowledgements

The Flenniken Laboratory is supported by the National Sciences Foundation CAREER Program, the United States Department of Agriculture National Institute of Food and Agriculture, Agriculture and Food Research Initiative (USDA-NIFA-AFRI) Program, Montana Department of Agriculture Specialty Crop Block Grant Program, Hatch Multistate Funding (NC-1173), and Project *Apis m*.. We would like to thank members of the Flenniken laboratory, Sandra Barroso Arevalo (Universidad Complutense de Madrid), Dr. Laura Brutscher (University of California - Davis), and Dr. Francesco Nazzi (Università di Udine) for reviewing this manuscript prior to publication.

BIBLIOGRAPHY

1. Michener, C. D. The Bees of the World. 2nd ed.; John Hopkins University Press: Baltimore and London, 2007.

2. Calderone, N. W. Insect Pollinated Crops, Insect Pollinators and US Agriculture: Trend Analysis of Aggregate Data for the Period 1992–2009. PLoS ONE **2012,** 7, (5), e37235.

3. Gallai, N.; Salles, J.-M.; Settele, J.; Vaissière, B. E. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological Economics **2009,** 68, (3), 810-821.

4. Cridland, J. M.; Tsutsui, N. D.; Ramirez, S. R. The Complex Demographic History and Evolutionary Origin of the Western Honey Bee, Apis Mellifera. Genome Biology and Evolution **2017,** 9, (2), 457-472.

5. Sheppard, W. S. A History of the Introduction of Honey Bee Races Into the United-States .2. Am Bee J **1989,** 129, (10), 664-667.

6. Han, F.; Wallberg, A.; Webster, M. T. From where did the Western honeybee (Apis mellifera) originate? Ecol Evol **2012,** 2, (8), 1949-1957.

7. Diao, Q.; Sun, L.; Zheng, H.; Zeng, Z.; Wang, S.; Xu, S.; Zheng, H.; Chen, Y.; Shi, Y.; Wang, Y.; Meng, F.; Sang, Q.; Cao, L.; Liu, F.; Zhu, Y.; Li, W.; Li, Z.; Dai, C.; Yang, M.; Chen, S.; Chen, R.; Zhang, S.; Evans, J. D.; Huang, Q.; Liu, J.; Hu, F.; Su, S.; Wu, J. Genomic and transcriptomic analysis of the Asian honeybee Apis cerana provides novel insights into honeybee biology. Sci Rep **2018,** 8, (1), 822.

8. Government, T. A. Status of the Asian honey bee in mainland Australia http://www.agriculture.gov.au/pests-diseases-weeds/bees/the-asian-honey-bee-in-australia (July 2, 2018),

9. Winston, M. L. The biology of the honey bee. Harvard University Press: Cambridge, USA, 1991.

10. vanEngelsdorp, D.; Meixner, M. D. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J Invertebr Pathol **2010,** 103, S80-S95.

11. California Almond Board Almond Almanac; 2014.

12. Traynor, K. S.; Rennich, K.; Forsgren, E.; Rose, R.; Pettis, J.; Kunkel, G.; Madella, S.; Evans, J.; Lopez, D.; vanEngelsdorp, D. Multiyear survey targeting disease incidence in US honey bees. Apidologie **2016,** 47, (3), 325-347.

13. Spleen, A. M.; Lengerich, E. J.; Rennich, K.; Caron, D.; Rose, R.; Pettis, J. S.; Henson, M.; Wilkes, J. T.; Wilson, M.; Stitzinger, J.; Lee, K.; Andree, M.; Snyder, R.; vanEngelsdorp, D.; for the Bee Informed, P. A national survey of managed honey bee 2011–12 winter colony losses in the United States: results from the Bee Informed Partnership. J Apicult Res **2015,** 52, (2), 44-53.

14. Steinhauer, N. A.; Rennich, K.; Wilson, M. E.; Caron, D. M.; Lengerich, E. J.; Pettis, J. S.; Rose, R.; Skinner, J. A.; Tarpy, D. R.; Wilkes, J. T.; vanEngelsdorp, D.; for the Bee Informed, P. A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. J Apicult Res **2015,** 53, (1), 1-18.

15. vanEngelsdorp, D.; Caron, D.; Hayes, J.; Underwood, R.; Henson, M.; Rennich, K.; Spleen, A.; Andree, M.; Snyder, R.; Lee, K.; Roccasecca, K.; Wilson, M.; Wilkes, J.; Lengerich, E.; Pettis, J.; for the Bee Informed, P. A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. J Apicult Res **2015,** 51, (1), 115-124.

16. vanEngelsdorp, D.; Evans, J. D.; Saegerman, C.; Mullin, C.; Haubruge, E.; Nguyen, B. K.; Frazier, M.; Frazier, J.; Cox-Foster, D.; Chen, Y.; Underwood, R.; Tarpy, D. R.; Pettis, J. S. Colony Collapse Disorder: A Descriptive Study. PLoS ONE **2009,** 4, (8), e6481.

17. vanEngelsdorp, D.; Hayes, J.; Underwood, R. M.; Pettis, J. A Survey of Honey Bee Colony Losses in the U.S., Fall 2007 to Spring 2008. PLoS ONE **2008,** 3, (12), e4071.

18. McMenamin, A. J.; Brutscher, L. M.; Glenny, W.; Flenniken, M. L. Abiotic and biotic factors affecting the replication and pathogenicity of bee viruses. Curr Opin Insect Sci **2016,** 16, 14-21.

19. Vaudo, A. D.; Tooker, J. F.; Grozinger, C. M.; Patch, H. M. Bee nutrition and floral resource restoration. Current Opinion in Insect Science **2015,** 10, 133-141.

20. DeGrandi-Hoffman, G.; Chen, Y. Nutrition, immunity and viral infections in honey bees. Curr Opin Insect Sci **2015,** 10, 170-176.

21. Barron, A. B. Death of the bee hive: understanding the failure of an insect society. Curr Opin Insect Sci **2015,** 10, 45-50.

22. Johnson, R. M. Honey Bee Toxicology. Annu Rev Entomol **2015,** 60, (1), 415-434.

23. Johnson, R. M.; Ellis, M. D.; Mullin, C. A.; Frazier, M. Pesticides and honey bee toxicity – USA. Apidologie **2010,** 41, (3), 312-331.

24. Simone-Finstrom, M.; Li-Byarlay, H.; Huang, M. H.; Strand, M. K.; Rueppell, O.; Tarpy, D. R. Migratory management and environmental conditions affect lifespan and oxidative stress in honey bees. Sci Rep **2016,** 6, (1), 810.

25. Velthuis, H. H. W.; van Doorn, A. A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie **2006,** 37, (4), 421-451.

26. Goulson, D. Bumblebees: Their Behaviour and Ecology. Oxford University Press: Oxford, UK and New York, USA, 2003.

27. Goulson, D. Effects of Introduced Bees on Native Ecosystems. Annu Rev Ecol Evol S **2003,** 34, (1), 1-26.

28. Amsalem, E.; Galbraith, D. A.; Cnaani, J.; Teal, P. E. A.; Grozinger, C. M. Conservation and modification of genetic and physiological toolkits underpinning diapause in bumble bee queens. Mol Ecol **2015,** 24, (22), 5596-5615.

29. Amsalem, E.; Grozinger, C. M.; Padilla, M.; Hefetz, A., The Physiological and Genomic Bases of Bumble Bee Social Behaviour. In Genomics, Physiology and Behaviour of Social Insects, Elsevier: London, UK, 2015; Vol. 48, pp 37-93.

30. Owen, R. E.; Otterstatter, M. C.; Carter, R. V.; Farmer, A.; Colla, S. R.; O'Toole, N. Significant expansion of the distribution of the bumble bee Bombus moderatus (Hymenoptera: Apidae) in Alberta over 20 years. Canadian Journal of Zoology-Revue Canadienne De Zoologie **2012,** 90, (1), 133-138.

31. Burkle, L. A.; Marlin, J. C.; Knight, T. M. Plant-Pollinator Interactions over 120 Years: Loss of Species, Co-Occurrence, and Function. Science **2013,** 339, (6127), 1611-1615.

32. Cameron, S. A.; Lozier, J. D.; Strange, J. P.; Koch, J. B.; Cordes, N.; Solter, L. F.; Griswold, T. L. Patterns of widespread decline in North American bumble bees. Proc Natl Acad Sci USA **2011,** 108, (2), 662-667.

33. Goulson, D.; Nicholls, E.; Botias, C.; Rotheray, E. L. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science **2015,** 347, (6229), 1255957-1255957.

34. Hatten, T. D.; Looney, C.; Strange, J. P.; Bosque-Perez, N. A. Bumble bee fauna of Palouse Prairie: Survey of native bee pollinators in a fragmented ecosystem. Journal of Insect Science **2013,** 13.

35. Woodard, S. H. Bumble bee ecophysiology: integrating the changing environment and the organism. Current Opinion in Insect Science **2017,** 22, 101-108.

36. Memmott, J.; Craze, P. G.; Waser, N. M.; Price, M. V. Global warming and the disruption of plant-pollinator interactions. Ecol Lett **2007,** 10, (8), 710-7.

37. Meeus, I.; Pisman, M.; Smagghe, G.; Piot, N. Interaction effects of different drivers of wild bee decline and their influence on host-pathogen dynamics. Current Opinion in Insect Science **2018,** 26, 136-141.

38. Koch, H.; Schmid-Hempel, P. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proc Natl Acad Sci USA **2011,** 108, (48), 19288-19292.

39. Barribeau, S. M.; Schmid-Hempel, P. Qualitatively different immune response of the bumblebee host, Bombus terrestris, to infection by different genotypes of the trypanosome gut parasite, Crithidia bombi. Infection, Genetics and Evolution **2013,** 20, 249-256.

40. Cordes, N.; Huang, W.-F.; Strange, J. P.; Cameron, S. A.; Griswold, T. L.; Lozier, J. D.; Solter, L. F. Interspecific geographic distribution and variation of the pathogens Nosema bombi and Crithidia species in United States bumble bee populations. J Invertebr Pathol **2012,** 109, (2), 209-216.

41. Barribeau, S. M.; Sadd, B. M.; du Plessis, L.; Schmid-Hempel, P. Gene expression differences underlying genotype-by-genotype specificity in a host–parasite system. Proc Natl Acad Sci USA **2014,** 111, (9), 3496-3501.

42. Imhoof, B.; Schmid-Hempel, P. Colony success of the bumble bee, Bombus terrestris, in relation to infections by two protozoan parasites, Crithidia bombi and Nosema bombi. Insect Soc **2014,** 46, (3), 233-238.

43. Parmentier, L.; Smagghe, G.; de Graaf, D. C.; Meeus, I. Varroa destructor Macula-like virus, Lake Sinai virus and other new RNA viruses in wild bumblebee hosts (Bombus pascuorum, Bombus lapidarius, and Bombus pratorum). J Invertebr Pathol **2016,** 134, (134), 6-11.

44. Niu, J.; Smagghe, G.; De Coninck, D. I. M.; Van Nieuwerburgh, F.; Deforce, D.; Meeus, I. In vivo study of Dicer-2-mediated immune response of the small interfering RNA pathway upon systemic infections of virulent and avirulent viruses in Bombus terrestris. Insect Biochem Mol Biol **2016,** 70, 127-137.

45. Meeus, I.; Brown, M. J. F.; de Graaf, D. C.; Smagghe, G. Effects of Invasive Parasites on Bumble Bee Declines. Conservation Biology **2011,** 25, (4), 662-671.

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

47. Piot, N.; Snoeck, S.; Vanlede, M.; Smagghe, G.; Meeus, I. The Effect of Oral Administration of dsRNA on Viral Replication and Mortality in Bombus terrestris. Viruses **2015,** 7, (6), 3172-3185.

48. Niu, J.; Cappelle, K.; de Miranda, J. R.; Smagghe, G.; Meeus, I. Analysis of reference gene stability after Israeli acute paralysis virus infection in bumblebees Bombus terrestris. J Invertebr Pathol **2014,** 115, 76-79.

49. Schoonvaere, K.; Smagghe, G.; Francis, F.; de Graaf, D. C. Study of the Metatranscriptome of Eight Social and Solitary Wild Bee Species Reveals Novel Viruses and Bee Parasites. Front Microbiol **2018,** 9, 754.

50. Sagili, R. R.; Burgett, D. M. Evaluating honey bee colonies for pollination: a guide for commercial growers and beekeepers; Oregon State University Extension Service, 2011; pp 1-8.

51. Amiri, E.; Meixner, M.; Nielsen, S. L.; Kryger, P. Four Categories of Viral Infection Describe the Health Status of Honey Bee Colonies. PLoS ONE **2015,** 10, (10), e0140272.

52. Pettis, J. S.; Rice, N.; Joselow, K.; vanEngelsdorp, D.; Chaimanee, V. Colony Failure Linked to Low Sperm Viability in Honey Bee (Apis mellifera) Queens and an Exploration of Potential Causative Factors. PLoS ONE **2016,** 11, (2), e0147220.

53. Nguyen, B. K.; Ribière, M.; vanEngelsdorp, D.; Snoeck, C.; Saegerman, C.; Kalkstein, A. L.; Schurr, F.; Brostaux, Y.; Faucon, J.-P.; Haubruge, E. Effects of honey bee virus prevalence, Varroa destructor load and queen condition on honey bee colony survival over the winter in Belgium. J Apicult Res **2015,** 50, (3), 195-202.

54. Glenny, W.; Cavigli, I.; Daughenbaugh, K. F.; Radford, R.; Kegley, S. E.; Flenniken, M. L. Honey bee (Apis mellifera) colony health and pathogen composition in migratory beekeeping operations involved in California almond pollination. PLoS ONE **2017,** 12, (8), e0182814.

55. Cavigli, I.; Daughenbaugh, K. F.; Martin, M.; Lerch, M.; Banner, K.; Garcia, E.; Brutscher, L. M.; Flenniken, M. L. Pathogen prevalence and abundance in honey bee colonies involved in almond pollination. Apidologie **2016,** 47, (2), 251-266.

56. Brosi, B. J. Pollinator specialization: from the individual to the community. New Phytol. **2016,** 210, (4), 1190-1194.

57. Garibaldi, L. A.; Steffan-Dewenter, I.; Winfree, R.; Aizen, M. A.; Bommarco, R.; Cunningham, S. A.; Kremen, C.; Carvalheiro, L. G.; Harder, L. D.; Afik, O.; Bartomeus, I.;

Benjamin, F.; Boreux, V.; Cariveau, D.; Chacoff, N. P.; Dudenhoffer, J. H.; Freitas, B. M.; Ghazoul, J.; Greenleaf, S.; Hipolito, J.; Holzschuh, A.; Howlett, B.; Isaacs, R.; Javorek, S. K.; Kennedy, C. M.; Krewenka, K. M.; Krishnan, S.; Mandelik, Y.; Mayfield, M. M.; Motzke, I.; Munyuli, T.; Nault, B. A.; Otieno, M.; Petersen, J.; Pisanty, G.; Potts, S. G.; Rader, R.; Ricketts, T. H.; Rundlof, M.; Seymour, C. L.; Schuepp, C.; Szentgyorgyi, H.; Taki, H.; Tscharntke, T.; Vergara, C. H.; Viana, B. F.; Wanger, T. C.; Westphal, C.; Williams, N.; Klein, A. M. Wild Pollinators Enhance Fruit Set of Crops Regardless of Honey Bee Abundance. Science **2013,** 339, (6127), 1608-1611.

58. Greenleaf, S. S.; Kremen, C. Wild bees enhance honey bees' pollination of hybrid sunflower. Proc Natl Acad Sci USA **2006,** 103, (37), 13890-13895.

59. Brittain, C.; Williams, N.; Kremen, C.; Klein, A. M. Synergistic effects of non-Apis bees and honey bees for pollination services. P R Soc B **2013,** 280, (1754), 20122767-20122767.

60. Winfree, R.; Williams, N. M.; Dushoff, J.; Kremen, C. Native bees provide insurance against ongoing honey bee losses. Ecol. Lett. **2007,** 10, (11), 1105-1113.

61. Dolezal, A. G.; Hendrix, S. D.; Scavo, N. A.; Carrillo-Tripp, J.; Harris, M. A.; Wheelock, M. J.; O'Neal, M. E.; Toth, A. L. Honey Bee Viruses in Wild Bees: Viral Prevalence, Loads, and Experimental Inoculation. PLoS ONE **2016,** 11, (11), e0166190.

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

63. McMahon, D. P.; Fürst, M. A.; Caspar, J.; Theodorou, P.; Brown, M. J. F.; Paxton, R. J. A sting in the spit: widespread cross-infection of multiple RNA viruses across wild and managed bees. J Anim Ecol **2015,** 84, (3), 615-624.

64. Fürst, M. A.; McMahon, D. P.; Osborne, J. L.; Paxton, R. J.; Brown, M. J. F. Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature **2014,** 506, (7488), 364-366.

65. Levitt, A. L.; Singh, R.; Cox-Foster, D. L.; Rajotte, E.; Hoover, K.; Ostiguy, N.; Holmes, E. C. Cross-species transmission of honey bee viruses in associated arthropods. Virus Res **2013,** 176, (1-2), 232-240.

66. Tehel, A.; Brown, M. J.; Paxton, R. J. Impact of managed honey bee viruses on wild bees. Curr Opin Virol **2016,** 19, 16-22.

67. Bigot, D.; Dalmon, A.; Roy, B.; Hou, C.; Germain, M.; Romary, M.; Deng, S.; Diao, Q.; Weinert, L. A.; Cook, J. M.; Herniou, E. A.; Gayral, P. The discovery of Halictivirus resolves the Sinaivirus phylogeny. Journal of General Virology **2017,** 98, (11), 2864-2875.

68. Brutscher, L. M.; McMenamin, A. J.; Flenniken, M. L. The Buzz about Honey Bee Viruses. Plos Pathog **2016,** 12, (8), e1005757.

69. Boncristiani, H. F.; Di Prisco, G.; Pettis, J. S.; Hamilton, M.; Chen, Y. Molecular approaches to the analysis of deformed wing virus replication and pathogenesis in the honey bee, Apis mellifera. Virol J **2009,** 6, (1), 221.

70. Chen, Y. P.; Siede, R., Honey Bee Viruses. In Adv Virus Res, Elsevier: 2007; Vol. 70, pp 33-80.

71. Genersch, E.; Aubert, M. Emerging and re-emerging viruses of the honey bee (Apis mellifera L.). Vet Res **2010,** 41, (6), 54.

72. McMenamin, A. J.; Flenniken, M. L. Recently identified bee viruses and their impact on bee pollinators. Curr Opin Insect Sci **2018,** 26, 120-129.

73. Remnant, E. J.; Shi, M.; Buchmann, G.; Blacquiere, T.; Holmes, E. C.; Beekman, M.; Ashe, A. A Diverse Range of Novel RNA Viruses in Geographically Distinct Honey Bee Populations. J Virol **2017,** 91, (16).

74. Gauthier, L.; Cornman, S.; Hartmann, U.; Cousserans, F.; Evans, J.; de Miranda, J.; Neumann, P. The Apis mellifera Filamentous Virus Genome. Viruses **2015,** 7, (7), 3798-3815.

75. Grozinger, C. M.; Flenniken, M. L. Bee Viruses: Ecology, Pathogenicity, and Impacts. Annu Rev Entomol **2018,** in press.

76. Mazzei, M.; Carrozza, M. L.; Luisi, E.; Forzan, M.; Giusti, M.; Sagona, S.; Tolari, F.; Felicioli, A. Infectivity of DWV Associated to Flower Pollen: Experimental Evidence of a Horizontal Transmission Route. PLoS ONE **2014,** 9, (11), e113448.

77. Chen, Y.; Evans, J.; Feldlaufer, M. Horizontal and vertical transmission of viruses in the honey bee, Apis mellifera. J Invertebr Pathol **2006,** 92, (3), 152-159.

78. Shen, M. Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite. Journal of General Virology **2005,** 86, (8), 2281-2289.

79. Shen, M.; Yang, X.; Cox-Foster, D.; Cui, L. The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. Virology **2005,** 342, (1), 141-149.

80. Yue, C.; Schroder, M.; Gisder, S.; Genersch, E. Vertical-transmission routes for deformed wing virus of honeybees (Apis mellifera). Journal of General Virology **2007,** 88, (8), 2329-2336.

81. Amiri, E.; Meixner, M.; Büchler, R.; Kryger, P. Chronic Bee Paralysis Virus in Honeybee Queens: Evaluating Susceptibility and Infection Routes. Viruses **2014,** 6, (3), 1188-1201.

82. Amiri, E.; Kryger, P.; Meixner, M. D.; Strand, M. K.; Tarpy, D. R.; Rueppell, O. Quantitative patterns of vertical transmission of deformed wing virus in honey bees. PLoS ONE **2018,** 13, (3), e0195283.

83. Gisder, S.; Aumeier, P.; Genersch, E. Deformed wing virus: replication and viral load in mites (Varroa destructor). Journal of General Virology **2009,** 90, (2), 463-467.

84. Nazzi, F.; Le Conte, Y. Ecology of Varroa destructor, the Major Ectoparasite of the Western Honey Bee, Apis mellifera. Annu Rev Entomol **2016,** 61, (1), 417-432.

85. Di Prisco, G.; Annoscia, D.; Margiotta, M.; Ferrara, R.; Varricchio, P.; Zanni, V.; Caprio, E.; Nazzi, F.; Pennacchio, F. A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. Proc Natl Acad Sci USA **2016,** 113, (12), 3203-3208.

86. Martin, S. J.; Highfield, A. C.; Brettell, L.; Villalobos, E. M.; Budge, G. E.; Powell, M.; Nikaido, S.; Schroeder, D. C. Global Honey Bee Viral Landscape Altered by a Parasitic Mite. Science **2012,** 336, (6086), 1304-1306.

87. Ryabov, E. V.; Wood, G. R.; Fannon, J. M.; Moore, J. D.; Bull, J. C.; Chandler, D.; Mead, A.; Burroughs, N.; Evans, D. J. A Virulent Strain of Deformed Wing Virus (DWV) of Honeybees (Apis mellifera) Prevails after Varroa destructor-Mediated, or In Vitro, Transmission. Plos Pathog **2014,** 10, (6), e1004230.

88. McMahon, D. P.; Natsopoulou, M. E.; Doublet, V.; Fürst, M.; Weging, S.; Brown, M. J. F.; Gogol-Döring, A.; Paxton, R. J. Elevated virulence of an emerging viral genotype as a driver of honeybee loss. P R Soc B **2016,** 283, (1833), 20160811.

89. de Miranda, J. R.; Genersch, E. Deformed wing virus. J Invertebr Pathol **2010,** 103, S48-S61.

90. Genersch, E.; von der Ohe, W.; Kaatz, H.; Schroeder, A.; Otten, C.; Büchler, R.; Berg, S.; Ritter, W.; Mühlen, W.; Gisder, S.; Meixner, M.; Liebig, G.; Rosenkranz, P. The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie **2010,** 41, (3), 332-352.

91. Annoscia, D.; Brown, S.; Di Prisco, G.; De Paoli, E.; Del Fabbro, S.; Zanni, V.; Galbraith, D.; Caprio, E.; Grozinger, C. M.; Pennacchio, F.; Nazzi, F. Haemolymph removal by the parasite Varroa destructor can trigger the proliferation of the Deformed Wing Virus in mite infested bees (Apis mellifera), contributing to enhanced pathogen virulence. bioRxiv **2018**, 257667.

92. Nazzi, F.; Pennacchio, F. Disentangling multiple interactions in the hive ecosystem. Trends Parasitol. **2014,** 30, (12), 556-561.

93. Nazzi, F.; Brown, S. P.; Annoscia, D.; Del Piccolo, F.; Di Prisco, G.; Varricchio, P.; Della Vedova, G.; Cattonaro, F.; Caprio, E.; Pennacchio, F. Synergistic Parasite-Pathogen Interactions Mediated by Host Immunity Can Drive the Collapse of Honeybee Colonies. Plos Pathog **2012,** 8, (6), e1002735.

94. Lee, K.; Steinhauer, N.; Travis, D. A.; Meixner, M. D.; Deen, J.; vanEngelsdorp, D. Honey bee surveillance: a tool for understanding and improving honey bee health. Current Opinion in Insect Science **2015,** 10, 37-44.

95. Asensio, I.; Vicente-Rubiano, M.; Munoz, M. J.; Fernandez-Carrion, E.; Sanchez-Vizcaino, J. M.; Carballo, M. Importance of Ecological Factors and Colony Handling for Optimizing Health Status of Apiaries in Mediterranean Ecosystems. PLoS ONE **2016,** 11, (10).

96. Olivier, V.; Blanchard, P.; Chaouch, S.; Lallemand, P.; Schurr, F.; Celle, O.; Dubois, E.; Tordo, N.; Thiéry, R.; Houlgatte, R.; Ribière, M. Molecular characterisation and phylogenetic analysis of Chronic bee paralysis virus, a honey bee virus. Virus Res **2008,** 132, (1-2), 59-68.

97. Chen, Y. P.; Pettis, J. S.; Corona, M.; Chen, W. P.; Li, C. J.; Spivak, M.; Visscher, P. K.; DeGrandi-Hoffman, G.; Boncristiani, H.; Zhao, Y.; vanEngelsdorp, D.; Delaplane, K.; Solter, L.; Drummond, F.; Kramer, M.; Lipkin, W. I.; Palacios, G.; Hamilton, M. C.; Smith, B.; Huang, S. K.; Zheng, H. Q.; Li, J. L.; Zhang, X.; Zhou, A. F.; Wu, L. Y.; Zhou, J. Z.; Lee, M.-L.; Teixeira, E. W.; Li, Z. G.; Evans, J. D. Israeli Acute Paralysis Virus: Epidemiology, Pathogenesis and Implications for Honey Bee Health. Plos Pathog **2014,** 10, (7), e1004261.

98. Bailey, L.; Fernando, E. F. Effects of Sacbrood Virus on Adult Honey-Bees. Annals of Applied Biology **1972,** 72, (1), 27-&.

99. Bailey, L. Acute bee-paralysis virus in adult honey bees injected with sacbrood virus. Virology 33, (2), 368.

100. Bailey, L. Honey bee pathology. Annu Rev Entomol.

101. Di Prisco, G.; Cavaliere, V.; Annoscia, D.; Varricchio, P.; Caprio, E.; Nazzi, F.; Gargiulo, G.; Pennacchio, F. Neonicotinoid clothianidin adversely affects insect immunity and

promotes replication of a viral pathogen in honey bees. Proc Natl Acad Sci USA **2013,** 110, (46), 18466-18471.

102. Brutscher, L. M.; Daughenbaugh, K. F.; Flenniken, M. L. Antiviral Defense Mechanisms in Honey Bees. Current Opinion in Insect Science **2015,** 10, 71-82.

103. Evans, J. D.; Aronstein, K.; Chen, Y. P.; Hetru, C.; Imler, J. L.; Jiang, H.; Kanost, M.; Thompson, G. J.; Zou, Z.; Hultmark, D. Immune pathways and defence mechanisms in honey bees Apis mellifera. Insect Mol Biol **2006,** 15, (5), 645-56.

104. Spain Ministry of Agriculture - Ministerio de Agricultura y Pesca, A. y. M. A. Informe de resultados del Programa de vigilancia sobre las pérdidas de colonias de abejas 2012 - 2015

08/31/2016, 2016.

105. Runckel, C.; Flenniken, M. L.; Engel, J. C.; Ruby, J. G.; Ganem, D.; Andino, R.; DeRisi, J. L. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, Nosema, and Crithidia. PLoS ONE **2011,** 6, (6), e20656.

106. Tentcheva, D.; Gauthier, L.; Zappulla, N.; Dainat, B.; Cousserans, F.; Colin, M. E.; Bergoin, M. Prevalence and Seasonal Variations of Six Bee Viruses in Apis mellifera L. and Varroa destructor Mite Populations in France. Appl Environ Microbiol **2004,** 70, (12), 7185-7191.

107. Nguyen, B. K.; Mignon, J.; Laget, D.; de Graaf, D. C.; Jacobs, F. J.; vanEngelsdorp, D.; Brostaux, Y.; Saegerman, C.; Haubruge, E. Honey bee colony losses in Belgium during the 2008–9 winter. J Apicult Res **2015,** 49, (4), 337-339.

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

109. Cornman, R. S.; Tarpy, D. R.; Chen, Y.; Jeffreys, L.; Lopez, D.; Pettis, J. S.; vanEngelsdorp, D.; Evans, J. D. Pathogen Webs in Collapsing Honey Bee Colonies. PLoS ONE **2012,** 7, (8), e43562.

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

111. (GRPC), G. P. R. C. GRPC Canadian National Honey Bee Health Survey. GPRC National Bee Diagnostic Centre (May 23, 2018),

112. Daughenbaugh, K. F.; Martin, M.; Brutscher, L. M.; Cavigli, I.; Garcia, E.; Lavin, M.; Flenniken, M. L. Honey Bee Infecting Lake Sinai Viruses. Viruses **2015,** 7, (6), 3285-3309.

113. van der Zee, R.; Pisa, L.; Andonov, S.; Brodschneider, R.; Charriere, J.-D.; Chlebo, R.; Coffey, M. F.; Crailsheim, K.; Dahle, B.; Gajda, A.; Gray, A.; Drazic, M. M.; Higes, M.; Kauko, L.; Kence, A.; Kence, M.; Kezic, N.; Kiprijanovska, H.; Kralj, J.; Kristiansen, P.; Hernandez, R. M.; Mutinelli, F.; Nguyen, B. K.; Otten, C.; Özkırım, A.; Pernal, S. F.; Peterson, M.; Ramsay, G.; Santrac, V.; Soroker, V.; Topolska, G.; Uzunov, A.; Vejsnaes, F.; Wei, S.; Wilkins, S. Managed honey bee colony losses in Canada, China, Europe, Israel and Turkey, for the winters of 2008–9 and 2009–10. J Apicult Res **2015,** 51, (1), 100-114.

114. van der Zee, R.; Gray, A.; Pisa, L.; de Rijk, T. An Observational Study of Honey Bee Colony Winter Losses and Their Association with Varroa destructor, Neonicotinoids and Other Risk Factors. PLoS ONE **2015,** 10, (7), e0131611.

115. de Miranda, J. R.; Bailey, L.; Ball, B. V.; Blanchard, P.; Budge, G. E.; Chejanovsky, N.; Chen, Y.-P.; Gauthier, L.; Genersch, E.; de Graaf, D. C.; Ribière, M.; Ryabov, E.; De Smet, L.; van der Steen, J. J. M. Standard methods for virus research in Apis mellifera. J Apicult Res **2015,** 52, (4), 1-56.

116. Bailey, L. Honey Bee Pathology. Annu Rev Entomol **1968,** 13, 191-212.

117. de Miranda, J. R.; Cordoni, G.; Budge, G. The Acute bee paralysis virus–Kashmir bee virus–Israeli acute paralysis virus complex. J Invertebr Pathol **2010,** 103, S30-S47.

118. Benaets, K.; Van Geystelen, A.; Cardoen, D.; De Smet, L.; de Graaf, D. C.; Schoofs, L.; Larmuseau, M. H. D.; Brettell, L. E.; Martin, S. J.; Wenseleers, T. Covert deformed wing virus infections have long-term deleterious effects on honeybee foraging and survival. P R Soc B **2017,** 284, (1848), 20162149.

119. Benjeddou, M.; Leat, N.; Allsopp, M.; Davison, S. Development of infectious transcripts and genome manipulation of Black queen-cell virus of honey bees. J Gen Virol **2002,** 83, (Pt 12), 3139-46.

120. Lamp, B.; Url, A.; Seitz, K.; Eichhorn, J.; Riedel, C.; Sinn, L. J.; Indik, S.; Köglberger, H.; Rümenapf, T. Construction and Rescue of a Molecular Clone of Deformed Wing Virus (DWV). PLoS ONE **2016,** 11, (11), e0164639.

121. Goblirsch, M. J.; Spivak, M. S.; Kurtti, T. J. A Cell Line Resource Derived from Honey Bee (Apis mellifera) Embryonic Tissues. PLoS ONE **2013,** 8, (7), e69831.

122. Carrillo-Tripp, J.; Dolezal, A. G.; Goblirsch, M. J.; Miller, W. A.; Toth, A. L.; Bonning, B. C. In vivo and in vitro infection dynamics of honey bee viruses. Sci Rep **2016,** 6, (1), S50.

123. Boncristiani, H. F.; Evans, J. D.; Chen, Y.; Pettis, J.; Murphy, C.; Lopez, D. L.; Simone-Finstrom, M.; Strand, M.; Tarpy, D. R.; Rueppell, O. In Vitro Infection of Pupae with Israeli Acute Paralysis Virus Suggests Disturbance of Transcriptional Homeostasis in Honey Bees (Apis mellifera). PLoS ONE **2013,** 8, (9), e73429.

124. Skubnik, K.; Novacek, J.; Fuzik, T.; Pridal, A.; Paxton, R. J.; Plevka, P. Structure of deformed wing virus, a major honey bee pathogen. Proc Natl Acad Sci U S A **2017,** 114, (12), 3210-3215.

125. Organtini, L. J.; Shingler, K. L.; Ashley, R. E.; Capaldi, E. A.; Durrani, K.; Dryden, K. A.; Makhov, A. M.; Conway, J. F.; Pizzorno, M. C.; Hafenstein, S. Honey Bee Deformed Wing Virus Structures Reveal that Conformational Changes Accompany Genome Release. J Virol **2017,** 91, (2).

126. Galbraith, D. A.; Yang, X.; Nino, E. L.; Yi, S.; Grozinger, C. Parallel Epigenomic and Transcriptomic Responses to Viral Infection in Honey Bees (Apis mellifera). Plos Pathog **2015,** 11, (3), e1004713.

127. Brutscher, L. M.; Daughenbaugh, K. F.; Flenniken, M. L. Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. Sci Rep **2017,** 7, (1), 6448.

128. Flenniken, M. L.; Andino, R. Non-specific dsRNA-mediated antiviral response in the honey bee. PLoS ONE **2013,** 8, (10), e77263.

129. O'Neal, S. T.; Swale, D. R.; Anderson, T. D. ATP-sensitive inwardly rectifying potassium channel regulation of viral infections in honey bees. Sci Rep **2017,** 7, (1), 614.

130. O'Neal, S. T.; Brewster, C. C.; Bloomquist, J. R.; Anderson, T. D. Amitraz and its metabolite modulate honey bee cardiac function and tolerance to viral infection. J Invertebr Pathol **2017,** 149, 119-126.

131. Evans, J. D.; Maleszka, R.; Hartfelder, K.; Worley, K. C.; Amdam, G. V.; Bitondi, M. M. G.; Collins, A. M.; Cristino, A. S.; Michael, H.; Lattorff, G.; Lobo, C. H.; Moritz, R. F. A.; Nunes, F. M. F.; Page, R. E.; Simões, Z. L. P.; Wheeler, D.; Carninci, P.; Fukuda, S.; Hayashizaki, Y.; Kai, C.; Kawai, J.; Sakazume, N.; Sasaki, D.; Tagami, M.; Albert, S.; Baggerman, G.; Beggs, K. T.; Bloch, G.; Cohen, M.; Drapeau, M. D.; Eisenhardt, D.; Ewing, M. A.; Fahrbach, S. E.; Hauser, F.; Hunt, G. J.; Huybrechts, J.; Jones, A. K.; Kaplan, N.; Leboulle, G.; Linial, M.; Littleton, J. T.; Mercer, A. R.; Robinson, G. E.; Richmond, T. A.; RodriguezZas, S. L.; Rubin, E. B.; Sattelle, D. B.; Schlipalius, D.; Schoofs, L.; Shemesh, Y.; Sweedler, J. V.; Velarde, R.; Verleyen, P.; Vierstraete, E.; Williamson, M. R.; Ament, S. A.; Brown, S. J.;

Corona, M.; Dearden, P. K.; Dunn, W. A.; Elekonich, M. M.; Foret, S.; Fujiyuki, T.; Gattermeier, I.; Gempe, T.; Kadowaki, T.; Kage, E.; Kamikouchi, A.; Kubo, T.; Kucharski, R.; Kunieda, T.; Lorenzen, M.; Morioka, M.; Ohashi, K.; Overbeek, R.; Robertson, H. M.; Ross, C. A.; Schioett, M.; Shippy, T.; Takeuchi, H.; Toth, A. L.; Willis, J. H.; Wilson, M. J.; Zdobnov, E. M.; Bork, P.; Gordon, K. H. J.; Letunic, I.; Hackett, K.; Peterson, J.; Felsenfeld, A.; Guyer, M.; Agarwala, R.; Cornuet, J. M.; Emore, C.; Mougel, F.; Vautrin, D.; Gillespie, J. J.; Cannone, J. J.; Gutell, R. R.; Elsik, C. G.; Cazzamali, G.; Eisen, M. B.; Grimmelikhuijzen, C. J. P.; Hummon, A. B.; Iyer, V. N.; Iyer, V.; Kosarev, P.; Mackey, A. J.; Reese, J. T.; Solovyev, V.; Souvorov, A.; Bilikova, K.; Chen, Y. P.; Clark, A. G.; Decanini, L. I.; Gelbart, W. M.; Hetru, C.; Hultmark, D.; Imler, J.-L.; Jiang, H.; Kanost, M.; Kimura, K.; Lazzaro, B. P.; Lopez, D. L.; Simuth, J.; Thompson, G. J.; Zou, Z.; De Jong, P.; Csűrös, M.; Milosavljevic, A.; Osoegawa, K.; Richards, S.; Shu, C.-L.; Duret, L.; Elhaik, E.; Graur, D.; Weaver, D. B.; Anzola, J. M.; Campbell, K. S.; Childs, K. L.; Collinge, D.; Crosby, M. A.; Dickens, C. M.; Gramates, L. S.; Grozinger, C. M.; Jones, P. L.; Jorda, M.; Ling, X.; Matthews, B. B.; Miller, J.; Mizzen, C.; Peinado, M. A.; Reid, J. G.; Russo, S. M.; Schroeder, A. J.; St Pierre, S. E.; Wang, Y.; Zhou, P.; Jiang, H.; Kitts, P.; Milshina, N. V.; Ruef, B.; Venkatraman, A.; Johnston, J. S.; Aquino-Perez, G.; Monnerot, M.; Solignac, M.; Whitfield, C. W.; Behura, S. K.; Berlocher, S. H.; Sheppard, W. S.; Smith, D. R.; Suarez, A. V.; Tsutsui, N. D.; Wei, X.; Wheeler, D.; Havlak, P.; Li, B.; Liu, Y.; Sodergren, E.; Zhang, L.; Beye, M.; Hasselmann, M.; Jolivet, A.; Lee, S.; Pu, L.-L.; Thorn, R.; Stolc, V.; Newman, T.; Samanta, M.; Tongprasit, W. A.; Aronstein, K. A.; Claudianos, C.; Berenbaum, M. R.; Biswas, S.; de Graaf, D. C.; Feyereisen, R.; Johnson, R. M.; Oakeshott, J. G.; Ranson, H.; Schuler, M. A.; Muzny, D.; Gibbs, R. A.; Weinstock, G. M.; Chacko, J.; Davis, C.; Dinh, H.; Gill, R.; Hernandez, J.; Hines, S.; Hume, J.; Jackson, L.; Kovar, C.; Lewis, L.; Miner, G.; Morgan, M.; Nazareth, L. V.; Nguyen, N.; Okwuonu, G.; Paul, H.; Santibanez, J.; Savery, G.; Svatek, A.; Villasana, D.; Wright, R. Insights into social insects from the genome of the honeybee Apis mellifera. Nature **2006,** 443, (7114), 931-949.

132. Sadd, B. M.; Barribeau, S. M.; Bloch, G.; de Graaf, D. C.; Dearden, P.; Elsik, C. G.; Gadau, J.; Grimmelikhuijzen, C. J. P.; Hasselmann, M.; Lozier, J. D.; Robertson, H. M.; Smagghe, G.; Stolle, E.; Van Vaerenbergh, M.; Waterhouse, R. M.; Bornberg-Bauer, E.; Klasberg, S.; Bennett, A. K.; Camara, F.; Guigo, R.; Hoff, K.; Mariotti, M.; Munoz-Torres, M.; Murphy, T.; Santesmasses, D.; Amdam, G. V.; Beckers, M.; Beye, M.; Biewer, M.; Bitondi, M. M. G.; Blaxter, M. L.; Bourke, A. F. G.; Brown, M. J.; Buechel, S. D.; Cameron, R.; Cappelle, K.; Carolan, J. C.; Christiaens, O.; Ciborowski, K. L.; Clarke, D. F.; Colgan, T. J.; Collins, D. H.; Cridge, A. G.; Dalmay, T.; Dreier, S.; du Plessis, L.; Duncan, E.; Erler, S.; Evans, J.; Falcon, T.; Flores, K.; Freitas, F. C. P.; Fuchikawa, T.; Gempe, T.; Hartfelder, K.; Hauser, F.; Helbing, S.; Humann, F. C.; Irvine, F.; Jermiin, L. S.; Johnson, C. E.; Johnson, R. M.; Jones, A. K.; Kadowaki, T.; Kidner, J. H.; Koch, V.; Köhler, A.; Kraus, F. B.; Lattorff, H. M. G.; Leask, M.; Lockett, G. A.; Mallon, E. B.; Antonio, D. S. M.; Marxer, M.; Meeus, I.; Moritz, R. F. A.; Nair, A.; Näpflin, K.; Nissen, I.; Niu, J.; Nunes, F. M. F.; Oakeshott, J. G.; Osborne, A.; Otte, M.; Pinheiro, D. G.; Rossié, N.; Rueppell, O.; Santos, C. G.; Schmid-Hempel, R.; Schmitt, B. D.; Schulte, C.; Simões, Z. L. P.; Soares, M. P. M.; Swevers, L.; Winnebeck, E. C.; Wolschin, F.; Yu, N.; Zdobnov, E. M.; Aqrawi, P. K.; Blankenburg, K. P.; Coyle, M.; Francisco, L.;

Hernandez, A. G.; Holder, M.; Hudson, M. E.; Jackson, L.; Jayaseelan, J.; Joshi, V.; Kovar, C.; Lee, S. L.; Mata, R.; Mathew, T.; Newsham, I. F.; Ngo, R.; Okwuonu, G.; Pham, C.; Pu, L.-L.; Saada, N.; Santibanez, J.; Simmons, D.; Thornton, R.; Venkat, A.; Walden, K. K. O.; Wu, Y.-Q.; Debyser, G.; Devreese, B.; Asher, C.; Blommaert, J.; Chipman, A. D.; Chittka, L.; Fouks, B.; Liu, J.; O'Neill, M. P.; Sumner, S.; Puiu, D.; Qu, J.; Salzberg, S. L.; Scherer, S. E.; Muzny, D. M.; Richards, S.; Robinson, G. E.; Gibbs, R. A.; Schmid-Hempel, P.; Worley, K. C. The genomes of two key bumblebee species with primitive eusocial organization. Genome Biol. **2015,** 16, (1), 227.

133. Munoz-Torres, M. C.; Reese, J. T.; Childers, C. P.; Bennett, A. K.; Sundaram, J. P.; Childs, K. L.; Anzola, J. M.; Milshina, N.; Elsik, C. G. Hymenoptera Genome Database: integrated community resources for insect species of the order Hymenoptera. Nucleic Acids Res **2010,** 39, (Database), D658-D662.

134. Merkling, S. H.; van Rij, R. P. Analysis of resistance and tolerance to virus infection in Drosophila. Nat Protoc **2015,** 10, (7), 1084-1097.

135. Merkling, S. H.; van Rij, R. P. Beyond RNAi: Antiviral defense strategies in Drosophila and mosquito. J Insect Physiol **2013,** 59, (2), 159-170.

136. Kingsolver, M. B.; Hardy, R. W. Making connections in insect innate immunity. Proc Natl Acad Sci USA **2012,** 109, (46), 18639-18640.

137. Lemaitre, B.; Hoffmann, J. The Host Defense of Drosophila melanogaster. Annu. Rev. Immunol. **2007,** 25, (1), 697-743.

138. Hoffmann, J. A. The immune response of Drosophila. Nature **2003,** 426, (6962), 33-38.

139. Imler, J. L.; Hoffmann, J. A. Toll receptors in innate immunity. Trends Cell Biol. 11, (7), 304-311.

140. Lamiable, O.; Imler, J.-L. Induced antiviral innate immunity in Drosophila. Current Opinion in Microbiology **2014,** 20, 62-68.

141. Samuel, G. H.; Adelman, Z. N.; Myles, K. M. Antiviral Immunity and Virus-Mediated Antagonism in Disease Vector Mosquitoes. Trends Microbiol **2018,** 26, (5), 447-461.

142. Blair, C.; Olson, K. The Role of RNA Interference (RNAi) in Arbovirus-Vector Interactions. Viruses **2015,** 7, (2), 820-843.

143. Imler, J.-L.; Bulet, P., Antimicrobial Peptides in Drosophila: Structures,Activities and Gene Regulation. In Mechanisms of Epithelial Defense, KARGER: Basel, 2005; Vol. 86, pp 1-21.

144. Lourenco, A. P.; Florecki, M. M.; Simões, Z. L. P.; Evans, J. D. Silencing of Apis mellifera dorsal genes reveals their role in expression of the antimicrobial peptide defensin-1. Insect Mol Biol **2018**.

145. Danihlík, J.; Aronstein, K.; Petřivalský, M. Antimicrobial peptides: a key component of honey bee innate immunity. J Apicult Res **2016,** 54, (2), 123-136.

146. Schlüns, H.; Crozier, R. H. Relish regulates expression of antimicrobial peptide genes in the honeybee, Apis mellifera, shown by RNA interference. Insect Mol Biol **2007,** 16, (6), 753-759.

147. Riddell, C.; Adams, S.; Schmid-Hempel, P.; Mallon, E. B. Differential Expression of Immune Defences Is Associated with Specific Host-Parasite Interactions in Insects. PLoS ONE **2009,** 4, (10), e7621.

148. Xu, P.; Shi, M.; Chen, X.-X. Antimicrobial Peptide Evolution in the Asiatic Honey Bee Apis cerana. PLoS ONE **2009,** 4, (1), e4239.

149. Choi, Y. S.; Choo, Y. M.; Lee, K. S.; Yoon, H. J.; Kim, I.; Je, Y. H.; Sohn, H. D.; Jin, B. R. Cloning and expression profiling of four antibacterial peptide genes from the bumblebee Bombus ignitus. Comp Biochem Phys B **2008,** 150, (2), 141-146.

150. Doublet, V.; Poeschl, Y.; Gogol-Döring, A.; Alaux, C.; Annoscia, D.; Aurori, C.; Barribeau, S. M.; Bedoya-Reina, O. C.; Brown, M. J. F.; Bull, J. C.; Flenniken, M. L.; Galbraith, D. A.; Genersch, E.; Gisder, S.; Grosse, I.; Holt, H. L.; Hultmark, D.; Lattorff, H. M. G.; Le Conte, Y.; Manfredini, F.; McMahon, D. P.; Moritz, R. F. A.; Nazzi, F.; Nino, E. L.; Nowick, K.; van Rij, R. P.; Paxton, R. J.; Grozinger, C. M. Unity in defence: honeybee workers exhibit conserved molecular responses to diverse pathogens. Bmc Genomics **2017,** 18, (1), 207.

151. Zanni, V.; Galbraith, D. A.; Annoscia, D.; Grozinger, C. M.; Nazzi, F. Transcriptional signatures of parasitization and markers of colony decline in Varroa-infested honey bees (Apis mellifera). Insect Biochem Mol Biol **2017,** 87, 1-13.

152. Ryabov, E. V.; Fannon, J. M.; Moore, J. D.; Wood, G. R.; Evans, D. J. The Iflaviruses Sacbrood virus and Deformed wing virus evoke different transcriptional responses in the honeybee which may facilitate their horizontal or vertical transmission. PeerJ **2016,** 4, e1591.

153. Steinmann, N.; Corona, M.; Neumann, P.; Dainat, B. Overwintering Is Associated with Reduced Expression of Immune Genes and Higher Susceptibility to Virus Infection in Honey Bees. PLoS ONE **2015,** 10, (6), e0129956.

154. Merkling, S. H.; Overheul, G. J.; van Mierlo, J. T.; Arends, D.; Gilissen, C.; van Rij, R. P. The heat shock response restricts virus infection in Drosophila. Sci Rep **2015,** 5, (1), 33.

155. Miyoshi, T.; Takeuchi, A.; Siomi, H.; Siomi, M. C. A direct role for Hsp90 in pre-RISC formation in Drosophila. Nat Struct Mol Biol **2010,** 17, (8), 1024-1026.

156. Sim, C.; Hong, Y. S.; Tsetsarkin, K. A.; Vanlandingham, D. L.; Higgs, S.; Collins, F. H. Anopheles gambiae heat shock protein cognate 70B impedes o'nyong-nyong virus replication. Bmc Genomics **2007,** 8, 231.

157. Mockel, N.; Gisder, S.; Genersch, E. Horizontal transmission of deformed wing virus: pathological consequences in adult bees (Apis mellifera) depend on the transmission route. Journal of General Virology **2011,** 92, (2), 370-377.

158. Bowen-Walker, P. L.; Martin, S. J.; Gunn, A. The transmission of deformed wing virus between honeybees (Apis mellifera L.) by the ectoparasitic mite Varroa jacobsoni Oud. J Invertebr Pathol **1999,** 73, (1), 101-106.

159. Nazzi, F.; Pennacchio, F. Honey Bee Antiviral Immune Barriers as Affected by Multiple Stress Factors: A Novel Paradigm to Interpret Colony Health Decline and Collapse. Viruses **2018,** 10, (4).

160. Kuster, R. D.; Boncristiani, H. F.; Rueppell, O. Immunogene and viral transcript dynamics during parasitic Varroa destructor mite infection of developing honey bee (Apis mellifera) pupae. J. Exp. Biol. **2014,** 217, (10), 1710-1718.

161. Chejanovsky, N.; Ophir, R.; Schwager, M. S.; Slabezki, Y.; Grossman, S.; Cox-Foster, D. Characterization of viral siRNA populations in honey bee colony collapse disorder. Virology **2014,** 454-455, 176-183.

162. Desai, S. D.; Eu, Y. J.; Whyard, S.; Currie, R. W. Reduction in deformed wing virus infection in larval and adult honey bees (Apis mellifera L.) by double-stranded RNA ingestion. Insect Mol Biol **2012,** 21, (4), 446-455.

163. Eleftherianos, I.; Won, S.; Chtarbanova, S.; Squiban, B.; Ocorr, K.; Bodmer, R.; Beutler, B.; Hoffmann, J. A.; Imler, J. L. ATP-sensitive potassium channel (KATP)-dependent regulation of cardiotropic viral infections. Proc Natl Acad Sci USA **2011,** 108, (29), 12024-12029.

164. Palmer, W. H.; Varghese, F. S.; van Rij, R. P. Natural Variation in Resistance to Virus Infection in Dipteran Insects. Viruses **2018,** 10, (3), 118.

165. Rückert, C.; Bell-Sakyi, L.; Fazakerley, J. K.; Fragkoudis, R. Antiviral responses of arthropod vectors: an update on recent advances. VirusDis. **2014,** 25, (3), 249-260.

166. Sanchez-Vargas, I.; Travanty, E. A.; Keene, K. M.; Franz, A. W. E.; Beaty, B. J.; Blair, C. D.; Olson, K. E. RNA interference, arthropod-borne viruses, and mosquitoes. Virus Res **2004,** 102, (1), 65-74.

167. Ding, S.-W. RNA-based antiviral immunity. Nat Rev Immunol **2010,** 10, (9), 632-644.

168. Fire, A. RNA-triggered gene silencing. Trends Genet **1999,** 15, (9), 358-363.

169. Flenniken, M. L.; Kunitomi, M.; Tassetto, M.; Andino, R., The Antiviral Role of RNA Interference. In Insect Virology, Asgari, S.; Johnson, K. N., Eds. Caister Academic Press: Norfolk, UK, 2010; pp 367-388.

170. Hammond, S. M. Argonaute2, a Link Between Genetic and Biochemical Analyses of RNAi. Science **2001,** 293, (5532), 1146-1150.

171. Blair, C. D. Mosquito RNAi is the major innate immune pathway controlling arbovirus infection and transmission. Future Microbiol **2011,** 6, (3), 265-277.

172. Bronkhorst, A. W.; van Rij, R. P. The long and short of antiviral defense: small RNA-based immunity in insects. Curr Opin Virol **2014,** 7, 19-28.

173. Brutscher, L. M.; Flenniken, M. L. RNAi and Antiviral Defense in the Honey Bee. Journal of Immunology Research **2015,** 2015, (1), 941897-10.

174. Wilkins, C.; Dishongh, R.; Moore, S. C.; Whitt, M. A.; Chow, M.; Machaca, K. RNA interference is an antiviral defence mechanism in Caenorhabditis elegans. Nature **2005,** 436, (7053), 1044-7.

175. Zamore, P. D. Ancient pathways programmed by small RNAs. Science **2002,** 296, (5571), 1265-9.

176. Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature **1998,** 391, (6669), 806-811.

177. Baulcombe, D. RNA silencing in plants. Nature **2004,** 431, (7006), 356-363.

178. Liu, Y.; Ye, X.; Jiang, F.; Liang, C.; Chen, D.; Peng, J.; Kinch, L. N.; Grishin, N. V.; Liu, Q. C3PO, an Endoribonuclease That Promotes RNAi by Facilitating RISC Activation. Science **2009,** 325, (5941), 750-753.

179. Li, H. Induction and Suppression of RNA Silencing by an Animal Virus. Science **2002,** 296, (5571), 1319-1321.

180. van Rij, R. P.; Saleh, M. C.; Berry, B.; Foo, C.; Houk, A.; Antoniewski, C.; Andino, R. The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes Dev **2006,** 20, (21), 2985-2995.

181. Saleh, M.-C.; van Rij, R. P.; Hekele, A.; Gillis, A.; Foley, E.; O'Farrell, P. H.; Andino, R. The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nat. Cell Biol. **2006,** 8, (8), 793-802.

182. Saleh, M.-C.; Tassetto, M.; van Rij, R. P.; Goic, B.; Gausson, V.; Berry, B.; Jacquier, C.; Antoniewski, C.; Andino, R. Antiviral immunity in Drosophila requires systemic RNA interference spread. Nature **2009,** 458, (7236), 346-350.

183. Goic, B.; Vodovar, N.; Mondotte, J. A.; Monot, C.; Frangeul, L.; Blanc, H.; Gausson, V.; Vera-Otarola, J.; Cristofari, G.; Saleh, M.-C. RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model Drosophila. Nat Immunol **2013,** 14, (4), 396-403.

184. Tassetto, M.; Kunitomi, M.; Andino, R. Circulating Immune Cells Mediate a Systemic RNAi-Based Adaptive Antiviral Response in Drosophila. Cell **2017,** 169, (2), 314-325.e13.

185. Poirier, E. Z.; Goic, B.; Tome-Poderti, L.; Frangeul, L.; Boussier, J.; Gausson, V.; Blanc, H.; Vallet, T.; Loyd, H.; Levi, L. I.; Lanciano, S.; Baron, C.; Merkling, S. H.; Lambrechts, L.; Mirouze, M.; Carpenter, S.; Vignuzzi, M.; Saleh, M. C. Dicer-2-Dependent Generation of Viral DNA from Defective Genomes of RNA Viruses Modulates Antiviral Immunity in Insects. Cell Host Microbe **2018,** 23, (3), 353-365 e8.

186. Liu, X.; Zhang, Y.; Yan, X.; Han, R. Prevention of Chinese Sacbrood Virus Infection in Apis cerana using RNA Interference. Curr Microbiol **2010,** 61, (5), 422-428.

187. Maori, E.; Paldi, N.; Shafir, S.; Kalev, H.; Tsur, E.; Glick, E.; Sela, I. IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. Insect Mol Biol **2009,** 18, (1), 55-60.

188. Nunes, F.; Aleixo, A.; Barchuk, A.; Bomtorin, A.; Grozinger, C.; Simões, Z. Non-Target Effects of Green Fluorescent Protein (GFP)-Derived Double-Stranded RNA (dsRNA-GFP) Used in Honey Bee RNA Interference (RNAi) Assays. Insects 2012, Vol. 3, Pages 601-615 **2013,** 4, (1), 90-103.

189. Lundgren, J. G.; Duan, J. J. RNAi-Based Insecticidal Crops: Potential Effects on Nontarget Species. Bioscience **2013,** 63, (8), 657-665.

190. Cappelle, K.; Smagghe, G.; Dhaenens, M.; Meeus, I. Israeli Acute Paralysis Virus Infection Leads to an Enhanced RNA Interference Response and Not Its Suppression in the Bumblebee Bombus terrestris. Viruses **2016,** 8, (12).

191. Jarosch, A.; Moritz, R. F. A. RNA interference in honeybees: off-target effects caused by dsRNA. Apidologie **2012,** 43, (2), 128-138.

192. Hunter, W.; Ellis, J.; vanEngelsdorp, D.; Hayes, J.; Westervelt, D.; Glick, E.; Williams, M.; Sela, I.; Maori, E.; Pettis, J.; Cox-Foster, D.; Paldi, N. Large-Scale Field Application of RNAi Technology Reducing Israeli Acute Paralysis Virus Disease in Honey Bees (Apis mellifera, Hymenoptera: Apidae). Plos Pathog **2010,** 6, (12), e1001160.

193. Beye, M.; Hartel, S.; Hagen, A.; Hasselmann, M.; Omholt, S. W. Specific developmental gene silencing in the honey bee using a homeobox motif. Insect Mol Biol **2002,** 11, (6), 527-32.

194. Aronstein, K.; Saldivar, E. Characterization of a honey bee Toll related receptor gene Am18w and its potential involvement in antimicrobial immune defense. Apidologie **2005,** 36, (1), 3-14.

195. Patel, A.; Fondrk, M. K.; Kaftanoglu, O.; Emore, C.; Hunt, G.; Frederick, K.; Amdam, G. V. The Making of a Queen: TOR Pathway Is a Key Player in Diphenic Caste Development. PLoS ONE **2007,** 2, (6), e509.

196. Barchuk, A. R.; Figueiredo, V. L. C.; Simões, Z. L. P. Downregulation of ultraspiracle gene expression delays pupal development in honeybees. J Insect Physiol **2008,** 54, (6), 1035-1040.

197. Nilsen, K. A.; Ihle, K. E.; Frederick, K.; Fondrk, M. K.; Smedal, B.; Hartfelder, K.; Amdam, G. V. Insulin-like peptide genes in honey bee fat body respond differently to manipulation of social behavioral physiology. J. Exp. Biol. **2011,** 214, (9), 1488-1497.

198. Wang, Y.; Baker, N.; Amdam, G. V. RNAi-mediated double gene knockdown and gustatory perception measurement in honey bees (Apis mellifera). J Vis Exp **2013**, (77).

199. Ihle, K. E.; Fondrk, M. K.; Page, R. E.; Amdam, G. V. Genotype effect on lifespan following vitellogenin knockdown. Exp. Gerontol. **2015,** 61, 113-122.

200. Nelson, C. M.; Ihle, K. E.; Fondrk, M. K.; Page, R. E.; Amdam, G. V. The Gene vitellogenin Has Multiple Coordinating Effects on Social Organization. Plos Biol **2007,** 5, (3), e62-677.

201. Deddouche, S.; Matt, N.; Budd, A.; Mueller, S.; Kemp, C.; Galiana-Arnoux, D.; Dostert, C.; Antoniewski, C.; Hoffmann, J. A.; Imler, J.-L. The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in Drosophila. Nat Immunol **2008,** 9, (12), 1425-1432.

202. Paradkar, P. N.; Trinidad, L.; Voysey, R.; Duchemin, J. B.; Walker, P. J. Secreted Vago restricts West Nile virus infection in Culex mosquito cells by activating the Jak-STAT pathway. Proc Natl Acad Sci USA **2012,** 109, (46), 18915-18920.

203. Paradkar, P. N.; Duchemin, J. B.; Voysey, R.; Walker, P. J. Dicer-2-dependent activation of Culex Vago occurs via the TRAF-Rel2 signaling pathway. PLoS Negl Trop Dis **2014,** 8, (4), e2823.

204. Pichlmair, A.; Schulz, O.; Tan, C. P.; Rehwinkel, J.; Kato, H.; Takeuchi, O.; Akira, S.; Way, M.; Schiavo, G.; Reis e Sousa, C. Activation of MDA5 Requires Higher-Order RNA Structures Generated during Virus Infection. J Virol **2009,** 83, (20), 10761-10769.

205. Takeuchi, O.; Akira, S. RIG-I-like antiviral protein in flies. Nat Immunol **2008,** 9, (12), 1327-1328.

206. Yoneyama, M.; Kikuchi, M.; Natsukawa, T.; Shinobu, N.; Imaizumi, T.; Miyagishi, M.; Taira, K.; Akira, S.; Fujita, T. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol **2004,** 5, (7), 730-737.

207. Kingsolver, M. B.; Huang, Z.; Hardy, R. W. Insect Antiviral Innate Immunity: Pathways, Effectors, and Connections. J Mol Biol **2013,** 425, (24), 4921-4936.

208. Cardinal, S.; Straka, J.; Danforth, B. N. Comprehensive phylogeny of apid bees reveals the evolutionary origins and antiquity of cleptoparasitism. Proc Natl Acad Sci USA **2010,** 107, (37), 16207-16211.

209. Terenius, O.; Papanicolaou, A.; Garbutt, J. S.; Eleftherianos, I.; Huvenne, H.; Kanginakudru, S.; Albrechtsen, M.; An, C.; Aymeric, J.-L.; Barthel, A.; Bebas, P.; Bitra, K.; Bravo, A.; Chevalier, F.; Collinge, D. P.; Crava, C. M.; de Maagd, R. A.; Duvic, B.; Erlandson, M.; Faye, I.; Felföldi, G.; Fujiwara, H.; Futahashi, R.; Gandhe, A. S.; Gatehouse, H. S.; Gatehouse, L. N.; Giebultowicz, J. M.; Gómez, I.; Grimmelikhuijzen, C. J. P.; Groot, A. T.; Hauser, F.; Heckel, D. G.; Hegedus, D. D.; Hrycaj, S.; Huang, L.; Hull, J. J.; Iatrou, K.; Iga, M.; Kanost, M. R.; Kotwica, J.; Li, C.; Li, J.; Liu, J.; Lundmark, M.; Matsumoto, S.; Meyering-Vos, M.; Millichap, P. J.; Monteiro, A.; Mrinal, N.; Niimi, T.; Nowara, D.; Ohnishi, A.; Oostra, V.; Ozaki, K.; Papakonstantinou, M.; Popadic, A.; Rajam, M. V.; Saenko, S.; Simpson, R. M.; Soberón, M.; Strand, M. R.; Tomita, S.; Toprak, U.; Wang, P.; Wee, C. W.; Whyard, S.; Zhang, W.; Nagaraju, J.; Ffrench-Constant, R. H.; Herrero, S.; Gordon, K.; Swevers, L.; Smagghe, G. RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design. J Insect Physiol **2011,** 57, (2), 231-245.

210. Boisson, B.; Jacques, J. C.; Choumet, V.; Martin, E.; Xu, J.; Vernick, K.; Bourgouin, C. Gene silencing in mosquito salivary glands by RNAi. Febs Lett **2006,** 580, (8), 1988-92.

211. Nayak, A.; Berry, B.; Tassetto, M.; Kunitomi, M.; Acevedo, A.; Deng, C.; Krutchinsky, A.; Gross, J.; Antoniewski, C.; Andino, R. Cricket paralysis virus antagonizes Argonaute 2 to modulate antiviral defense in Drosophila. Nat Struct Mol Biol **2010,** 17, (5), 547-554.

212. Wang, H.; Smagghe, G.; Meeus, I. The role of a single gene encoding the Single von Willebrand factor C-domain protein (SVC) in bumblebee immunity extends beyond antiviral defense. Insect Biochem Mol Biol **2017,** 91, 10-20.

213. Ronald, P. C.; Beutler, B. Plant and Animal Sensors of Conserved Microbial Signatures. Science **2010,** 330, (6007), 1061-1064.

214. Coffman, S. R.; Lu, J.; Guo, X.; Zhong, J.; Jiang, H.; Broitman-Maduro, G.; Li, W.-X.; Lu, R.; Maduro, M.; Ding, S.-W. Caenorhabditis elegans RIG-I Homolog Mediates Antiviral RNA Interference Downstream of Dicer-Dependent Biogenesis of Viral Small Interfering RNAs. MBio **2017,** 8, (2), e00264-17.

215. Ashe, A.; Sarkies, P.; Le Pen, J.; Tanguy, M.; Miska, E. A. Antiviral RNA Interference against Orsay Virus Is neither Systemic nor Transgenerational in Caenorhabditis elegans. J Virol **2015,** 89, (23), 12035-12046.

216. Lu, R.; Maduro, M.; Li, F.; Li, H. W.; Broitman-Maduro, G.; Li, W. X.; Ding, S. W. Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. Nature **2005,** 436, (7053), 1040-1043.

217. Schnettler, E.; Tykalová, H.; Watson, M.; Sharma, M.; Sterken, M. G.; Obbard, D. J.; Lewis, S. H.; McFarlane, M.; Bell-Sakyi, L.; Barry, G.; Weisheit, S.; Best, S. M.; Kuhn, R. J.; Pijlman, G. P.; Chase-Topping, M. E.; Gould, E. A.; Grubhoffer, L.; Fazakerley, J. K.; Kohl, A. Induction and suppression of tick cell antiviral RNAi responses by tick-borne flaviviruses. Nucleic Acids Res **2014,** 42, (14), 9436-9446.

218. Green, T. J.; Speck, P. Antiviral Defense and Innate Immune Memory in the Oyster. Viruses **2018,** 10, (3), 133.

219. Robalino, J.; Bartlett, T. C.; Chapman, R. W.; Gross, P. S.; Browdy, C. L.; Warr, G. W. Double-stranded RNA and antiviral immunity in marine shrimp: Inducible host mechanisms and evidence for the evolution of viral counter-responses. Dev Comp Immunol **2007,** 31, (6), 539-547.

220. Pitaluga, A. N.; Mason, P. W.; Traub-Cseko, Y. M. Non-specific antiviral response detected in RNA-treated cultured cells of the sandfly, Lutzomyia longipalpis. Dev Comp Immunol **2008,** 32, (3), 191-197.

221. Martins-da-Silva, A.; Telleria, E.; Batista, M.; Marchini, F.; Traub-Csekö, Y.; Tempone, A. Identification of Secreted Proteins Involved in Nonspecific dsRNA-Mediated Lutzomyia longipalpis LL5 Cell Antiviral Response. Viruses **2018,** 10, (1), 43.

222. van Mierlo, J. T.; van Cleef, K. W. R.; van Rij, R. P., Defense and Counterdefense in the RNAi-Based Antiviral Immune System in Insects. In Drosophila, Springer New York

Humana Press: Totowa, NJ, 2011; Vol. 721, pp 3-22.

223. De Maio, F. A.; Risso, G.; Iglesias, N. G.; Shah, P.; Pozzi, B.; Gebhard, L. G.; Mammi, P.; Mancini, E.; Yanovsky, M. J.; Andino, R.; Krogan, N.; Srebrow, A.; Gamarnik, A. V. The Dengue Virus NS5 Protein Intrudes in the Cellular Spliceosome and Modulates Splicing. Plos Pathog **2016,** 12, (8), e1005841.

224. Wang, Y.; Jorda, M.; Jones, P. L.; Maleszka, R.; Ling, X.; Robertson, H. M.; Mizzen, C. A.; Peinado, M. A.; Robinson, G. E. Functional CpG Methylation System in a Social Insect. Science **2006,** 314, (5799), 645-647.

225. Tidbury, H. J.; Pedersen, A. B.; Boots, M. Within and transgenerational immune priming in an insect to a DNA virus. Proc Biol Sci **2011,** 278, (1707), 871-6.

226. Gourbal, B.; Pinaud, S.; Beckers, G. J. M.; Van Der Meer, J. W. M.; Conrath, U.; Netea, M. G. Innate immune memory: An evolutionary perspective. Immunol Rev **2018,** 283, (1), 21-40.

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

CHAPTER FOUR

THE HEAT SHOCK RESPONSE IN THE WESTERN HONEY BEE (*APIS MELLIFERA*) IS ANTIVIRAL

Contributions of Authors and Co-Authors

Manuscript in Chapter Four

Author: Alexander J. McMenamin

Contributions: Acquired funding, designed the study, collected and analyzed data, interpreted the results, wrote and revised the manuscript

Co-author: Katie F. Daughenbaugh

Contributions: Collected and analyzed data, interpreted the results, revised the manuscript

Co-author: Michelle L. Flenniken

Contributions: Supervised the study, acquired funding, designed the study, interpreted the results, wrote and revised the manuscript

Manuscript Information

Alexander J. McMenamin, Katie F. Daughenbaugh, Michelle L. Flenniken

The heat shock response in the Western honey bee (*Apis mellifera*) is antiviral

Viruses

Status of Manuscript
___ Prepared for submission to a peer-reviewed journal
___ Officially submitted to a peer-reviewed journal
___ Accepted by a peer-reviewed journal
_X_ Published in a peer-reviewed journal

Feb; 12(2): 245. doi: 10.3390/v12020245

The Heat Shock Response in the Western Honey Bee (Apis mellifera) is Antiviral

Alexander J. McMenamin [1,2,3], Katie F. Daughenbaugh [1,3] and Michelle L. Flenniken [1,2,3]*

[1] Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717, USA; alexmcmenamin2@gmail.com (A.J.M.); kdaughenbaugh@gmail.com (K.F.D.)

[2] Department of Microbiology and Immunology, Montana State University, Bozeman, MT 59717, USA

[3] Pollinator Health Center, Montana State University, Bozeman, MT 59717, USA

* Correspondence: michelle.flenniken@montana.edu; Tel.: +1-406-994-7229

Received: 23 January 2020; Accepted: 19 February 2020; Published: 22 February 2020

Abstract: Honey bees (*Apis mellifera*) are an agriculturally important pollinator species that live in easily managed social groups (i.e., colonies). Unfortunately, annual losses of honey bee colonies in many parts of the world have reached unsustainable levels. Multiple abiotic and biotic stressors, including viruses, are associated with individual honey bee and colony mortality. Honey bees have evolved several antiviral defense mechanisms including conserved immune pathways (e.g., Toll, Imd, JAK/STAT) and dsRNA-triggered responses including RNA interference and a non-sequence specific dsRNA-mediated response. In addition, transcriptome analyses of virus-infected honey bees implicate an antiviral role of stress response pathways, including the heat shock response. Herein, we demonstrate that the heat shock response is antiviral in honey bees. Specifically, heat-shocked honey bees (i.e., 42 °C for 4 h) had reduced levels of the model virus, Sindbis-GFP, compared with bees maintained at a constant temperature. Virus-infection and/or heat shock resulted in differential expression of six heat shock protein encoding genes and three immune genes, many of which are positively correlated. The heat shock protein encoding and immune gene transcriptional responses observed in virus-infected bees were not completely recapitulated by administration of double stranded RNA (dsRNA), a virus-associated molecular

pattern, indicating that additional virus–host interactions are involved in triggering antiviral stress response pathways.

Keywords: honey bee; *Apis mellifera*; heat shock; thermal stress; antiviral immunity; RNA virus; insect virus; insect antiviral defense

---

1. Introduction

Honey bees (*Apis mellifera*) are eusocial insects in the order Hymenoptera that live in colonies consisting of one reproductive queen, hundreds of reproductive males (drones), and approximately 30,000 non-reproductive sterile female workers. Honey bees are generalist pollinators of numerous plant species, including fruit, nut, and vegetable crops [1-3] valued at $14.6 billion annually in the United States [4]. Unfortunately, the U.S. and parts of Europe have experienced unsustainable annual losses of honey bee colonies (e.g., 33% average yearly losses since 2006 in the U.S.) [5-11]. Colony losses are attributed to numerous biotic and abiotic stressors, including inadequate nutrition, the *Varroa destructor* mite, and pathogens [12,13]. There are numerous viruses that infect honey bees and the prevalence and abundance of several of them, including deformed wing virus, have been associated with colony losses globally [10,11,14-26].

Positive-sense single-stranded RNA viruses comprise the largest group of known honey bee pathogens (reviewed in [27,28]). These include members of *Iflaviridae* (i.e., deformed wing virus, sacbrood virus, and slow bee paralysis virus), *Dicistroviridae* (i.e., black queen cell virus, Israeli acute paralysis virus, acute bee paralysis virus, and Kashmir bee virus), chronic bee paralysis virus

[29], and Lake Sinai viruses [15,30,31], as well as a growing list of other viruses and virus families (reviewed in [27,28,32]). Viruses are transmitted vertically from parents to offspring and horizontally between bees within the crowded environment of the colony via contact and trophallaxis (mouth to mouth food transfer), as well as via contact with contaminated floral resources while foraging [33,34] (reviewed in [28,35]). In addition, several viruses are transmitted by the ectoparasitic mite, *Varroa destructor* (reviewed in [22,27,28]). Like other organisms, honey bees have evolved sophisticated immune systems to limit virus infections [36] (reviewed in [37,38]). Unlike many organisms, honey bees also have social immune mechanisms [39,40] (reviewed in [41-43]). Although it is unclear how social immunity affects viral infections, hygienic behaviors that reduce mite infestation likely reduce mite-mediated virus transmission (reviewed in [41,43]).

Antiviral defense at the individual bee level impacts the health of the entire colony. Honey bee colonies are considered superorganisms and, therefore, require a sufficient number of workers to keep the colony warm, feed the developing brood, and forage. Honey bee antiviral defense mechanisms include autophagy, endocytosis, apoptosis, eicosanoid signaling, melanization, and the Toll and Imd (Immune Deficiency) pathways involving NF-κB (Nuclear Factor κB) homologues Dorsal and Relish, JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription), JNK (c-Jun N-terminal kinase), MAPK (Mitogen-Activated Protein Kinases), and RNA interference (RNAi) pathways [14,20,44-51]. RNAi is a sequence-specific post-transcriptional gene silencing mechanism. The short-interfering RNA (siRNA) directed RNAi pathway is the primary insect antiviral response, and is induced upon recognition of virus-derived

double stranded RNA (dsRNA) [37,44,45,51-60]. In honey bees and bumble bees, administration of dsRNA, a virus-associated molecular pattern (VAMP), results in a non-sequence specific antiviral response and impacts gene expression [46,61-66].

Transcriptome analyses of honey bees identified hundreds of differentially expressed genes in response to dsRNA [46,61,66] and virus infection including RNAi pathway members and genes encoding antimicrobial peptides and heat shock proteins (HSPs) [14,48,49,61,67]. A recombinant Sindbis virus that expresses green fluorescent protein (SINV-GFP) has been used to investigate antiviral defense in a wide range of insects including fruit flies [68], mosquitos [69], and honey bees [46,61]. SINV-GFP is easily produced in cultured cells, trackable, and does not encode an RNAi suppressor protein, and thus facilitates investigation of this and other insect antiviral response pathways. Honey bees infected with SINV-GFP exhibited increased expression of *ago-2*, *dcr-like*, *mf116383*, and numerous heat shock stress response pathway members including *heat shock protein 90, activator of hsp90, hsp60, heat shock protein 83-like, dnaj shv-like, protein lethal(2)essential for life-like,* and *hsp10* [61]. Whereas *heat shock protein 70 cognate 3* expression was increased in bees naturally infected with Israeli acute paralysis virus (IAPV) in one study, its expression was decreased in bees experimentally inoculated with IAPV in a second study [14,48]. Additional experiments are needed to elucidate the role of the heat shock stress response pathway in honey bee antiviral defense.

In general, heat shock proteins (HSPs) are involved in maintaining cellular protein homeostasis—protein synthesis, folding, function, and regulation of degradation (i.e., proteostasis) [70]. The heat shock response and broader proteostasis network in honey bees are conserved and the expression

of core heat shock factor target genes and several members of the broader proteostasis network are induced by heat-stress [71]. There is a positive relationship between the heat shock response and immune response in other invertebrates, including *Drosophila melanogaster* and *Caenorhabditis elegans* [72-76]. However, McKinstry et al. determined that heat shock factor target gene expression was reduced in response to wounding and bacterial infection in honey bees and thus concluded that the heat shock response and humoral immune responses are mutually antagonistic [71]. In the context of viral infection, HSPs can both positively and negatively affect viral replication [76-81]. For example, heat shock protein 90 (Hsp90) facilitates the assembly of Flock House virus (FHV) replicase in *Drosophila* S2 cells, while heat shock protein 40 (Hsp40) and Hsp70 family members have contrasting effects on FHV replication in yeast [79,80]. Additionally, HSPs are required for assembly of the RNAi-induced silencing complex (RISC) and effective RNAi defense against viruses in *Drosophila melanogaster* [77,78]. Intriguingly, heat-stress (temperatures >37 °C) reduced deformed wing virus (DWV) loads in naturally infected honey bees [82,83].

Therefore, given the transcriptional regulation of HSP-encoding genes in virus-infected honey bees, the role of HSPs in insect RNAi, and the experimental reduction of naturally occurring DWV infections in heat-stressed honey bees, we hypothesized that HSPs and the heat shock response are involved in honey bee antiviral defense [14,48,49,61,77,78,82,83]. To test this hypothesis, we examined the effect of heat shock (42 °C for 4 h) on honey bees experimentally infected with a model virus SINV-GFP, via intrathoracic injection, an infection route that mimics mite-mediated virus transmission and ensures precise delivery of a known dose. The relative expression of HSPs

and immune genes was assessed by quantitative PCR (qPCR). We determined that both viral infection and heat shock induced the expression of HSPs, though expression in bees exposed to heat shock alone varied. The relative expression of HSPs and immune genes was positively correlated, suggesting potential co-regulation of these genes in response to stressors. Lastly, double stranded RNA, which is a hallmark of virus infection, was not sufficient to recapitulate the HSP induction pattern observed in virus-infected honey bees. This indicates that virus–host interactions beyond molecular pattern recognition are required to induce the expression of some HSP-encoding genes in honey bees.

2. Materials and Methods

2.1. Honey Bees

Honey bee (*Apis mellifera)* colonies were established from packages (~1.5 kg of worker bees and a naturally-mated queen) of primarily *Apis mellifera carnica* stock purchased from a commercial producer in Montana in April 2018. Honey bees were kept in Langstroth hives located on Montana State University's Horticulture Farm in Bozeman, MT, USA. Colonies were maintained using standard apicultural practices, including bi-monthly evaluation of *Varroa destructor* mite infestation levels using the powdered sugar roll method [84]. Colonies were treated with formic acid polysaccharide gel strips (Mite Away Quick Strips®, Nature's Own Design Apiary Products, Frankford, Ontario, Canada) when mite infestation was greater than 3% (3 mites per 100 bees) [84,85].

Honey bees for laboratory-based experiments were obtained from frames of newly emerging bees, which were collected one day prior to each experiment and maintained at 32 °C in a laboratory incubator overnight. Young, age-matched (~24 h post-emergence), female adult bees were utilized for all experiments. For the duration of the experiment, honey bees were housed in modified deli-containers at 32 °C and fed bee candy (powdered sugar mixed with corn syrup until pliable) and water *ad libitum* [46,86].

2.2. dsRNA Preparation

Double stranded RNA was generated by in vitro transcription with T7 RNA polymerase [61,68]. T7 promoter-containing PCR-products were amplified using primers listed in Table S1, with the following thermocycler program: pre-incubation of 95 °C (5 min), 35 cycles of 95 °C (30 s), 60 °C (30 s), and 72 °C (1 min) followed by a final elongation at 72 °C (5 min). PCR products were used as template for T7 polymerase transcription (100 µL reactions: NTPs (each 7.5 mM final), RNase OUT (40 units) (Invitrogen, Carlsbad, California, USA), buffer (400 mM HEPES pH 7.5, 120 mM $MgCl_2$, 10 mM spermidine, 200 mM DTT); reactions were carried out at 37 °C overnight (8–10 h). DNA was removed by adding 1 unit of RQ1 DNAse (Promega, Madison, Wisconsin, USA) and incubating for 15 min at 37 °C. dsRNA products were precipitated with 500 µL ethanol and 1:10 volume 3 M sodium acetate (pH 5.2), suspended in 200 µL RNase-free water, and annealed at 100 °C for 5 min and then slowly cooled to room temperature. dsRNA products were purified by phenol:chloroform extraction and subsequent precipitation with 600 µL ethanol and 80 µL 5 M ammonium acetate. RNA pellets were dissolved in 60–100 µL 10 mM Tris HCl (pH 7.5).

Quality was assessed by agarose gel electrophoresis and spectrophotometry. The dsRNA quantity based on gel band intensity relative to a standard was determined using ImageJ version 1.50i [87].

2.3. Virus Infection and Heat Shock Protocol

Glass needles for honey bee intra-thoracic injections were made by pulling borosilicate glass capillary tubes (100 mm long, 1 mL capacity, Kimble-Chase) with a coil temperature of 61 °C on the PC-10 Dual-Stage Glass Micropipette Puller (Narishige, East Meadow, New York, USA). Prior to injection, age-matched honey bees (~24 h post-emergence) were cold anesthetized for 10 min at 4 °C. Honey bees were infected with SINV-GFP (3750 plaque forming units (PFU) in 2 µL 10 mM Tris HCl buffer pH 7.5) via intra-thoracic injection using a Harbo syringe (Honey bee Insemination Service) and microcapillary glass needles; mock-infected bees were injected with 2 µL buffer (10 mM Tris HCl, pH 7.5) [46,61]. The dose of 3750 PFUs SINV-GFP per honey bee was based on our previous studies that determined that this dose allowed for a natural progression of infection over the course of the experiment [46]. By 72 h post-infection virus could be visualized by fluorescence microscopy and relatively quantified by Western blot analysis and qPCR [46,61]. Furthermore by 72 h post-infection. changes in gene expression reflected honey bee antiviral defense mechanisms [46,61]. This dose is modest compared to drosophila studies which typically utilize 250–2500 PFUs per fly; a newly emerged female worker honey bee (~150 mg) weighs ~200× more than an adult female fruit fly (0.8 mg) [68].

Experimental treatment groups that were subjected to temperature stress were intrathoracically injected with either buffer or virus, allowed to recover for 6 h at 32 °C (i.e., ample time for post-

injection recovery, but less than the amount of time estimated for a virus replication cycle), exposed to heat shock (i.e., 42 °C for 4 h) [88], and then transferred back to 32 °C for the remainder of the study. The heat shock experiments were carried out using three independent honey bee cohorts obtained from three different colonies on distinct dates (i.e., replicate 1 in June 2018, replicate 2 in August 2018, and replicate 3 in July 2019).

2.4. RNA Isolation

Honey bee samples were dissected into head, thorax, and abdomen. The abdomen was chosen for further analysis as it is the primary site of immune cell-generating fat bodies and it is distal from the site of injection, and thus virus infection naturally spread to that tissue. Honey bee abdomens were homogenized in 300 µL of deionized water with a sterile steel ball (5 mm) using a Tissue Lyser II (Qiagen, Germantown, Maryland, USA) at 30 Hz for 2 min. Then, 300 µL of TRIzol reagent (Invitrogen) was added to the homogenate, vortexed for 15 s and incubated at room temperature for 5 min. Next, 100 µL of chloroform was added, samples were shaken by hand for 15 s and incubated on the benchtop for another 2 min. Samples were then centrifuged at 12,000× *g* at 4 °C for 15 min and the aqueous phase was transferred to a clean centrifuge tube. One volume of isopropanol was added to the aqueous phase, mixed by inversion, and nucleic acid was precipitated by incubation at room temperature for 10 min. The precipitate was pelleted by centrifugation at 12,000× *g* at 4 °C for 10 min. Pellets were then washed with 500 µL of 75% ethanol and centrifuged at 7500× *g* at 4 °C for 5 min, then air dried for 10 min at room temperature and dissolved in 30 µL of deionized water. RNA concentrations and quality were assessed on a Nanodrop 2000 spectrophotometer (Thermo Fisher). When quality was low, RNA was precipitated

a second time by addition of 4 volumes of cold ethanol and 1:10 of a volume 3 M sodium acetate (pH 5.5) and incubation at −20 °C overnight. Nucleic acids were pelleted by centrifugation at 12,000× *g* at 4 °C for 10 min and pellets were washed one time with 500 µL 70% ethanol and centrifuged at 12,000× *g* at 4 °C for 5 min. Pellets were air dried and suspended in 30 µL deionized H_2O. Samples were stored at −80 °C until analysis.

2.5. Reverse Transcription/cDNA Synthesis

Reverse transcription reactions were performed by incubating 2000 ng total RNA, 200 units M-MLV reverse-transcriptase (Promega) and 500 ng random hexamer primers (IDT) for 1 h at 37 °C, according to the manufacturer's instructions. cDNA was diluted 1:2 and 2 µL was used for PCR or qPCR analysis.

2.6. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used to test replicate experiments for pre-existing infections with honey bee infecting viruses since bees were obtained from honey bee colonies which are subject to naturally occurring infections. Pools were made from the cDNA of 4 bees (n = 8–12 bees total), that received no injection but were age-matched to the experimental bees, which were tested for pre-existing/confounding infections by pathogen-specific PCR (Figure S3). Honey bees were tested for black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV), deformed wing virus (DWV), Israeli acute bee paralysis virus (IAPV), Kashmir bee virus (KBV), sacbrood virus (SBV) and Lake Sinai viruses (LSVs 1–4). PCR was performed according to standard methods [89]. In brief, 2 µL cDNA template was combined with

10 pmol of each forward and reverse primer (Table S1), and amplified with Denville ChoiceTaq polymerase (Thomas Scientific, Swedesboro, New Jersey, USA) according to the manufacturer's instructions using the following cycling conditions: 95 °C for 5 min, 95 °C for 30 s, 57 °C for 30 s, 72 °C for 30s, 35 cycles, followed by final elongation at 72 °C for 4 min.

2.7. Quantitative PCR (qPCR)

Quantitative PCR was used to analyze the abundance of SINV-GFP and the relative abundances of honey bee transcripts expressed by immune and heat shock protein encoding genes. All qPCR reactions were performed in triplicate with 2 µL of cDNA template. Each 20 µL reaction contained 1× Denville ChoiceTaq Mastermix, 0.4 µM each forward and reverse primer, 1× SYBR Green (Life Technologies, Carlsbad, CA, USA), and 3 mM $MgCl_2$. A CFX Connect Real Time instrument (BioRad, Hercules, California, USA) was used for the following thermo-profile: pre-incubation 95 °C for one minute followed by 40 cycles of 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 15 s, with a final melt curve analysis at 65 °C for 5 s to 95 °C.

To quantify viral genome copy numbers in the samples, SINV-GFP plasmid standards were used as templates, with concentrations ranging from 10^3 to 10^9 copies per reaction to create a linear standard curve. The detection limit was 10^3 copies of the SINV cDNA using primers qSindbisFW4495 and qSindbisREV4635. The host gene *Am rpl8* was amplified in triplicate for each sample for comparison, using primers Rpl8-Fw1 and Rpl8-Rev1 (Table S1). Reactions without template were carried out as negative controls. qPCR specificity was verified through melt point analysis and via gel electrophoresis, and all products had previously been verified by

sequencing. The linear equation for the plasmid standard for SINV was: Ct = −3.348x + 40.25 (R^2 = 0.996, efficiency = 98.9%) where 'x' is the log(SINV genome equivalents). The relative expression of host genes was determined by a ranked ΔΔCt method in which the ΔCt was calculated by subtracting the *rpl8* Ct value from the Ct of the gene of interest. Then the ΔCt values were ranked to control for natural inter-individual variation in gene expression and the matching mock-infected ΔCt was subtracted from the treatment group ΔCt to obtain the ΔΔCt. The fold-change in cDNA abundance was calculated by the equation $2^{-\Delta\Delta Ct}$; see Tables S2-S7 for qPCR data presented in Figures 1-3, 5, and S1.

2.8. Statistical Analysis

All analyses were carried out using R v3.5.1 in RStudio v1.1.456 [90]. Unless otherwise stated, pairwise comparisons of gene expression were evaluated using the *pairwise.wilcox.test* function in the base R *stats* package (v3.6.2) to perform a Wilcoxon Rank Sums with a Benjamini–Hochberg correction for multiple comparisons. The correlation matrix was calculated and visualized using the *corrplot* package (v0.84) [91].

3. Results

3.1. Heat Treatment Reduces Viral Abundance in Adult Bees

To examine the impact of short duration temperature stress on the outcome of virus infection in honey bees, bees were infected via intrathoracic injection with the model virus Sindbis virus-GFP (SINV-GFP), allowed to recover for 6 h at 32 °C, exposed to heat shock (i.e., 42 °C for 4 h [88]), and then transferred back to 32 °C for the remainder of the study. Virus abundance in individual

honey bees was assessed at 72 h post-infection by qPCR, and in three independent experiments heat shock significantly reduced SINV-GFP RNA equivalents (transcripts and genomes) (Figure 1). In replicate 1, heat-shocked bees had a 74% lower mean virus abundance ($5.44 \times 10^7 \pm 6.72 \times 10^6$ SINV-GFP RNA copies) compared to bees maintained at a constant temperature ($2.1 \times 10^8 \pm 2.82 \times 10^7$ SINV-GFP RNA copies; ANOVA, F = 27.16, $p = 3.48 \times 10^{-5}$). In replicate 2, heat-shocked bees had a 90% lower mean virus abundance ($4.9 \times 10^7 \pm 1.1 \times 10^7$ SINV-GFP RNA copies) compared to bees maintained at a constant temperature ($4.28 \times 10^8 \pm 1.28 \times 10^8$ RNA copies; ANOVA, F = 9.93, $p = 0.0058$). And, lastly, in replicate 3 heat-shocked bees had an 87% lower mean virus abundance ($1.84 \times 10^7 \pm 6.14 \times 10^6$ SINV RNA copies) compared to bees maintained at a constant temperature ($1.32 \times 10^8 \pm 5.4 \times 10^7$; ANOVA, F = 4.43, $p = 0.048$).

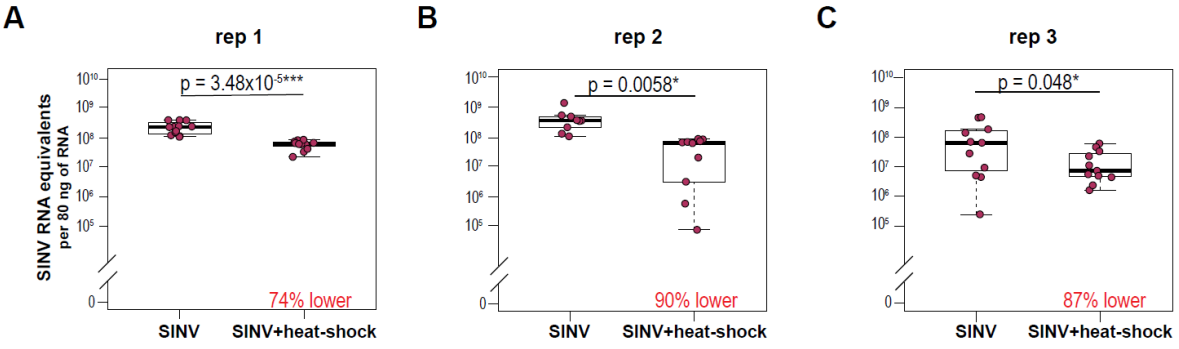


Figure 1. **Heat shock reduces virus abundance in adult honey bees.** Virus (SINV-GFP) abundance in individual honey bees at 72 h post-infection was assessed by qPCR. Data from three biological replicates with honey bees (n = 9–12) obtained from different colonies (rep 1, 2, 3) illustrate that bees subjected to heat shock (42 °C for 4 h) following SINV-GFP injection harbored less virus than bees maintained at 32 °C for the duration of the experiment. (**A**) Replicate 1, SINV-infected bees maintained at 32 °C had a mean viral abundance of $2.1 \times 10^8 \pm 2.8 \times 10^7$ (n = 12) SINV RNA copies, compared to $5.44 \times 10^7 \pm 6.72 \times 10^6$ (n = 11) SINV RNA copies in heat-shocked bees (ANOVA, F = 27.16, $p = 3.48 \times 10^{-5}$), (**B**) Replicate 2, SINV-infected bees maintained at 28˚C had a mean viral abundance of $4.28 \times 10^8 \pm 1.28 \times 10^8$ (n = 9) compared to the 90% lower mean virus abundance in heat-shocked bees of $4.9 \times 10^7 \pm 1.1 \times 10^7$ (n = 9)

SINV RNA copies (ANOVA, F = 9.93, $p = 0.0058$) and (**C**) Replicate 3, SINV injected bees had a mean viral abundance of 1.32×10^8.
Significance levels: * = p <0.05; ** = p <0.005; *** = p <0.0005.

3.2. Virus Infection and Heat Shock Induce the Expression of Heat Shock Protein Encoding Genes

In order to identify the heat shock proteins (HSPs) potentially involved in reducing virus levels in heat-shocked honey bees, qPCR was used to measure the expression of six candidate HSP-encoding genes 72 h post-injection in virus-infected bees relative to mock-infected (buffer-injected) bees that were either maintained at a constant temperature or exposed to heat shock post-infection (Figure 2, Figure S1). The panel of honey bee heat shock protein encoding genes examined in these experiments included several genes that exhibited increased expression in previous transcriptome level analyses of virus-infected bees (i.e., *protein lethal(2)essential for life-like, dnaj shv-like,* and *hsp83-like*) [61], a gene involved in the unfolded protein response (i.e., *hsc70-3*), and core heat-shock response genes (i.e., *hsc70-4*, *hsp83-like*, and *hsp90*) [71].

Protein lethal(2)essential for life-like (pl2) is a small heat shock protein that contains an Hsp20-domain. Furthermore, *pl2* was the sole differentially expressed gene common to five transcriptome studies of virus-infected bees [14,48,49,61,67], including symptomatic bees that were orally infected with Israeli acute paralysis virus (IAPV) [48], naturally IAPV-infected bees [14], bees infected with deformed wing virus and sacbrood virus via oral inoculation [49], SINV-GFP-infected bees [61], and *Varroa destructor* parasitized and/or virus-infected bees [67].

In this study, *pl2* expression was greater in SINV-GFP infected bees compared to mock-infected bees in two of the three biological replicates (rep1, 1.55 fold, $p = 5.1 \times 10^{-5}$; rep2, 10.59 fold, $p = 0.00013$) but not in replicate 3 ($p = 0.715$) (Figure 2, Figure S1). Heat shock of mock-infected bees also resulted in greater *pl2* expression in two of three biological replicates (i.e., rep1, 2.86 fold, $p = 5.1 \times 10^{-5}$; rep2, 2.24 fold, $p = 0.033$), but decreased expression in replicate 3 (0.52 fold, $p = 0.033$) relative to mock-infected bees maintained at a constant temperature. For the majority of the samples, combining both stressors, virus infection and heat shock, induced *pl2* gene expression. Specifically, *pl2* expression in virus-infected heat-shocked bees was higher relative to mock-infected bees maintained at a constant temperature in two biological replicates (rep1, 4.93 fold, $p = 0.00097$; rep2, 40.5 fold, $p = 0.00013$), but not in replicate 3 ($p = 0.715$). Similarly, *pl2* expression in dual-stressed bees was greater than virus-infected bees maintained at a constant temperature in two of the replicates (rep1, $p = 0.036$; rep2, $p = 0.00063$), but not in replicate 3 ($p = 0.715$).

Previous transcriptome analyses of virus-infected honey bees determined that the expression of an Hsp40 gene *dnaj shv-like* was greater in virus-infected bees relative to mock-infected bees [61]. Likewise, in the experiments described herein, *dnaj shv-like* expression was greater in virus-infected bees relative to mock-infected bees (rep1, 1.39 fold, $p = 9.2 \times 10^{-5}$; rep2, 3.67 fold $p = 0.00013$; rep3, 3.74 fold, $p = 5.4 \times 10^{-5}$) (Figure 2, Figure S1). Heat shock also induced *dnaj shv-like* expression in mock-infected bees (rep1, 2.99 fold, $p = 6.2 \times 10^{-5}$; rep2, 1.39 fold, $p = 0.00014$; rep3, 2.81 fold, $p = 5.4 \times 10^{-5}$). Additionally, viral infection in conjunction with heat shock resulted in increased expression of *dnaj shv-like* relative to mock-infected bees maintained at a constant

temperature (rep1, 1.46 fold, $p = 0.018$; rep2, 8.19 fold, $p = 0.00013$; rep3, 4.05 fold, $p = 5.4 \times 10^{-5}$). Combining stressors increased expression of *dnaj shv-like* over heat shock treatment alone in two replicates (rep2, $p = 0.0074$; rep3, $p = 0.0028$), in which virus-infected bees had an overall greater expression of this gene, and higher expression compared to heat-treated mock-infected bees (rep2, $p = 0.00025$; rep3, $p = 0.0023$). In contrast, mock-infected heat-shocked bees had greater *dnaj shv-like* expression in the first biological replicate.

Heat shock 70-kDa protein cognate 3 (*hsc70-3*) is a conserved endoplasmic reticulum chaperone that functions in the unfolded protein response [71,92-94]. It is induced in honey bees exposed to heat stress (i.e., 45 °C for 4 h) [71]. In *Drosophila melanogaster* S2 cells *hsc70-3* is required for effective RNA-interference [78]. In the honey bee experiments described herein, SINV-GFP infection induced expression of *hsc70-3* over mock-infection in all three biological replicates (i.e., rep1, 1.76 fold, $p = 6.8 \times 10^{-5}$; rep2, 3.07 fold, $p = 0.00013$; rep3, 2.66 fold, $p = 6.8 \times 10^{-5}$). Though virus infection alone induced *hsc70-3* expression, heat shock alone did not result in increased expression in the majority of the bees and was only observed in one replicate (rep 1, 2.57 fold, $p = 6.2 \times 10^{-5}$). However, honey bees that were both virus-infected and heat-shocked had greater *hsc70-3* expression relative to mock-infected heat-shocked bees (rep1, 1.59 fold, $p = 0.014$; rep2, 3.25 fold, $p = 0.00013$; rep3, 2.90 fold, $p = 6.8 \times 10^{-5}$) (Figure 2, Figure S1).

Heat shock 70-kDa protein cognate 4 (*hsc70-4*) is a core heat shock response gene that is induced by exposing honey bees to heat stress (i.e., 42 °C and 45 °C for 4 h) [71,95-97]. It is also an important chaperone for the assembly of the RNA-induced silencing complex (RISC) in *Drosophila* S2 cells and in flies [77,78]. Similar to previous studies, we determined that heat-

shocked honey bees exhibited increased expression of *hsc70-4* relative to bees maintained at a constant temperature (mock-infected heat-shocked vs. mock-infected: rep1, 3.63 fold change, $p = 5.1 \times 10^{-5}$; rep2, 1.43 fold change, $p = 0.00014$; rep3, 2.44 fold change, $p = 5.4 \times 10^{-5}$). Virus infection also induced *hsc70-4* expression in two of the three replicates (rep2, 1.44 fold, $p = 0.00013$; rep3, 2.61 fold, $p = 5.4 \times 10^{-5}$). Combining stressors resulted in considerable heterogeneity in *hsc70-4* expression. In replicate 1, *hsc70-4* expression in bees that were virus-infected and heat-shocked was similar to mock-infected bees ($p = 1.00$) and significantly lower than bees exposed to heat shock alone ($p = 4.4 \times 10^{-6}$). In replicate 2, virus-infected bees that were heat shocked had 3.8 fold higher *hsc70-4* expression than mock-infected bees ($p = 0.0013$) and had higher expression than virus-infected bees ($p = 0.0049$) and bees that were only heat shocked ($p = 0.0049$). In replicate 3, bees that received both stressors had 2.28 fold higher expression of *hsc70-4* compared to mock-infected bees ($p = 5.4 \times 10^{-5}$), but no differences in expression compared to bees that were only virus-infected or only heat-shocked (Figure 2, Figure S1).

Heat shock protein 83-like (*hsp83-like),* a core heat shock response gene, exhibited increased expression in SINV-GFP infected bees [61,71,95,96]. It is also the homologue of *hsp83,* another chaperone of the RISC assembly in *Drosophila melanogaster* [77]. As expected, *hsp83-like* expression was greater in virus-infected bees than in mock-infected bees in three replicates (rep1, 1.20 fold, $p = 0.038$; rep2, 2.31 fold, $p = 0.00012$; rep3, 2.37 fold, $p = 6.8 \times 10^{-5}$). In contrast, *hsp83-like* expression was induced by heat shock in replicate 1, and specifically mock-infected heat-shocked bees exhibited 1.75 greater expression than mock-infected bees maintained at a constant temperature ($p = 0.01$), but had reduced expression in replicate 2 (0.27 fold change, $p =$

0.00012) and a trend toward reduced expression in replicate 3 (0.69 fold change, $p = 0.05$). However, reduced *hsp83-like* expression in virus-infected heat-shocked bees compared to virus-infected bees maintained at a constant temperature was observed in all three replicates (rep1, $p = 0.0034$; rep2, $p = 0.00012$; rep3, $p = 0.00051$) (Figure 2, Figure S1).

Heat shock protein 90 (hsp90), another core heat-shock response gene, is induced in honey bees exposed to temperature stress (i.e., heat shock at 45 °C for 4 h) [71,95]. In this study, *hsp90* was consistently induced by viral infection (virus-infected vs. mock-infected bees, rep1, 1.24 fold, $p = 0.01$; rep2, 1.79 fold, $p = 0.00012$; rep3, 2.36 fold, $p = 5.4 \times 10^{-5}$). Heat shock resulted in higher *hsp90* expression relative to mock-infected bees in two of the three replicates (rep1, 3.59 fold, $p = 5.1 \times 10^{-5}$; rep3, 2.16 fold, $p = 5.4 \times 10^{-5}$), but expression was reduced in one replicate (rep2, 0.44 fold, $p = 0.00012$). Similar to the *hsc70-4* results, combining stressors resulted in considerable heterogeneity. *Hsp90* expression in honey bees that were both virus-infected and heat-shocked had mildly increased levels compared to mock-infected bees maintained at a constant temperature in replicate 1 (1.24 fold, $p = 0.057$), but had reduced expression in replicate 2 (0.68 fold, $p = 0.00012$) and increased expression in replicate 3 (2.55 fold, $p = 5.4 \times 10^{-5}$) (Figure 2, Figure S1).

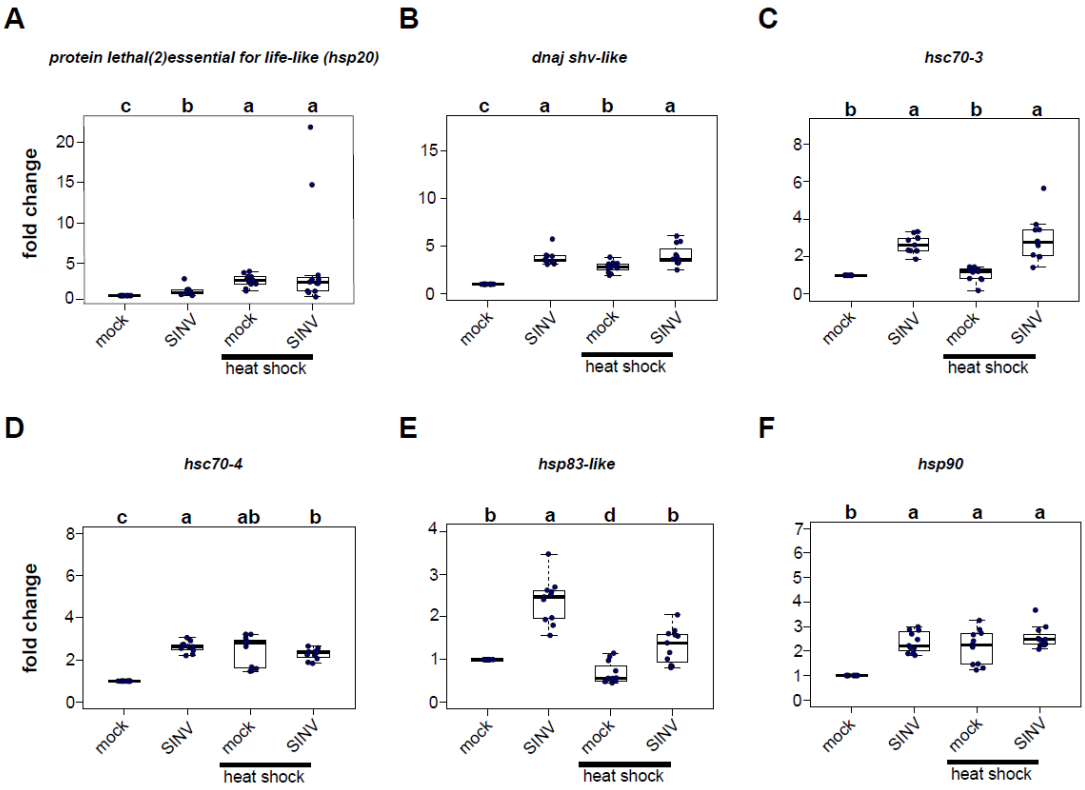


Figure 2. Induction of heat shock genes by virus infection and heat shock. The relative expression of honey bee heat shock protein encoding genes was assessed by qPCR using the ΔΔ*C*t method with normalization to *rpl8* and relative to mock-infected bees maintained at a constant temperature, unless otherwise stated. (**A**) *Protein lethal(2)essential for life-like (hsp20)* expression is increased by both viral infection and heat shock, including when stressors are combined. (**B**) *Dnaj shv-like* is increased in expression by both viral infection and heat shock, including when stressors are combined. (**C**) *Heat shock 70-kDa protein cognate 3 (hsc70-3)* expression was induced by viral infection, and in bees subjected to both stressors, but not by heat shock alone. (**D**) *Heat shock 70-kDa protein cognate 4 (hsc70-4)* expression was increased in all three treatment groups (i.e., virus infection, heat shock, and combined stressors). (**E**) *Heat shock protein 83-like* (*hsp83-like)* expression was induced by virus infection, while heat shock reduced expression in two out of three biological replicates. The combination of virus infection and heat shock also reduced *hsp83-like* expression relative to viral infection alone in all three replicates, indicating heat shock may suppress expression. (**F**) *Heat shock protein 90 (hsp90)* expression was increased in virus-infected bees, heat-shocked mock-infected bees, and by the combination of both stressors. Data were analyzed by a pairwise Wilcoxon Rank Sums with a Benjamini–Hochberg correction for multiple comparisons within replicates (n = 9–12 bees for each sample group). Shared letters above two treatments denote there is no difference while different letters denote a statistical difference. Figure 2 includes results from a representative biological replicate

for each gene (i.e., rep1 for panel **A**, and rep3 for **B**–**F**). The data for all three biological replicates are presented in Figure S1.

3.3. Impact of Heat Shock on Expression of Honey Bee Antiviral Defense Genes

To discern whether the reduced virus abundance in heat-shocked honey bees was due to higher expression of honey bee immune genes, we assessed the relative expression of three honey bee antiviral defense genes (i.e., *mf116383, dcr-like*, and *ago2*, Figure 3) at 72 h post-infection [37,51,61]. These genes were identified in previous transcriptome level analyses that determined that their expression was greater in SINV-GFP infected honey bees [61]. Furthermore, the antiviral role of *dcr-like* and *mf116383* was confirmed in vivo [61]. Increased expression of *dcr-like* and *ago2* was also reported in transcriptome level analyses of IAPV-infected bees [48]. However, transcription of these key RNAi genes was not induced by deformed wing virus infection in honey bees [50] or virus infection of the model insect, *Drosophila melanogaster* [98-101]. *Mf116383* was not evaluated in these studies, as it was not well-annotated prior to 2017.

We identified and annotated the honey bee gene *mf116383* (GenBank) due to its increased expression in SINV-GFP infected bees relative to mock-infected bees and confirmed its role in limiting virus infection in honey bees [61]. Though the specific mechanism by which *mf116383* reduces virus infection is unknown, it was originally referred to as a probable cyclin-dependent serine/threonine-protein kinase based on prior partial annotation of LOC725387 (XM_001121241.4) [102-104]. In this study, *mf116383* was consistently increased in expression in virus-infected honey bees (rep1, 1.65 fold, $p = 4.1 \times 10^{-5}$; rep2, 4.76 fold, $p = 0.00015$; rep 3, 3.90 fold change, $p = 6.8 \times 10^{-5}$) (Figure 3A). *Mf116383* expression was also induced in mock-

infected bees exposed to heat shock relative to mock-infected bees that were kept at a constant temperature (rep1, 1.86 fold, $p = 4.1 \times 10^{-5}$; rep2, 1.78 fold, $p = 0.006$; rep3, 2.66 fold, $p = 0.0015$). This is intriguing since virus-infected bees that were heat shocked harbored less virus than bees maintained at constant temperature post virus infection (Figure 1). Virus infection coupled with heat shock resulted in increased *mf116383* expression in two replicates relative to expression in mock-infected bees held at constant temperature (rep1, 3.5 fold, $p = 4.1 \times 10^{-5}$; rep3, 3.42 fold, $p = 6.8 \times 10^{-5}$), but showed no difference in replicate 2 (Figure 3A). However, a comparison of dual-stressed honey bees compared to mock-infected heat-shocked bees determined that *mf116383* expression was only appreciably greater in one replicate (rep1, $p = 0.0012$) and trended toward higher expression in another replicate (rep3, $p = 0.056$) (Figure 3A).

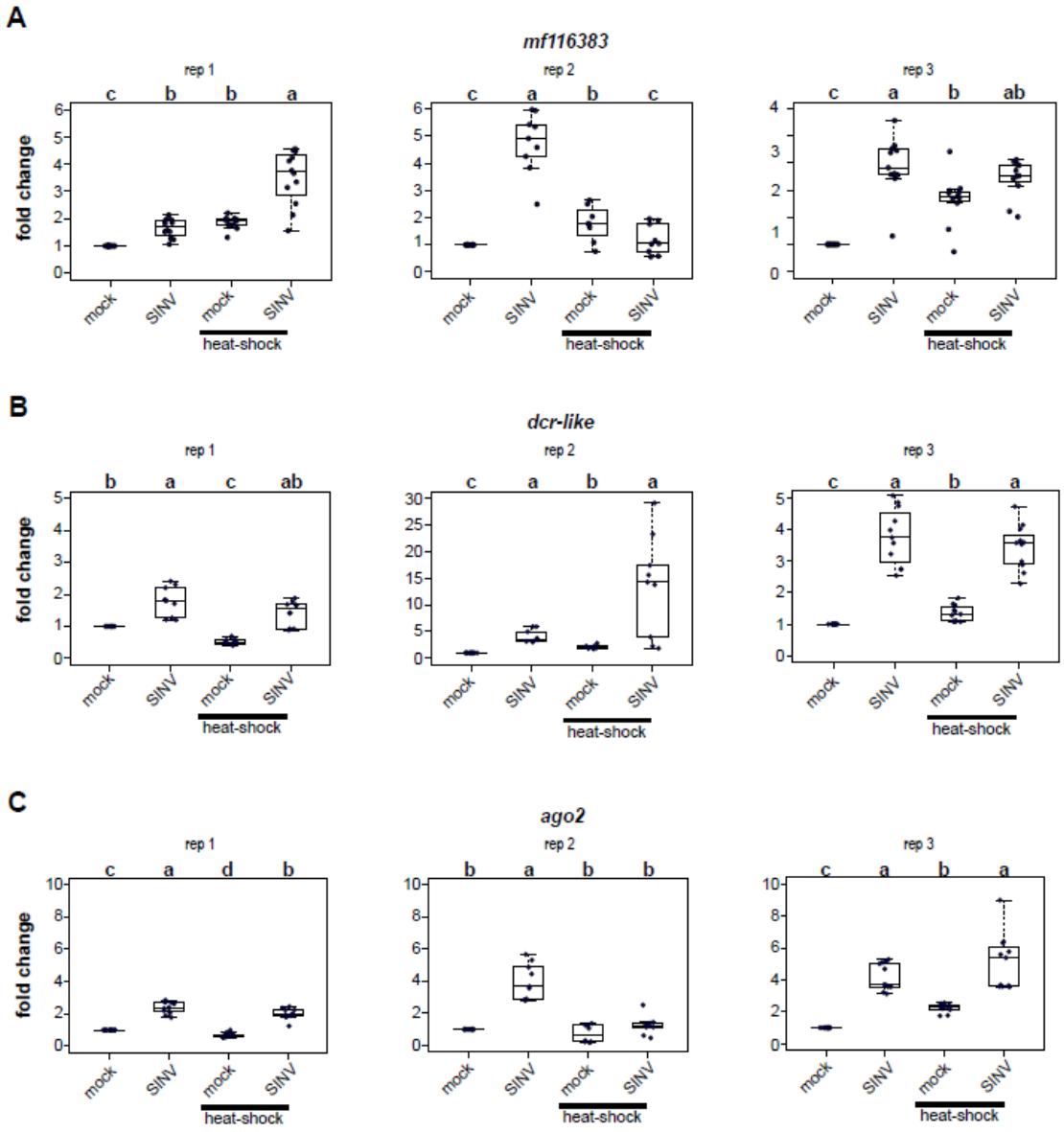


Figure 3. Effect of virus infection and heat shock on honey bee antiviral gene expression. The relative expression of three honey bee antiviral genes was assessed using qPCR using the ΔΔ*C*t method with normalization to *rpl8* and relative to expression in mock-infected bees kept at constant temperature. (**A**) *Dicer (dcr)-like* expression in virus-infected honey bees was consistently increased, while heat shock resulted in decreased expression in replicate 1 and increased expression in replicates 2 and 3. (**B**) Similarly, *ago2* expression was consistently increased in virus-infected bees relative to mock-infected bees, but heat shock resulted in a variable response. (**C**) *Mf116383* expression was consistently induced in virus-infected bees relative to those that were mock-infected. *Mf116383* was the only honey bee antiviral gene that

exhibited increased expression in mock-infected heat shocked bees relative to mock-infected bees kept at a constant temperature in all three replicates (n = 9–12 for each group). All pairwise comparisons were analyzed by a Wilcoxon rank sums test with a Benjamini–Hochberg correction for multiple comparisons. Shared letters above two treatments denote no difference while different letters denote a statistical difference, and p-values for specific comparisons are reported in the text.

Dcr-like is an RNA helicase domain-containing endonuclease that recognizes dsRNA and processes it into short 21–22 nucleotide double-stranded short interfering RNAs (siRNAs) [45,105,106]. In this study, *dcr-like* expression was increased in virus-infected honey bees (rep1, 1.78 fold, $p = 0.00032$; rep2, 4.09 fold, $p = 0.00013$; rep3, 3.76 fold, $p = 2.7 \times 10^{-5}$) (Figure 3B). *Dcr-like* expression was also greater in mock-infected heat shocked honey bees compared to mock-infected bees maintained at constant temperature in two replicates (rep2, 2.11 fold, $p = 0.00014$; rep3, 1.35 fold, $p = 2.7 \times 10^{-5}$), but it was decreased in one replicate (rep1, 0.52 fold, $p = 0.00032$). Similarly, combining stressors resulted in increased expression compared to mock-infected bees in two replicates (rep2, 13.5 fold, $p = 0.00013$; rep3, 3.45 fold, $p = 2.7 \times 10^{-5}$), but not in replicate 1 ($p = 0.115$) (Figure 3B).

Ago2 is the core endonuclease of the RISC, which when loaded with single-stranded siRNA targets complementary sequences in viral or cellular RNAs for cleavage [45,105,107]. As in previous studies, *ago2* expression was greater in virus-infected bees compared to mock-infected bees (rep1, 2.39 fold, $p = 4.1 \times 10^{-5}$; rep2, 4.0 fold, $p = 0.00015$; rep3, 4.18 fold, $p = 2.7 \times 10^{-5}$) (Figure 3C) [61]. Likewise, the expression of *ago2* was increased in virus-infected and heat-shocked bees compared to mock-infected bees held at constant temperature in two replicates (rep 1, 1.99 fold, $p = 4.1 \times 10^{-5}$; rep3, 5.13 fold, $p = 2.7 \times 10^{-5}$) (Figure 3C). However, *ago2* expression in heat-shocked mock-infected bees compared to mock-infected bees held at constant temperature was

variable (i.e., rep1, 0.68 fold reduction, $p = 4.1 \times 10^{-5}$; rep2, no difference, $p = 1.00$; rep3, 2.23 fold increase, $p = 2.7 \times 10^{-5}$).

3.4. Honey Bee Immune Gene and Heat Shock Protein Gene Expression Positively Correlate

Heat shock proteins play important roles in the insect antiviral response. Specifically, *hsp90* and *hsc70-4* act as chaperones for RISC assembly, thereby facilitating RNAi-mediated antiviral defense [77,78]. Therefore, to examine potential co-regulation of immune genes with HSP-encoding genes, a Pearson's correlation coefficient matrix was calculated for each gene measured in this study, including data from four treatment groups (i.e., mock-infected, SINV-GFP-infected, mock-infected and heat shocked, and SINV-GFP-infected and heat shocked) with nine to twelve bees per biological replicate. In this analysis, *ago2* expression was positively correlated with the expression of three HSP-encoding genes, including *hsp83-like* ($r = 0.41$, $p = 0.02$), *hsc70-3* ($r = 0.29$, $p < 0.0001$) and *hsp90* ($r = 0.27$, $p = 0.01$) (Figure 4). *Dcr-like* expression was positively correlated with the expression of four HSP-encoding genes, including *pl2* ($r = 0.46$, $p < 0.0001$), *hsc70-3* ($r = 0.65$, $p < 0.0001$), *hsc70-4* ($r = 0.54$, $p < 0.0001$), and *dnaj shv-like* ($r = 0.89$, $p < 0.0001$). Lastly, *mf116383* expression positively correlated with the expression of two HSP-encoding genes, including *hsp83-like* ($r = 0.46$, $p < 0.0001$) and *hsp90* ($r = 0.24$, $p = 0.02$) (Figure 4 and Figure S2).

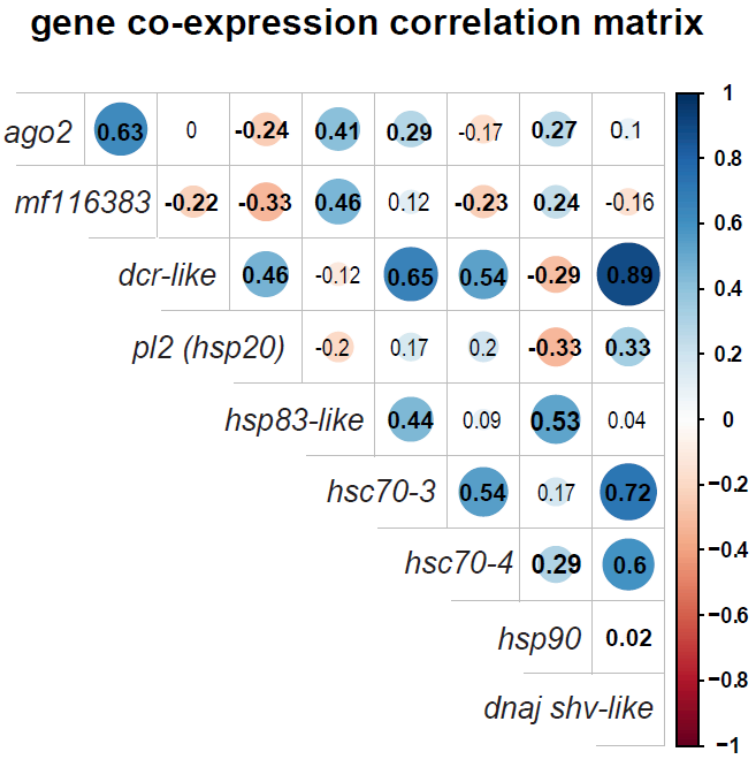


Figure 4. Honey bee gene co-expression correlation matrix. The co-expression of genes in virus-infected heat-shocked honey bees was analyzed by calculating the correlation coefficient for each pairwise comparison, which are listed in each cell. Correlation coefficients (*r*-values) quantify the strength and direction of the relationship between the expression of two genes. Shaded blue circles represent positive correlations while shaded red circles denote negative correlations. The larger the circle, the stronger the association and statistically significant correlations ($p < 0.05$) are indicated by a bold *r*-value. The matrix illustrates several significant positive correlations between immune genes (*dcr-like*, *ago2,* and *mf116383*) and heat shock proteins (*pl2, hsp83-like, hsc70-3, hsc70-4, hsp90, dnaj shv-like*), including *dcr-like* and *dnaj shv-like*, *hsc70-3* and *dnaj shv-like*, and *dcr-like* and *hsc70-3*.

3.5. Increased Expression of Heat Shock Protein Encoding Genes Post Virus Infection Is Not Completely Recapitulated by dsRNA-Treatment

To determine whether dsRNA, a virus-associated molecular pattern (VAMP), is necessary and sufficient to induce heat shock protein gene expression, honey bees were either injected with buffer

or with buffer containing 1 μg of dsRNA, with sequence corresponding to the Drosophila C virus genome, and thus not specific to any honey bee gene or honey bee-infecting virus. As a control, we assessed the expression of *mf116383*, which is induced by dsRNA in a previous study [61], and, indeed, expression was induced at 72 h post dsRNA-injection (rep1, 3.29 fold, $p = 1.36 \times 10^{-5}$; rep2, 1.20 fold, $p = 0.0014$) (Figure 5). Likewise, the expression of five HSP-encoding genes 72 h post dsRNA-injection was measured by qPCR and the results varied for each gene assessed.

Specifically, dsRNA-treated bees exhibited greater expression of *hsc70-3* (rep1, 1.15 fold, $p = 0.05$; rep2, 1.30 fold, $p = 6.29 \times 10^{-5}$), and *hsp83-like* in replicate 1 (2.91 fold, $p = 1.4 \times 10^{-5}$), but not in replicate 2 ($p = 0.51$). In contrast, dsRNA-treated bees exhibited reduced expression of *dnaj shv-like* (rep1, 0.91 fold, $p = 0.0059$; rep2, 0.66 fold, $p = 6.29 \times 10^{-5}$) and *hsc70-4* in one of two replicates (rep2, 0.66 fold, $p = 6.34 \times 10^{-5}$). Injection with dsRNA had a variable impact on *hsp90* expression, increasing it 1.17-fold in the first replicate ($p = 0.00038$) and decreasing 0.83-fold in the second replicate ($p = 8.46 \times 10^{-5}$). Together, these results indicate that the HSP expression profile induced by virus infection is not completely recapitulated by exposure to dsRNA. Instead, there are likely other aspects of the virus-honey bee host interaction that result in differential regulation of genes in the heat shock stress response pathway.

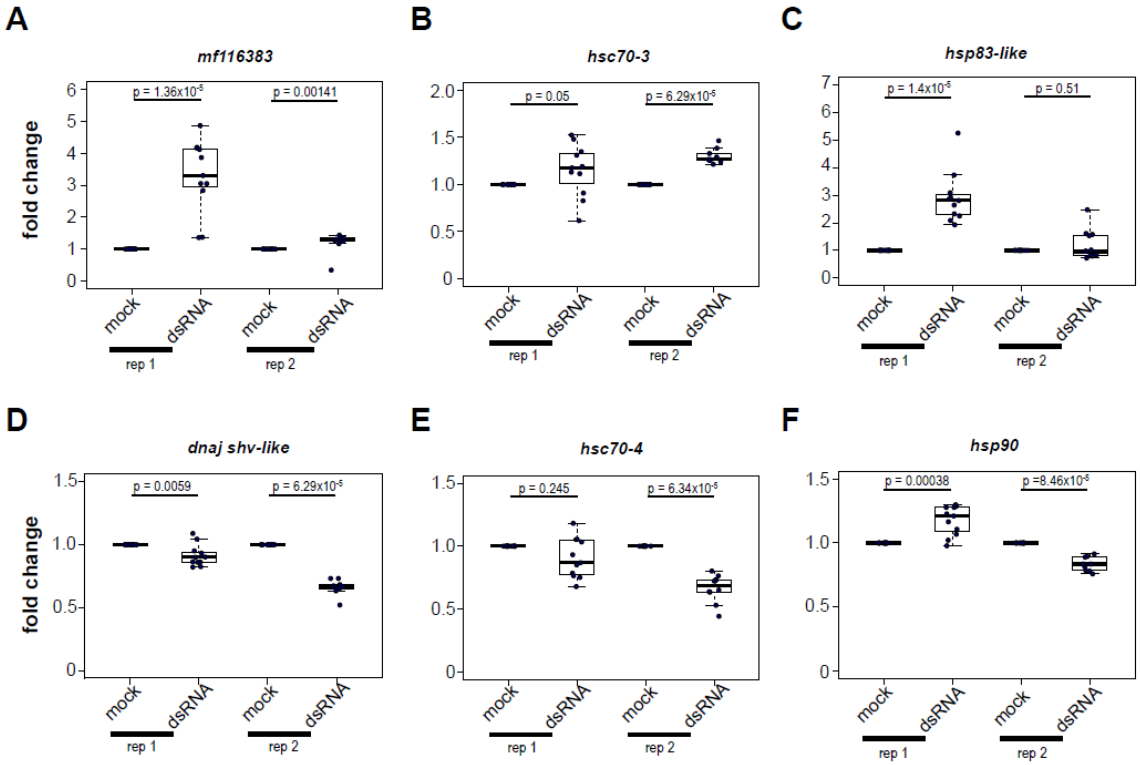


Figure 5. Treatment of honey bees with dsRNA, a virus-associated molecular pattern, differentially impacts heat shock protein encoding gene expression. To examine the impact of dsRNA, a virus-associated molecular pattern, on honey bee gene expression, bees were intrathoracically injected with dsRNA or buffer (n = 10–12 bees for each sample group). The relative expression of heat shock protein encoding genes was assessed by qPCR using the ΔΔCt method with normalization to the internal control gene *rpl8* and relative to mock-infected bees and pairwise comparisons were analyzed by a Wilcoxon rank sums test. (**A**) *MF116383* expression was greater in dsRNA-treated honey bees for both replicates. (**B**) *Hsc70-3* expression was greater in dsRNA-treated bees in both biological replicates. (**C**) *Hsp83-like* in dsRNA treated bees was increased in expression in replicate 1, but not in replicate 2. (**D**) *Dnaj shv-like* expression was reduced in dsRNA-treated bees in both biological replicates. (**E**) *Hsc70-4* expression was reduced in dsRNA-treated bees replicate 2, but not in replicate 1. (**F**) *Hsp90* expression in dsRNA-treated bees was variable with greater expression in replicate 1 and lower expression in replicate 2.

4. Discussion

Honey bees have evolved a wide range of social and molecular strategies to control pathogens [37-39,51,108]. Viruses are associated with honey bee colony losses and individual mortality [14,20,22,24-27,47-50]. The outcomes of viral infections in individual honey bees are primarily governed by cellular immune responses, which include dsRNA-triggered immune responses (i.e., RNAi and non-sequence specific dsRNA mediated antiviral mechanisms) [14,46,48,50,59-61,109,110] and canonical immune signaling pathways, including the JAK/STAT, JNK, and Imd pathways, and the NF-κB/Dorsal mediated Toll pathway, which has been further characterized in vivo [20,111]. In addition, in several insect species behavioral fever reduces parasitic burden in individuals (reviewed in [112]). In honey bees, a group-level behavior termed "social fever" is hypothesized to function as a colony-level immune response. One study determined that colony temperature was increased in response to inoculation with fungal spores [40], although subsequent pathogen loads were not evaluated and the response was not consistently observed, perhaps due to environmental conditions [113]. Therefore, though "social fever" is an interesting aspect of honey bee biology, it requires further investigation and has not been observed in response to viral pathogens.

The mechanisms and genes involved in honey bee antiviral responses require further investigation. Herein, we present evidence that the heat shock response is involved in honey bee antiviral defense. First, we demonstrated that heat shock (i.e., exposure to 42 °C for 4 h) reduced the abundance of the model virus, SINV-GFP, compared to bees maintained at a constant temperature. One hypothesis that might explain the 74–90% reduction in virus abundance in heat-shocked

honey bees is that there is a general disruption of protein synthesis in heat-stressed bees. However, drosophila cells completely recover normal protein synthesis upon return to physiologically normal temperatures after heat shock (four treatments at 37 °C for 25 min each) [114]. Furthermore, honey bee thoraces can reach in excess of 45 °C during foraging and aggression, suggesting they are adapted to cope with these high temperatures for short durations of time [115-118]. Therefore, disruption of protein synthesis is unlikely to be sufficient to explain the near log-reduction in viral abundance in heat-shocked bees at 72 h post-infection. Instead, it is likely that the transcriptional regulation of heat shock proteins is at least partially responsible for the antiviral effect of heat shock.

The expression of most of the heat shock protein encoding genes examined in this study was induced by virus infection in three biological replicates, except *protein lethal(2) essential for life-like* and *hsc70-4*, which were induced in two biological replicates. Though in general the expression of heat shock protein genes was induced by heat-treatment alone or with the combination of both stressors, there was some heterogeneity in expression in these treatment groups. These differences could be explained by either stochasticity, or by real differences in independent biological replicates for which we used three separate outbred honey bee colonies. These colonies include individual half-sisters of different genetic lineages that likely represent several different genetic sub-species prevalent in the U.S. [119]. Indeed, different honey bee subspecies (i.e., *A. mellifera carnica* and *A. mellifera ligustica*) have different thermotolerances and metabolic responses to heat stress [120]. Therefore, variation in honey bee genetic lineages could result in differential transcriptional regulation in response to heat shock. In addition, the

biological replicates were carried out at different dates across two summers (i.e., June 2018, August 2018, and July 2019) when all the colonies were actively rearing brood, though there may have been variation in the physiological states of the colonies due to differences in weather and forage availability.

Virus infection induces the expression of numerous honey bee immune genes [14,46,48,49,61,67]. Similar to our previous study that examined the honey bee transcriptional response to SINV-GFP infection [61], we determined that virus infection resulted in increased expression of honey bee immune genes including *ago-2*, *dcr-like*, and *mf116383* [61]. However, *mf116383* was the only gene consistently induced by heat-treatment alone. Therefore, these data reveal a novel aspect of this recently described immune gene and suggests that *mf116383* may serve as one point of cross-talk between the generalized antiviral immune response and the heat shock response in honey bees. Since only ~35% of the honey bee genome has well-annotated orthologues with genes in other species including *Drosophila melanogaster*, there are numerous uncharacterized genes, like *mf116383*. It is exciting to further understand the biological role(s) of these genes in honey bees and other model and non-model organisms.

In contrast to the impact of heat shock on *mf116383* expression, heat shock had a variable effect on the expression of RNAi machinery across replicates. In some cases, the expression of *dcr-like* and *ago2* was reduced in heat shocked honey bees when compared to honey bees that were maintained at 32 °C. This implies that the mitigating effect of heat-treatment on virus infection is not simply explained by greater expression of the RNAi machinery. Instead, the protective effect of HSPs may be in part due to more efficient chaperone-mediated loading of the RISC [77,78].

Increased availability of chaperone proteins following heat shock may also ensure there are chaperones available for host-proteins as opposed to being occupied by viral proteins [79,80]. In addition to the expression of heat shock protein encoding genes correlating with each other, as expected, the expression of some HSP-encoding genes (e.g., *hsc70-3*, *hsc70-4,* and *hsp90*) was positively correlated with *dcr-like* and *ago2*. This suggests co-regulation of these genes. Though further studies are needed to determine the mechanisms leading to co-regulation of immune genes and HSP-encoding genes, it may be advantageous to co-regulate HSPs and HSP client proteins [77]. In addition to HSPs serving as chaperones for RNAi proteins, they may also play a direct antiviral role by interacting with viral proteins or may participate in, or mediate, broader stress-response pathways involved in antiviral defense.

The majority of viruses produce dsRNA molecules during their replication cycle (i.e., replicative intermediates of ssRNA viruses and secondary RNA structures, including internal ribosomal entry sites (IRES) and tRNA-like structures). Therefore, most host organisms have evolved mechanisms to detect dsRNA and subsequently trigger antiviral defense pathways. As expected based on a previous transcriptome analysis [61], *mf116383* expression was induced by dsRNA in the experiments described herein. Intriguingly, dsRNA-treatment did not fully recapitulate the HSP gene expression pattern that was observed in virus-infected bees. For example, *dnaj shv-like* and *hsc70-4* were both consistently induced by viral infection, but they had reduced expression in bees exposed to dsRNA alone. In contrast, the expression of both *hsc70-3* and *hsp83-like* was increased in virus-infected bees and dsRNA-treated bees. It is unclear which protein might be mediating transcriptional regulation of heat shock protein encoding genes in response to dsRNA, but it may

be a protein like the mammalian dsRNA-dependent Protein kinase R (PKR), which is essential for the murine heat shock response and the expression of *hsp70* and *hsp84* [121]. Alternatively, an unidentified DEAD box helicase domain-containing protein, like those involved in cytosolic detection of dsRNA in mammalian cells (e.g., RIG-I, LGP2, MDA-5) may be mediating this response [122,123] (reviewed in [124]). There is some precedence for DEAD box helicase domain-containing proteins regulating gene expression. For example, the DEAD/H-box helicase Dicer-2 detects dsRNA and regulates the expression of the secreted antiviral peptide Vago in drosophila and mosquitoes [101,125,126]. Future studies will identify which honey bee protein or proteins are mediating differential expression of heat shock protein encoding genes in virus infection.

In summary, the work described herein indicates that stress response proteins, including those in the heat shock response and proteostasis network, are involved in honey bee antiviral defense. Further biochemical analyses are needed to confidently demonstrate their role in antiviral defense and the protective effect of heat shock. Future studies will aim to identify modes of coordination between stress and immune response pathways and proteins, as well as other potential antiviral functions of heat shock proteins, such as direct interaction with viral proteins.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1

Author Contributions: Conceptualization, A.J.M. and M.L.F.; methodology, A.J.M., K.F.D., M.L.F.; formal analysis A.J.M., K.F.D., and M.L.F.; investigation, A.J.M., K.F.D., and M.L.F.; resources, M.L.F.; data curation, A.J.M., K.F.D. and M.L.F.; writing—original draft preparation, A.J.M. and M.L.F.; writing—review and editing, A.J.M., K.F.D., and M.L.F.; visualization, A.J.M., and M.L.F.; supervision, M.L.F.; project administration, M.L.F.; funding acquisition, M.L.F. All authors have read and agreed to the published version of the manuscript.

Funding: The Flenniken laboratory is supported by the National Science Foundation CAREER Program (Award# 1651561), the United States Department of Agriculture

National Institute of Food and Agriculture, Agriculture and Food Research Initiative (USDA-NIFA-AFRI) program, Montana Department of Agriculture Specialty Crop Block Grant Program, the National Institutes of Health IDeA Program COBRE grant GM110732, Hatch Multistate Funding (NC-1173), and Project Apis m.-Costco Scholar Fellowship Program for Honey Bee Health. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Acknowledgments: The authors would like to thank members of the Flenniken Laboratory for discussions throughout these experiments and for valuable editing and feedback on this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gallai, N.; Salles, J.-M.; Settele, J.; Vaissière, B.E. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological Economics **2009**, 68, 810-821, doi:10.1016/j.ecolecon.2008.06.014.

2. Eilers, E.J.; Kremen, C.; Smith Greenleaf, S.; Garber, A.K.; Klein, A.-M. Contribution of Pollinator-Mediated Crops to Nutrients in the Human Food Supply. PLOS ONE **2011**, 6, e21363, doi:10.1371/journal.pone.0021363.

3. Klein, A.M.; Vaissiere, B.E.; Cane, J.H.; Steffan-Dewenter, I.; Cunningham, S.A.; Kremen, C.; Tscharntke, T. Importance of pollinators in changing landscapes for world crops. Proc Biol Sci **2007**, 274, 303-313, doi:10.1098/rspb.2006.3721.

4. Calderone, N.W. Insect pollinated crops, insect pollinators and US agriculture: Trend analysis of aggregate data for the period 1992-2009. PLoS ONE **2012**, 7, 24-28, doi:10.1371/journal.pone.0037235.

5. vanEngelsdorp, D.; Hayes, J.; Underwood, R.; Pettis, J. A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. Journal of Apicultural Research **2010**, 49, 7, doi:10.3896/IBRA.1.49.1.03.

6. Chauzat, M.-P.; Jacques, A.; Laurent, M.; Bougeard, S.; Hendrikx, P.; Ribière-Chabert, M.; De Graaf, D.; Méroc, E.; Nguyen, B.K.; Roelandt, S., et al. Risk indicators affecting honeybee colony survival in Europe: one year of surveillance. Apidologie **2016**, 47, 348-378, doi:10.1007/s13592-016-0440-z.

7. Seitz, N.; Traynor, K.S.; Steinhauer, N.; Rennich, K.; Wilson, M.E.; Ellis, J.D.; Rose, R.; Tarpy, D.R.; Sagili, R.R.; Caron, D.M., et al. A national survey of managed honey bee 2014–2015 annual colony losses in the USA. Journal of Apicultural Research **2015**, 54, 292-304, doi:10.1080/00218839.2016.1153294.

8. Kulhanek, K.; Steinhauer, N.; Rennich, K.; Caron, D.M.; Sagili, R.R.; Pettis, J.S.; Ellis, J.D.; Wilson, M.E.; Wilkes, J.T.; Tarpy, D.R., et al. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. Journal of Apicultural Research **2017**, 56, 328-340, doi:10.1080/00218839.2017.1344496.

9. Lee, K.V.; Steinhauer, N.; Rennich, K.; Wilson, M.E.; Tarpy, D.R.; Caron, D.M.; Rose, R.; Delaplane, K.S.; Baylis, K.; Lengerich, E.J., et al. A national survey of managed honey bee 2013-2014 annual colony losses in the USA. Apidologie **2015**, 46, 292-305, doi:10.1007/s13592-015-0356-z.

10. Traynor, K.S.; Rennich, K.; Forsgren, E.; Rose, R.; Pettis, J.; Kunkel, G.; Madella, S.; Evans, J.; Lopez, D.; vanEngelsdorp, D. Multiyear survey targeting disease incidence in US honey bees. Apidologie **2016**, 47, 325-347, doi:10.1007/s13592-016-0431-0.

11. Genersch, E.; Von Der Ohe, W.; Kaatz, H.; Schroeder, A.; Otten, C.; Büchler, R.; Berg, S.; Ritter, W.; Mühlen, W.; Gisder, S., et al. The German bee monitoring project: A long term study to understand periodically high winter losses of honey bee colonies. Apidologie **2010**, 41, 332-352, doi:10.1051/apido/2010014.

12. Cornman, R.S.; Tarpy, D.R.; Chen, Y.; Jeffreys, L.; Lopez, D.; Pettis, J.S.; VanEngelsdorp, D.; Evans, J.D. Pathogen webs in collapsing honey bee colonies. PLoS ONE **2012**, 7, e43562, doi:10.1371/journal.pone.0043562.

13. McMenamin, A.J.; Brutscher, L.M.; Glenny, W.; Flenniken, M.L. Abiotic and biotic factors affecting the replication and pathogenicity of bee viruses. Current Opinion in Insect Science **2016**, 16, 14-21, doi:10.1016/j.cois.2016.04.009.

14. Chen, Y.P.; Pettis, J.S.; Corona, M.; Chen, W.P.; Li, C.J.; Spivak, M.; Visscher, P.K.; DeGrandi-Hoffman, G.; Boncristiani, H.; Zhao, Y., et al. Israeli Acute Paralysis Virus: Epidemiology, Pathogenesis and Implications for Honey Bee Health. PLOS Pathogens **2014**, 10, e1004261, doi:10.1371/journal.ppat.1004261.

15. Daughenbaugh, K.F.; Martin, M.; Brutscher, L.M.; Cavigli, I.; Garcia, E.; Lavin, M.; Flenniken, M.L. Honey bee infecting Lake Sinai viruses. Viruses **2015**, 7, 3285-3309, doi:10.3390/v7062772.

16. van der Zee, R.; Pisa, L.; Andonov, S.; Brodschneider, R.; Charrière, J.-D.; Chlebo, R.; Coffey, M.F.; Crailsheim, K.; Dahle, B.; Gajda, A., et al. Managed honey bee colony losses in Canada, China, Europe, Israel and Turkey, for the winters of 2008–9 and 2009–10. Journal of Apicultural Research **2012**, 51, 100-114, doi:10.3896/IBRA.1.51.1.12.

17. Antúnez, K.; Anido, M.; Branchiccela, B.; Harriet, J.; Campa, J.; Invernizzi, C.; Santos, E.; Higes, M.; Martín-Hernández, R.; Zunino, P. Seasonal Variation of Honeybee Pathogens and

its Association with Pollen Diversity in Uruguay. Microbial Ecology **2015**, 70, 522-533, doi:10.1007/s00248-015-0594-7.

18. Budge, G.E.; Pietravalle, S.; Brown, M.; Laurenson, L.; Jones, B.; Tomkies, V.; Delaplane, K.S. Pathogens as Predictors of Honey Bee Colony Strength in England and Wales. PLOS ONE **2015**, 10, e0133228, doi:10.1371/journal.pone.0133228.

19. Granberg, F.; Vicente-Rubiano, M.; Rubio-Guerri, C.; Karlsson, O.E.; Kukielka, D.; Belák, S.; Sánchez-Vizcaíno, J.M. Metagenomic Detection of Viral Pathogens in Spanish Honeybees: Co-Infection by Aphid Lethal Paralysis, Israel Acute Paralysis and Lake Sinai Viruses. PLoS ONE **2013**, 8, e57459, doi:10.1371/journal.pone.0057459.

20. Nazzi, F.; Brown, S.P.; Annoscia, D.; Del Piccolo, F.; Di Prisco, G.; Varricchio, P.; Vedova, G.D.; Cattonaro, F.; Caprio, E.; Pennacchio, F. Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. PLoS Pathogens **2012**, 8, doi:10.1371/journal.ppat.1002735.

21. Porrini, C.; Mutinelli, F.; Bortolotti, L.; Granato, A.; Laurenson, L.; Roberts, K.; Gallina, A.; Silvester, N.; Medrzycki, P.; Renzi, T., et al. The Status of Honey Bee Health in Italy: Results from the Nationwide Bee Monitoring Network. PLOS ONE **2016**, 11, e0155411, doi:10.1371/journal.pone.0155411.

22. McMenamin, A.J.; Genersch, E. Honey bee colony losses and associated viruses. Current Opinion in Insect Science **2015**, 8, 121-129, doi:10.1016/j.cois.2015.01.015.

23. Di Prisco, G.; Cavaliere, V.; Annoscia, D.; Varricchio, P.; Caprio, E.; Nazzi, F.; Gargiulo, G.; Pennacchio, F. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. Proceedings of the National Academy of Sciences of the United States of America **2013**, 110, 18466-18471, doi:10.1073/pnas.1314923110.

24. Dainat, B.; Evans, J.D.; Chen, Y.P.; Gauthier, L.; Neumann, P. Dead or alive: deformed wing virus and Varroa destructor reduce the life span of winter honeybees. Appl Environ Microbiol **2012**, 78, 981-987, doi:10.1128/AEM.06537-11.

25. Dainat, B.; Evans, J.D.; Chen, Y.P.; Gauthier, L.; Neumann, P. Predictive markers of honey bee colony collapse. PloS one **2012**, 7, e32151, doi:10.1371/journal.pone.0032151.

26. Dainat, B.; Neumann, P. Clinical signs of deformed wing virus infection are predictive markers for honey bee colony losses. Journal of invertebrate pathology **2013**, 112, 278-280, doi:10.1016/j.jip.2012.12.009.

27. Brutscher, L.M.; McMenamin, A.J.; Flenniken, M.L. The buzz about honey bee viruses. PLoS Pathogens **2016**, 12, e1005757, doi:10.1371/journal.ppat.1005757.

28. Grozinger, C.M.; Flenniken, M.L. Bee Viruses: Ecology, Pathogenicity, and Impacts. Annual Review of Entomology **2019**, 64, 205-226, doi:10.1146/annurev-ento-011118-111942.

29. Olivier, V.; Blanchard, P.; Chaouch, S.; Lallemand, P.; Schurr, F.; Celle, O.; Dubois, E.; Tordo, N.; Thiéry, R.; Houlgatte, R., et al. Molecular characterisation and phylogenetic analysis of Chronic bee paralysis virus, a honey bee virus. Virus Research **2008**, 132, 59-68, doi:https://doi.org/10.1016/j.virusres.2007.10.014.

30. Bigot, D.; Dalmon, A.; Roy, B.; Hou, C.; Germain, M.; Romary, M.; Deng, S.; Diao, Q.; Weinert, L.A.; Cook, J.M., et al. The discovery Halictivirus resolves the Sinaivirus phylogeny. Journal of General Virology **2017**, 98, 2864-2875, doi:10.1099/jgv.0.000957.

31. Cornman, R.S. Relative abundance and molecular evolution of Lake Sinai Virus (Sinaivirus) clades. PeerJ **2019**, 7, e6305, doi:https://doi.org/10.7717/peerj.6305.

32. McMenamin, A.J.; Flenniken, M.L. Recently identified bee viruses and their impact on bee pollinators. Current Opinion in Insect Science **2018**, 26, 120-129, doi:https://doi.org/10.1016/j.cois.2018.02.009.

33. Singh, R.; Levitt, A.L.; Rajotte, E.G.; Holmes, E.C.; Ostiguy, N.; Vanengelsdorp, D.; Lipkin, W.I.; Depamphilis, C.W.; Toth, A.L.; Cox-Foster, D.L. RNA viruses in hymenopteran pollinators: evidence of inter-Taxa virus transmission via pollen and potential impact on non-Apis hymenopteran species. PloS one **2010**, 5, e14357, doi:10.1371/journal.pone.0014357.

34. Mazzei, M.; Carrozza, M.L.; Luisi, E.; Forzan, M.; Giusti, M.; Sagona, S.; Tolari, F.; Felicioli, A. Infectivity of DWV associated to flower pollen: experimental evidence of a horizontal transmission route. PLoS One **2014**, 9, e113448, doi:10.1371/journal.pone.0113448.

35. Chen, Y.P.; Evans, J.; Feldlaufer, M. Horizontal and vertical transmission of viruses in the honey bee, Apis mellifera. Journal of Invertebrate Pathology **2006**, 92, 152-159, doi:10.1016/j.jip.2006.03.010.

36. Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L., Jiang, H., Kanost, M., Thompson, G.J., Zou, Z., Hultmark, D., J D. Immune pathways and defence mechanisms in honey bees Apis mellifera. Insect Molecular Biology **2006**, 15, 645-656, doi:10.1111/j.1365-2583.2006.00682.x.

37. Brutscher, L.M.; Daughenbaugh, K.F.; Flenniken, M.L. Antiviral defense mechanisms in honey bees. Current Opinion in Insect Science **2015**, 2, 1-12, doi:10.1016/j.cois.2015.04.016.

38. McMenamin, A.J.; Daughenbaugh, K.F.; Parekh, F.; Pizzorno, M.C.; Flenniken, M.L. Honey Bee and Bumble Bee Antiviral Defense. Viruses **2018**, 10, 395, doi:10.3390/v10080395.

39. Simone, M.; Evans, J.D.; Spivak, M. Resin collection and social immunity in honey bees. Evolution; international journal of organic evolution **2009**, 63, 3016-3022, doi:10.1111/j.1558-5646.2009.00772.x.

40. Starks, P.T.; Blackie, C.A.; Seeley, T.D. Fever in honeybee colonies. Naturwissenschaften **2000**, 87, 229-231, doi:10.1007/s001140050709.

41. Evans, J.D.; Spivak, M. Socialized medicine: Individual and communal disease barriers in honey bees. Journal of Invertebrate Pathology **2010**, 103, S62-S72, doi:https://doi.org/10.1016/j.jip.2009.06.019.

42. Spivak, M.; Goblirsch, M.; Simone-Finstrom, M. Social-medication in bees: the line between individual and social regulation. Current Opinion in Insect Science **2019**, 33, 49-55, doi:https://doi.org/10.1016/j.cois.2019.02.009.

43. Simone-Finstrom, M. Social Immunity and the Superorganism: Behavioral Defenses Protecting Honey Bee Colonies from Pathogens and Parasites. Bee World **2017**, 94, 21-29, doi:10.1080/0005772X.2017.1307800.

44. Kingsolver, M.B.; Hardy, R.W. Making connections in insect innate immunity. Proceedings of the National Academy of Sciences of the United States of America **2012**, 109, 18639-18640, doi:10.1073/pnas.1216736109.

45. Kingsolver, M.B.; Huang, Z.; Hardy, R.W. Insect antiviral innate immunity: Pathways, effectors, and connections. Journal of Molecular Biology **2013**, 425, 4921-4936, doi:10.1016/j.jmb.2013.10.006.

46. Flenniken, M.L.; Andino, R. Non-Specific dsRNA-Mediated Antiviral Response in the Honey Bee. PLoS ONE **2013**, 8, 1-16, doi:10.1371/journal.pone.0077263.

47. Bull, J.C.; Ryabov, E.V.; Prince, G.; Mead, A.; Zhang, C.; Baxter, L.A.; Pell, J.K.; Osborne, J.L.; Chandler, D. A Strong Immune Response in Young Adult Honeybees Masks Their Increased Susceptibility to Infection Compared to Older Bees. PLoS Pathogens **2012**, 8, 11-14, doi:10.1371/journal.ppat.1003083.

48. Galbraith, D.A.; Yang, X.; Nino, E.L.; Yi, S.; Grozinger, C. Parallel epigenomic and transcriptomic responses to viral infection in honey bees (Apis mellifera). PLoS Pathog **2015**, 11, e1004713, doi:10.1371/journal.ppat.1004713.

49. Ryabov, E.V.; Fannon, J.M.; Moore, J.D.; Wood, G.R.; Evans, D.J. The Iflaviruses Sacbrood virus and Deformed wing virus evoke different transcriptional responses in the honeybee which may facilitate their horizontal or vertical transmission. PeerJ **2016**, 4, e1591, doi:10.7717/peerj.1591.

50. Ryabov, E.V.; Wood, G.R.; Fannon, J.M.; Moore, J.D.; Bull, J.C.; Chandler, D.; Mead, A.; Burroughs, N.; Evans, D.J. A Virulent Strain of Deformed Wing Virus (DWV) of Honeybees (Apis mellifera) Prevails after Varroa destructor-Mediated, or In Vitro, Transmission. PLoS Pathogens **2014**, 10, doi:10.1371/journal.ppat.1004230.

51. Brutscher, L.M.; Flenniken, M.L. RNAi and antiviral defense in the honey bee. Journal of Immunology Research **2015**, 2015, doi:10.1155/2015/941897.

52. Bronkhorst, A.W.; van Rij, R.P. The long and short of antiviral defense: small RNA-based immunity in insects. Current Opinion in Virology **2014**, 7, 19-28, doi:https://doi.org/10.1016/j.coviro.2014.03.010.

53. Galiana-Arnoux, D.; Dostert, C.; Schneemann, A.; Hoffmann, J.A.; Imler, J.-L. Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. Nature Immunology **2006**, 7, 590-597, doi:10.1038/ni1335.

54. van Rij, R.P.; Saleh, M.-C.; Berry, B.; Foo, C.; Houk, A.; Antoniewski, C.; Andino, R. The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes & Development **2006**, 20, 2985-2995, doi:10.1101/gad.1482006.

55. Zambon, R.A.; Vakharia, V.N.; Wu, L.P. RNAi is an antiviral immune response against a dsRNA virus in Drosophila melanogaster. Cellular Microbiology **2006**, 8, 880-889, doi:10.1111/j.1462-5822.2006.00688.x.

56. Wang, X.-H.; Aliyari, R.; Li, W.-X.; Li, H.-W.; Kim, K.; Carthew, R.; Atkinson, P.; Ding, S.-W. RNA Interference Directs Innate Immunity Against Viruses in Adult Drosophila. Science **2006**, 312, 452-454, doi:10.1126/science.1125694.

57. Myles, K.M.; Wiley, M.R.; Morazzani, E.M.; Adelman, Z.N. Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. Proceedings of the National Academy of Sciences **2008**, 105, 19938, doi:10.1073/pnas.0803408105.

58. Brackney, D.E.; Beane, J.E.; Ebel, G.D. RNAi Targeting of West Nile Virus in Mosquito Midguts Promotes Virus Diversification. PLOS Pathogens **2009**, 5, e1000502, doi:10.1371/journal.ppat.1000502.

59. Maori, E.; Paldi, N.; Shafir, S.; Kalev, H.; Tsur, E.; Glick, E.; Sela, I. IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. Insect Molecular Biology **2009**, 18, 55-60.

60. Chejanovsky, N.; Ophir, R.; Schwager, M.S.; Slabezki, Y.; Grossman, S.; Cox-Foster, D. Characterization of viral siRNA populations in honey bee colony collapse disorder. Virology **2014**, 454-455, 176-183, doi:10.1016/j.virol.2014.02.012.

61. Brutscher, L.M.; Daughenbaugh, K.F.; Flenniken, M.L. Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. Scientific Reports **2017**, 7, 6448, doi:10.1038/s41598-017-06623-z.

62. Piot, N.; Snoeck, S.; Vanlede, M.; Smagghe, G.; Meeus, I. The effect of oral administration of dsRNA on viral replication and mortality in Bombus terrestris. Viruses **2015**, 7, 3172-3185, doi:10.3390/v7062765.

63. Niu, J.; Meeus, I.; Cappelle, K.; Piot, N.; Smagghe, G. The immune response of the small interfering RNA pathway in the defense against bee viruses. Current Opinion in Insect Science **2014**, 6, 22-27, doi:10.1016/j.cois.2014.09.014.

64. Niu, J.; Smagghe, G.; De Coninck, D.I.; Van Nieuwerburgh, F.; Deforce, D.; Meeus, I. In vivo study of Dicer-2-mediated immune response of the small interfering RNA pathway upon systemic infections of virulent and avirulent viruses in Bombus terrestris. Insect Biochem Mol Biol **2016**, 70, 127-137, doi:10.1016/j.ibmb.2015.12.006.

65. Jarosch, A.; Moritz, R.F.A. RNA interference in honeybees: off-target effects caused by dsRNA. Apidologie **2012**, 43, 128-138, doi:10.1007/s13592-011-0092-y.

66. Nunes, F.M.F.; Aleixo, A.C.; Barchuk, A.R.; Bomtorin, A.D.; Grozinger, C.M.; Simões, Z.L.P. Non-Target Effects of Green Fluorescent Protein (GFP)-Derived Double-Stranded RNA (dsRNA-GFP) Used in Honey Bee RNA Interference (RNAi) Assays. Insects **2013**, 4, 90-103.

67. Doublet, V.; Poeschl, Y.; Gogol-Döring, A.; Alaux, C.; Annoscia, D.; Aurori, C.; Barribeau, S.M.; Bedoya-Reina, O.C.; Brown, M.J.F.; Bull, J.C., et al. Unity in defence: honeybee workers exhibit conserved molecular responses to diverse pathogens. BMC Genomics **2017**, 18, 207, doi:10.1186/s12864-017-3597-6.

68. Saleh, M.C.; Tassetto, M.; van Rij, R.P.; Goic, B.; Gausson, V.; Berry, B.; Jacquier, C.; Antoniewski, C.; Andino, R. Antiviral immunity in Drosophila requires systemic RNA interference spread. Nature **2009**, 458, 346-350, doi:10.1038/nature07712.

69. Venticinque, L.; Meruelo, D. Sindbis viral vector induced apoptosis requires translational inhibition and signaling through Mcl-1 and Bak. Molecular Cancer **2010**, 9, 37, doi:10.1186/1476-4598-9-37.

70. Taylor, R.C.; Berendzen, K.M.; Dillin, A. Systemic stress signalling: understanding the cell non-autonomous control of proteostasis. Nature Reviews Molecular Cell Biology **2014**, 15, 211-217, doi:10.1038/nrm3752.

71. McKinstry, M.; Chung, C.; Truong, H.; Johnston, B.A.; Snow, J.W. The heat shock response and humoral immune response are mutually antagonistic in honey bees. Scientific Reports **2017**, 7, 8850, doi:10.1038/s41598-017-09159-4.

72. Prithika, U.; Deepa, V.; Balamurugan, K. External induction of heat shock stimulates the immune response and longevity of Caenorhabditis elegans towards pathogen exposure. Innate Immunity **2016**, 22, 466-478, doi:10.1177/1753425916654557.

73. Singh, V.; Aballay, A. Heat Shock and Genetic Activation of HSF-1 Enhance Immunity to Bacteria. Cell Cycle **2006**, 5, 2443-2446, doi:10.4161/cc.5.21.3434.

74. Singh, V.; Aballay, A. Heat-shock transcription factor (HSF)-1 pathway required for Caenorhabditis elegans immunity. Proceedings of the National Academy of Sciences **2006**, 103, 13092, doi:10.1073/pnas.0604050103.

75. Chen, J.; Xie, C.; Tian, L.; Hong, L.; Wu, X.; Han, J. Participation of the p38 pathway in Drosophila host defense against pathogenic bacteria and fungi. Proceedings of the National Academy of Sciences **2010**, 107, 20774, doi:10.1073/pnas.1009223107.

76. Merkling, S.H.; Overheul, G.J.; Mierlo, J.T.V.; Arends, D.; Gilissen, C.; Rij, R.P.V. The heat shock response restricts virus infection in Drosophila. Nature Scientific Reports **2015**, 5, 1-15, doi:10.1038/srep12758.

77. Iwasaki, S.; Kobayashi, M.; Yoda, M.; Sakaguchi, Y.; Katsuma, S.; Suzuki, T.; Tomari, Y. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. Mol Cell **2010**, 39, 292-299, doi:10.1016/j.molcel.2010.05.015.

78. Dorner, S.; Lum, L.; Kim, M.; Paro, R.; Beachy, P.A.; Green, R. A genomewide screen for components of the RNAi pathway in Drosophila cultured cells. Proceedings of the National Academy of Sciences **2006**, 103, 11880–11885.

79. Kampmueller, K.M.; Miller, D.J. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells. Journal of virology **2005**, 79, 6827-6837, doi:10.1128/JVI.79.11.6827-6837.2005.

80. Weeks, S.A.; Shield, W.P.; Sahi, C.; Craig, E.A.; Rospert, S.; Miller, D.J. A Targeted Analysis of Cellular Chaperones Reveals Contrasting Roles for Heat Shock Protein 70 in Flock House Virus RNA Replication. Journal of Virology **2010**, 84, 330, doi:10.1128/JVI.01808-09.

81. Glotzer, J.B.; Saltik, M.; Chiocca, S.; Michou, A.-I.; Moseley, P.; Cotten, M. Activation of heat-shock response by an adenovirus is essential for virus replication. Nature **2000**, 407, 207-211, doi:10.1038/35025102.

82. Dalmon, A.; Peruzzi, M.; Le Conte, Y.; Alaux, C.; Pioz, M. Temperature-driven changes in viral loads in the honey bee Apis mellifera. Journal of Invertebrate Pathology **2019**, 160, 87-94, doi:https://doi.org/10.1016/j.jip.2018.12.005.

83. Bordier, C.; Dechatre, H.; Suchail, S.; Peruzzi, M.; Soubeyrand, S.; Pioz, M.; Pélissier, M.; Crauser, D.; Conte, Y.L.; Alaux, C. Colony adaptive response to simulated heat waves and consequences at the individual level in honeybees (Apis mellifera). Scientific Reports **2017**, 7, 3760, doi:10.1038/s41598-017-03944-x.

84. Milbrath, M. Varroa Mite Monitoring: Using a sugar roll to identify populations of Varroa destructor in honey bee colonies. Availabe online: https://pollinators.msu.edu/resources/beekeepers/varroa-mite-monitoring1/ (accessed on 2 January 2020).

85. Healthy Bees, Healthy People, Healthy Planet. Tools for Varroa Management: A Guide to Effective Varroa Sampling and Control, 1st ed.; The Honey Bee Health Coalition: Keystone, United States. 2015; p. 8.

86. Evans, J.D.; Chen, Y.P.; Prisco, G.d.; Pettis, J.; Williams, V. Bee cups: single-use cages for honey bee experiments. Journal of Apicultural Research **2009**, 48, 300-302, doi:10.1080/00218839.2009.11101548.

87. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. Nature Methods **2012**, 9, 671-675, doi:10.1038/nmeth.2089.

88. Severson, D.W.; Erickson, E.H.; Williamson, J.L.; Aiken, J.M. Heat stress induced enhancement of heat shock protein gene activity in the honey bee (Apis mellifera). Experientia **1990**, 46, 737-739, doi:10.1007/BF01939951.

89. de Miranda, J.R.; Bailey, L.; Ball, B.V.; Blanchard, P.; Budge, G.E.; Chejanovsky, N.; Chen, Y.-P.; Gauthier, L.; Genersch, E.; de Graaf, D.C., et al. Standard methods for virus research in Apis mellifera. Journal of Apicultural Research **2013**, 52, 1-56, doi:10.3896/IBRA.1.52.4.22.

90. Team, R. RStudio: Integrated Development Environment for R, 1.1.456; RStudio, Inc.: Vienna, Austria, 2016.

91. Taiyun Wei [cre, a.; [aut], V.S.; [ctb], M.L.; [ctb], Y.X.; [ctb], Y.J.; [ctb], J.Z. Package 'corrplot', 0.84; CRAN:Vienna, Austria, 2017.

92. Johnston, B.A.; Hooks, K.B.; McKinstry, M.; Snow, J.W. Divergent forms of endoplasmic reticulum stress trigger a robust unfolded protein response in honey bees. Journal of Insect Physiology **2016**, 86, 1-10, doi:https://doi.org/10.1016/j.jinsphys.2015.12.004.

93. Walter, P.; Ron, D. The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation. Science **2011**, 334, 1081, doi:10.1126/science.1209038.

94. Pincus, D.; Chevalier, M.W.; Aragón, T.; van Anken, E.; Vidal, S.E.; El-Samad, H.; Walter, P. BiP Binding to the ER-Stress Sensor Ire1 Tunes the Homeostatic Behavior of the Unfolded Protein Response. PLOS Biology **2010**, 8, e1000415, doi:10.1371/journal.pbio.1000415.

95. Solís, Eric J.; Pandey, Jai P.; Zheng, X.; Jin, Dexter X.; Gupta, Piyush B.; Airoldi, Edoardo M.; Pincus, D.; Denic, V. Defining the Essential Function of Yeast Hsf1 Reveals a Compact Transcriptional Program for Maintaining Eukaryotic Proteostasis. Molecular Cell **2016**, 63, 60-71, doi:https://doi.org/10.1016/j.molcel.2016.05.014.

96. Mahat, Dig B.; Salamanca, H.H.; Duarte, Fabiana M.; Danko, Charles G.; Lis, John T. Mammalian Heat Shock Response and Mechanisms Underlying Its Genome-wide Transcriptional Regulation. Molecular Cell **2016**, 62, 63-78, doi:https://doi.org/10.1016/j.molcel.2016.02.025.

97. Elekonich, M.M. Extreme thermotolerance and behavioral induction of 70-kDa heat shock proteins and their encoding genes in honey bees. Cell Stress and Chaperones **2009**, 14, 219-226, doi: https://doi.org/10.1007/s12192-008-0063-z.

98. Dostert, C.; Jouanguy, E.; Irving, P.; Troxler, L.; Galiana-Arnoux, D.; Hetru, C.; Hoffmann, J.A.; Imler, J.-L. The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. Nature Immunology **2005**, 6, 946-953, doi:10.1038/ni1237.

99. Zhu, F.; Ding, H.; Zhu, B. Transcriptional profiling of Drosophila S2 cells in early response to Drosophila C virus. Virology Journal **2013**, 10, 210, doi:10.1186/1743-422X-10-210.

100. Kemp, C.; Mueller, S.; Goto, A.; Barbier, V.; Paro, S.; Bonnay, F.; Dostert, C.; Troxler, L.; Hetru, C.; Meignin, C., et al. Broad RNA Interference–Mediated Antiviral Immunity and Virus-Specific Inducible Responses in Drosophila. The Journal of Immunology **2013**, 190, 650, doi:10.4049/jimmunol.1102486.

101. Deddouche, S.; Matt, N.; Budd, A.; Mueller, S.; Kemp, C.; Galiana-Arnoux, D.; Dostert, C.; Antoniewski, C.; Hoffmann, J.A.; Imler, J.-L. The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in drosophila. Nature Immunology **2008**, 9, 1425-1432, doi:10.1038/ni.1664.

102. Weinstock, G.M.; Robinson, G.E.; Gibbs, R.A.; Weinstock, G.M.; Weinstock, G.M.; Robinson, G.E.; Worley, K.C.; Evans, J.D.; Maleszka, R.; Robertson, H.M., et al. Insights into social insects from the genome of the honeybee Apis mellifera. Nature **2006**, 443, 931-949, doi:10.1038/nature05260.

103. Elsik, C.G.; Worley, K.C.; Bennett, A.K.; Beye, M.; Camara, F.; Childers, C.P.; de Graaf, D.C.; Debyser, G.; Deng, J.; Devreese, B., et al. Finding the missing honey bee genes: lessons learned from a genome upgrade. BMC Genomics **2014**, 15, 86, doi:10.1186/1471-2164-15-86.

104. Wallberg, A.; Bunikis, I.; Pettersson, O.V.; Mosbech, M.-B.; Childers, A.K.; Evans, J.D.; Mikheyev, A.S.; Robertson, H.M.; Robinson, G.E.; Webster, M.T. A hybrid de novo genome assembly of the honeybee, Apis mellifera, with chromosome-length scaffolds. BMC Genomics **2019**, 20, 275, doi:10.1186/s12864-019-5642-0.

105. Kim, K.; Lee, Y.S.; Harris, D.; Nakahara, K.; Carthew, R.W. The RNAi pathway initiated by Dicer-2 in Drosophila. Cold Spring Harbor symposia on quantitative biology **2006**, 71, 39-44, doi:10.1101/sqb.2006.71.008.

106. Kemp, C.; Imler, J.-L. Antiviral immunity in drosophila. Current Opinion in Immunology **2009**, 21, 3-9, doi:https://doi.org/10.1016/j.coi.2009.01.007.

107. Kawamura, Y.; Saito, K.; Kin, T.; Ono, Y.; Asai, K.; Sunohara, T.; Okada, T.N.; Siomi, M.C.; Siomi, H. Drosophila endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature **2008**, 453, 793-797, doi:10.1038/nature06938.

108. Simone-Finstrom, M.D.; Spivak, M. Increased resin collection after parasite challenge: a case of self-medication in honey bees? PloS one **2012**, 7, e34601, doi:10.1371/journal.pone.0034601.

109. Desai, S.D.; Eu, Y.J.; Whyard, S.; Currie, R.W. Reduction in deformed wing virus infection in larval and adult honey bees (Apis mellifera L.) by double-stranded RNA ingestion. Insect Molecular Biology **2012**, 21, 446-455, doi:10.1111/j.1365-2583.2012.01150.x.

110. Liu, X.; Zhang, Y.; Yan, X.; Han, R. Prevention of chinese sacbrood virus infection in apis cerana using rna interference. Current Microbiology **2010**, 61, 422-428, doi:10.1007/s00284-010-9633-2.

111. Di Prisco, G.; Annoscia, D.; Margiotta, M.; Ferrara, R.; Varricchio, P.; Zanni, V.; Caprio, E.; Nazzi, F.; Pennacchio, F. A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. Proceedings of the National Academy of Sciences **2016**, 113, 201523515, doi:10.1073/pnas.1523515113.

112. Stahlschmidt, Z.R.; Adamo, S.A. Context dependency and generality of fever in insects. Naturwissenschaften **2013**, 100, 691-696, doi:10.1007/s00114-013-1057-y.

113. Simone-Finstrom, M.; Foo, B.; Tarpy, D.R.; Starks, P.T. Impact of Food Availability, Pathogen Exposure, and Genetic Diversity on Thermoregulation in Honey Bees (Apis mellifera). Journal of Insect Behavior **2014**, 27, 527-539, doi:10.1007/s10905-014-9447-3.

114. Lindquist, S. Regulation of protein synthesis during heat shock. Nature **1981**, 293, 311-314, doi:10.1038/293311a0.

115. Cooper, P.D.; Schaffer, W.M. Temperature Regulation of Honey Bees (Apis mellifera) foraging in the Sonoran Desert. Journal of Experimental Biology **1985**, 1-15.

116. Stabentheiner, A.; Kovac, H.; Schmaranzer, S. Honeybee nestmate recognition: the thermal behaviour of guards and their examinees. Journal of Experimental Biology **2002**, 205, 2637.

117. Stabentheiner, A.; Kovac, H.; Schmaranzer, S. Thermal Behaviour of Honeybees During Aggressive Interactions. Ethology **2007**, 113, 995-1006, doi:10.1111/j.1439-0310.2007.01403.x.

118. Kovac, H.; Stabentheiner, A.; Schmaranzer, S. Thermoregulation of water foraging honeybees—Balancing of endothermic activity with radiative heat gain and functional requirements. Journal of Insect Physiology **2010**, 56, 1834-1845, doi:https://doi.org/10.1016/j.jinsphys.2010.08.002.

119. Cobey, S.; Sheppard, W.S.; Tarpy, D.R. Honey Bee Colony Health: Challenges and Sustainable Solutions; Sammataro, D., Yoder, J.A., Eds.; CRC Press: Boca Raton, United States, 2011.

120. Kovac, H.; Käfer, H.; Stabentheiner, A.; Costa, C. Metabolism and upper thermal limits of Apis mellifera carnica and A. m. ligustica. Apidologie **2014**, 45, 664-677, doi:10.1007/s13592-014-0284-3.

121. Zhao, M.; Tang, D.; Lechpammer, S.; Hoffman, A.; Asea, A.; Stevenson, M.A.; Calderwood, S.K. Double-stranded RNA-dependent Protein Kinase (pkr) Is Essential for Thermotolerance, Accumulation of HSP70, and Stabilization of ARE-containing HSP70 mRNA during Stress. Journal of Biological Chemistry **2002**, 277, 44539-44547.

122. Schoggins, J.W.; Rice, C.M. Interferon-stimulated genes and their antiviral effector functions. Current Opinion in Virology **2011**, 1, 519-525, doi:10.1016/j.coviro.2011.10.008.

123. Yoneyama, M.; Kikuchi, M.; Natsukawa, T.; Shinobu, N.; Imaizumi, T.; Miyagishi, M.; Taira, K.; Akira, S.; Fujita, T. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nature Immunology **2004**, 5, 730-737, doi:10.1038/ni1087.

124. Cordin, O.; Banroques, J.; Tanner, N.K.; Linder, P. The DEAD-box protein family of RNA helicases. Gene **2006**, 367, 17-37, doi:https://doi.org/10.1016/j.gene.2005.10.019.

125. Paradkar, P.N.; Trinidad, L.; Voysey, R.; Duchemin, J.B.; Walker, P.J. Secreted Vago restricts West Nile virus infection in Culex mosquito cells by activating the Jak-STAT pathway. Proc Natl Acad Sci U S A **2012**, 109, 18915-18920, doi:10.1073/pnas.1205231109.

126. Paradkar, P.N.; Duchemin, J.-B.; Voysey, R.; Walker, P.J. Dicer-2-Dependent Activation of Culex Vago Occurs via the TRAF-Rel2 Signaling Pathway. PLOS Neglected Tropical Diseases **2014**, 8, e2823, doi:10.1371/journal.pntd.0002823.

CHAPTER FIVE

THE HONEY BEE GENE *BEE ANTIVIRAL PROTEIN-1* (*BAP1*) IS A TAXONOMICALLY RESTRICTED ANTIVIRAL IMMUNE GENE

Contributions of authors and Co-Authors

Manuscript in Chapter Five

Author: Alexander J McMenamin

Contributions: Designed the study, collected and analyzed data, interpreted the results, wrote and revised the manuscript

Co-author: Laura Brutscher

Contributions: Designed the study, collected and analyzed data, revised the manuscript

Co-author: Katie F. Daughenbaugh

Contributions: Collected and analyzed data, revised the manuscript

Co-author: Michell L. Flenniken

Contributions: Supervised the study, acquired funding, designed the study, interpreted the results, wrote and revised the manuscript

Manuscript Information

Alexander J. McMenamin, Laura Brutscher, Katie F. Daughenbaugh, Michelle L. Flenniken

The honey bee gene *bee antiviral protein-1* (*bap1*) is a taxonomically restricted antiviral immune gene

Status of Manuscript
___ Prepared for submission to a peer-reviewed journal
_X_ Officially submitted to a peer-reviewed journal
___ Accepted by a peer-reviewed journal
___ Published in a peer-reviewed journal

Submitted to: Frontiers in Insect Science

The honey bee gene *bee antiviral protein-1 (bap1)* is a taxonomically restricted antiviral immune gene

Alexander J. McMenamin[1,2,3], Laura Brutscher[3], Katie F. Daughenbaugh[1,3], and Michelle L. Flenniken[1,2,3*]

[1]Department of Plant Sciences and Plant Pathology, [2]Department of Microbiology and Immunology,[3]Pollinator Health Center, Montana State University, Bozeman, MT, USA

*** Correspondence:**

Corresponding Author

michelle.flenniken@montana.edu

Keywords: honey bee$_1$, antiviral$_2$, MF116383$_3$, bee antiviral protein-1$_4$, bap1$_5$, honey bee virus$_6$, honey bee immune system$_7$, ago2$_8$.

Abstract

Insects have evolved a wide range of strategies to combat invading pathogens, including viruses. Genes that encode proteins involved in immune responses often evolve under positive selection due to their co-evolution with pathogens. Insect antiviral defense includes the RNA interference (RNAi) mechanism, which is triggered by recognition of non-self, virally produced, double-stranded RNAs. Indeed, insect RNAi genes (e.g., *dicer* and *argonaute-2*) are under high selective pressure. Honey bees (*Apis mellifera*) are eusocial insects that respond to viral infections via both sequence specific RNAi and a non-sequence specific dsRNA triggered pathway, which is less well characterized. A transcriptome-level study of virus-infected and/or dsRNA-treated

honey bees revealed increased expression of a novel antiviral gene, GenBank: MF116383, and *in vivo* experiments confirmed its antiviral function. Due to *in silico* annotation and sequence similarity, MF116383 was originally annotated as a probable *cyclin-dependent serine/threonine-protein kinase.* In this study, we confirmed that MF116383 limits virus infection, and carried out further bioinformatic and phylogenetic analyses to better characterize this important gene – which we renamed *bee antiviral protein-1* (*bap1*). Phylogenetic analysis revealed that *bap1* is taxonomically restricted to Hymenoptera and *Blatella germanica* (the German cockroach) and that the majority of *bap1* amino acids are evolving under neutral selection. This is in-line with the results from structural prediction tools that indicate Bap1 is a highly disordered protein, which likely has relaxed structural constraints. Assessment of honey bee gene expression using a weighted gene correlation network analysis revealed that *bap1* expression was highly correlated with several immune genes – most notably *argonaute-2*. The coexpression of *bap1* and *argonaute-2* was confirmed in an independent dataset that accounted for the effect of virus abundance. Together, these data demonstrate that *bap1* is a taxonomically restricted, rapidly evolving antiviral immune gene. Future work will examine the signal cascade responsible for regulating the expression of *bap1* and other honey bee antiviral defense genes, including coexpressed ago-2, and determine whether the virus limiting function of *bap1* acts in parallel or in tandem with RNAi.

1 Introduction

Conflict is the engine of evolution by natural selection and ecological diversification. Inter-organism conflict is most apparent when one organism parasitizes another, thus pitting the interests of two organisms against one another. Conflict between hosts and parasites drives the evolution of a wide range of strategies to (1) distinguish self from non-self and (2) defend against invading pathogens. Together, these systems comprise an organism's immune system (Müller and Müller, 2003). Metazoan immune systems are subdivided into the innate and adaptive responses which are further classified as humoral or cellular responses (Müller and Müller, 2003; Cooper and Alder, 2006; Ronald and Beutler, 2010; Kingsolver and Hardy, 2012; Kingsolver et al., 2013; McMenamin et al., 2018). Both humoral and cellular responses have been implicated in antiviral immunity in insects, including *Apis mellifera* – the western honey bee, a eusocial, cavity-nesting insect that is an important plant pollinator (Klein et al., 2007; Gallai et al., 2009; Eilers et al., 2011). The innate antibacterial and antifungal humoral responses in insects are induced by Pathogen Recognition Receptors (PRRs) which recognize Pathogen Associated Molecular Patterns (PAMPs) (Ferrandon et al., 2007). The recognition of PAMPs by PRRs activates immune cascades – such as the Toll (NF-kB/Dorsal), Imd (NF-kB/Relish), and Jak/STAT pathways - which regulate the production and secretion of soluble antimicrobial peptides (AMPs) into the hemolymph (reviewed in (Hoffmann, 2003; Ferrandon et al., 2007; Kingsolver and Hardy, 2012; Kingsolver et al., 2013; Buchon et al., 2014; Brutscher and Flenniken, 2015; McMenamin et al., 2018)). These pathways are also involved in insect antiviral immunity, although the primary antiviral immune response is the RNA interference (RNAi)

response (reviewed in (Kingsolver et al., 2013)). The RNAi response is a sequence-specific post-transcriptional gene silencing mechanism induced upon the recognition of double stranded RNAs (dsRNAs), including the replicative forms of single-stranded positive sense RNA viruses, by Dicer endoribonucleases. Dicer cleaves long dsRNAs into 21-22 bp short interfering RNAs, one strand of which (the guide strand) is retained by Argonaute-2 (Ago2), which is part of the RNAi-induced silencing complex (RISC). The RISC surveils the cell for complementary target RNAs, including viral genomes and transcripts, that are recognized, bound, and cleaved – which in turn limits virus infections (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006; Deddouche et al., 2008; Myles et al., 2008) (reviewed in (Kingsolver and Hardy, 2012; Kingsolver et al., 2013; Bronkhorst and van Rij, 2014)).

Historically the invertebrate immune system was considered strictly innate (i.e., hosts respond naïvely to each encounter with a pathogen), while it was thought that the adaptive immune system, which includes pathway-specific and memory responses, was limited to jawed vertebrates (reviewed in (Hoffmann, 2003; Brennan and Anderson, 2004; Cooper and Alder, 2006; Buchon et al., 2014)). However, from an ecological and evolutionary perspective, it would be expected that insect hosts would have evolved immune systems with plasticity and specificity to respond to repeated exposure to pathogens within and across generations. Indeed, recent studies indicate that *Drosophila melanogaster* exhibits an adaptive immune response to viral infection (Saleh et al., 2009; Tassetto et al., 2017; Poirier et al., 2018; Suzuki et al., 2020). This response is predicated on the production of virus genome-derived DNA (vDNA) by reverse transcriptase, followed by vDNA transcription, and cleavage of the RNA transcripts into short

interfering RNAs (siRNAs) (Tassetto et al., 2017; Poirier et al., 2018). Because RNAi proteins, including *dicer* and *ago2,* are involved in the conflict between hosts and viruses one would hypothesize that they are under positive selection and evolving rapidly (Paterson et al., 2010; Daugherty and Malik, 2012). In fact, analysis of dN/dS ratios of RNAi genes across multiple insect/arthropods indicate that these proteins have higher rates of adaptive evolution and selective sweeps compared to paralogous "housekeeping" genes, including in fruit flies and honey bees (Obbard et al., 2006; Palmer et al., 2018).

Similar to other insects, RNA interference is antiviral in honey bees, as co-administration of a virus with virus-sequence specific dsRNA results in a reduction of virus abundance (Maori et al., 2009; Liu et al., 2010; Desai et al., 2012; Flenniken and Andino, 2013; Chejanovsky et al., 2014; Niu et al., 2014; Brutscher et al., 2017; Leonard et al., 2020). For example, larval and adult honey bees fed diets with deformed wing virus (DWV) and DWV-specific dsRNA had greater longevity and lower DWV levels than bees exposed to DWV alone (Desai et al., 2012). In addition to sequence-specific RNAi, honey bees and bumble bees mount a virus-limiting response when exposed to dsRNA of any sequence (Flenniken and Andino, 2013; Piot et al., 2015; Cappelle et al., 2016; Niu et al., 2016b; Brutscher et al., 2017). In contrast, this non-sequence specific dsRNA-triggered immune response has not been observed in highly investigated, solitary insects, including fruit-flies and mosquitos. Although the details of this non-sequence specific dsRNA-triggered antiviral mechanism have yet to be fully elucidated, the transcriptional level response to dsRNA both independently and in the context of virus infection

have been well-characterized in honey bees, and the expression of select genes, including those involved in RNAi have also been examined in bumble bees (Flenniken and Andino, 2013; Cappelle et al., 2016; Niu et al., 2016a; Niu et al., 2016b; Wang et al., 2016; Brutscher et al., 2017; Niu et al., 2017; Wang et al., 2017). Specifically, a honey bee transcriptome sequencing study identified hundreds of genes differentially expressed in response to virus infection and/or dsRNA injection (Brutscher et al., 2017). The antiviral roles of two of the genes that exhibited greater expression in response to virus and/or dsRNA, *dicer-like* and the gene encoded by GenBank: MF116383, herein renamed *bee antiviral protein-1* (*bap1*), were confirmed *in vivo*. Specifically, RNAi-mediated reduction of expression of either of these genes in honey bees resulted in greater levels of the model virus Sindbis virus (SINV) compared to virus levels in bees treated with a non-specific dsRNA control (Brutscher et al., 2017). As described above, Dicer is an endoribonuclease that processes dsRNA into siRNAs that are bound by Ago2/RISC and serve as sequence-specific guides for the targeted cleavage of cognate RNAs, including viral genomes and transcripts. The precise antiviral function of *bee antiviral protein-1* (*bap1*) (GenBank: MF116383.1) is still unknown. It was originally described as putative cyclin-dependent serine/threonine kinase based on *in silico* analysis (Elsik et al., 2014; Brutscher et al., 2017). It was one of the most highly differentially expressed genes in response to virus-infection (Brutscher et al., 2017). Therefore, to further characterize the gene, the full-length cDNA was Sanger sequenced revealing that the transcript length was longer than predicted (i.e., 5,158 nucleotide) and shared 91% nucleotide identity with an *Apis cerana* probable cyclin-dependent

serine/threonine-protein kinase transcript (XM_017051141.1) and therefore it was similarly described in a previous publication (Brutscher et al., 2017).

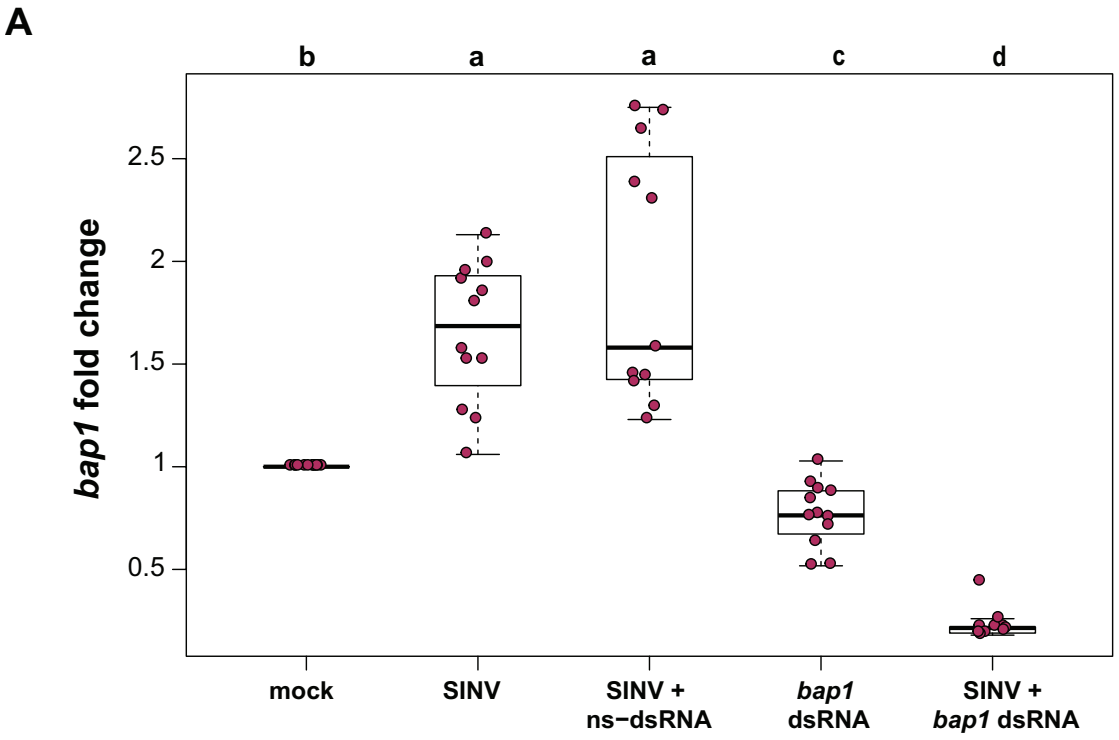
In this study, we replicated the finding that the gene encoded by GenBank: MF116383 restricts virus infection and further characterized this gene. Sequence analysis using HMMER and domain searches failed to identify a putative kinase domain, therefore we renamed this gene *bee antiviral protein-1* (*bap1*) to better reflect its function. Analysis of the phylogenetic distribution of *bap1* and its orthologues revealed that it is taxonomically restricted to Hymenopteran insects and the German cockroach (*Blatella germanica*). To evaluate the potential evolutionary selective pressures on this gene, we calculated the site-wise dN/dS ratios and determined that the high substitution rate indicated by Bayesian phylogenetic inference is likely indicative of neutral selection across the majority of the gene, although some sites are under purifying and positive selection. To identify the pathways and proteins with which *bap1* may be associated, we used a weighted gene correlation network analysis (WGCNA) to identify highly coexpressed genes. This analysis revealed that *bap1* is coexpressed with several immune genes, most notably *ago2*, which we confirmed in an independent dataset. Together these data demonstrate that *bap1* is an important honey bee antiviral defense gene that is taxonomically restricted to primarily social insects (i.e., members of Apidae, Formicoidea, and *Blatella germanica*).

2 Results and Discussion

2.1 Virus-limiting role of honey bee *bap1* (GenBank: MF116383) confirmed

ln honey bees, dsRNA triggers both sequence specific and non-sequence specific virus limiting mechanisms that reduce virus abundance (Maori et al., 2009; Liu et al., 2010; Desai et al., 2012;

Flenniken and Andino, 2013; Chejanovsky et al., 2014; Niu et al., 2014; Ryabov et al., 2014; Brutscher et al., 2017). To confirm that *bap1* restricts virus infection, we carried out honey bee virus infection studies in the context of either non-sequence specific dsRNA (ns-dsRNA) or *bap1*-specific dsRNA and quantified the relative expression of *bap1* and virus abundance using quantitative PCR (qPCR). In this study, similar to a previous report, honey bees infected with the model virus Sindbis-GFP (SINV) exhibited 1.65x greater expression of *bap1* compared to mock-infected bees (Dunnett's test, $p < 0.001$) (Figure 1A) (Brutscher et al., 2017). Likewise, co-administration of virus and ns-dsRNA, which is a viral-associated molecular pattern (VAMP), also resulted in a 1.92x increase of *bap1* expression at 72 hours post-infection (hpi) compared to levels in mock-infected honey bees (Figure 1A). RNAi-mediated gene knock-down was achieved by injecting *bap1*-seqeunce specific dsRNA which resulted in a mean 0.77x reduction, and a 0.23x reduction in *bap1* expression in bees co-injected with *bap1* dsRNA and virus relative to mock ($p <0.001$) (Figure 1A).


A
b
a
a
c
d
bap1 fold change
2.5
2
1.5
1
0.5
mock
SINV
SINV +
ns−dsRNA
bap1
dsRNA
SINV +
bap1 dsRNA

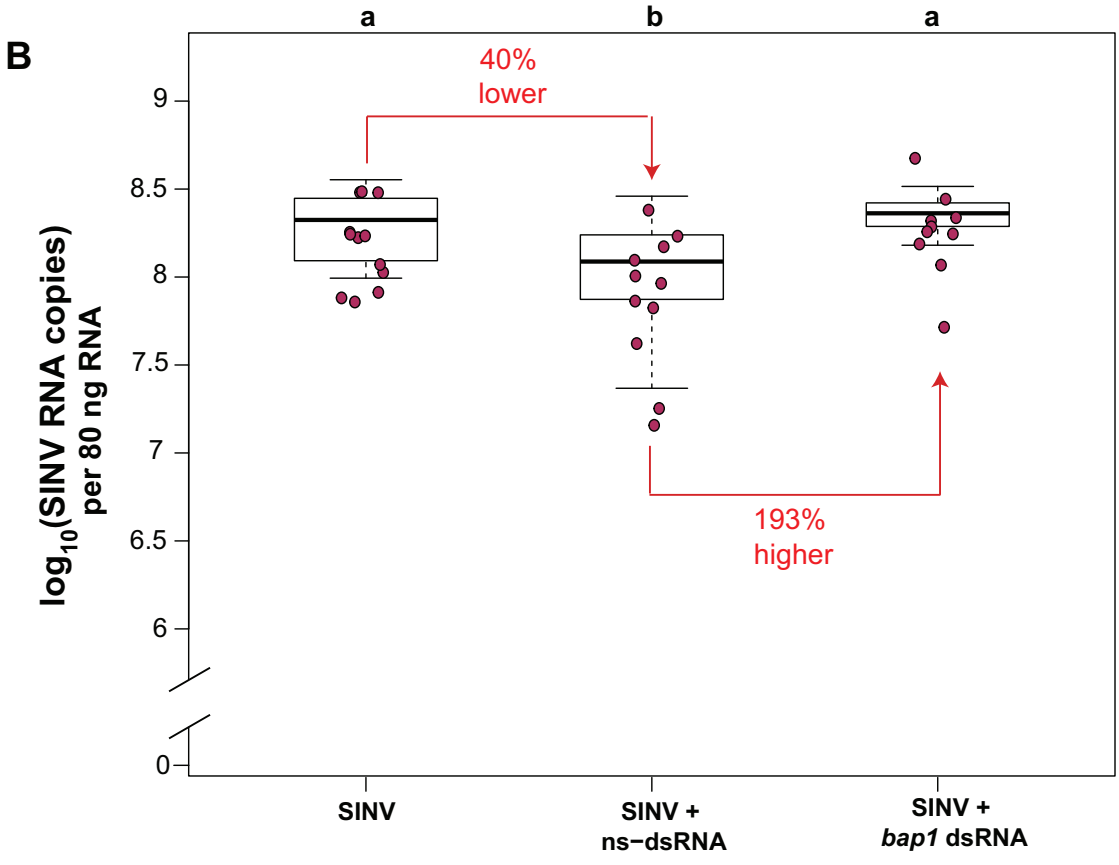

B
a
b
a
40%
lower
193%
higher
log10(SINV RNA copies)
per 80 ng RNA
9
8.5
8
7.5
7
6.5
6
0
SINV
SINV +
ns−dsRNA
SINV +
bap1 dsRNA

Figure 1. *Bee antiviral protein-1 (bap1)* is an antiviral immune gene.
(A) Relative expression of *bap1* was assessed by qPCR, normalized to *rpl8* and fold changes were calculated relative to buffer-injected bees (mock). Bees infected with Sindbis-GFP (SINV) exhibited 1.65x greater expression of *bap1* compared to mock-infected bees (Dunnett's test, $p < 0.001$). Co-injection of virus and ns-dsRNA also resulted in a 1.92x increase of *bap1* expression at 72 hours post-infection (hpi) compared to levels in mock-infected honey bees. RNAi-mediated gene knock-down was achieved by injecting *bap1*-seqeunce specific dsRNA which resulted in a mean 0.77x reduction, and a 0.23 fold reduction in *bap1* expression in bees co-injected with *bap1* dsRNA and virus relative to mock ($p <0.001$). **(B)** Virus abundance was measured by determining the viral RNA copies using qPCR. Bees coinjected with ns-dsRNA and SINV and 40% lower SINV levels compared to SINV-only injected ($p = 0.045$) ($p = 0.045$). Bees with reduced *bap1* expression had 193% higher SINV levels relative to bees coinjected with SINV and ns-dsRNA ($p = 0.016$). Different letters above the treatments groups indicates a statistically significant difference.

Virus abundance in virus-infected bees compared to virus-infected bees co-injected with either ns-dsRNA or *bap1*-dsRNA was examined by determining the viral RNA copies (which represent both viral genomes and transcripts during active infections) using qPCR. In this study, similar to previous studies, co-injection of ns-dsRNA reduced SINV levels by 40% relative to levels in virus-infected bees ($p = 0.045$, Figure 1B). Whereas virus abundance was not reduced in bees that were co-injected with *bap1*-dsRNA, as compared to virus-only infected bees (Figure 1B). Furthermore, honey bees with reduced *bap1* expression harbored 193% more virus than control bees which received ns-dsRNA ($p = 0.016$) (Figure 1B). Together, these results indicate that antiviral defense was hindered in honey bees with reduced *bap1* expression and confirm the findings of Brutscher *et al*. that *bap1* has an antiviral function in honey bees (Brutscher et al., 2017).

2.2 Phylogenetic analysis of *bee antiviral protein-1* (*bap1*) reveal it is a taxonomically restricted, divergent gene that is evolving under neutral selection

Bee antiviral protein-1 (GenBank: MF116383) is a recently described honey bee antiviral gene transcribed from LOC725387 to produce a 5,158 nucleotide (nt) long transcript that produces a 1,511 amino acid protein (Supplemental Figure S1) (Brutscher et al., 2017). Although, transcriptome and *in vivo* studies indicate it is important for honey bee antiviral defense, the mechanistic and functional roles of *bap1* have not yet been elucidated. Therefore, we utilized HHpred with default parameters to identify any putative conserved amino acid domains to infer Bap1 function at the cellular level (Zimmermann et al., 2018; Gabler et al., 2020). Hidden Markov Model searches against the protein data bank (PBD), Pfam-A_34, Swissprot, COG_KOG, or NCBI Conserved Domains databases all failed to identify any conserved domains. Therefore, in contrast to initial reports based on automated annotations, the gene encoded by GenBank: MF116383 does not likely have a kinase domain, and therefore *bap1* is a more accurate name for this gene and associated protein (ASQ15625).

To gain insight on the phylogenetic distribution of *bap1*, which may also provide functional insights, we identified orthologous proteins in other insects and assessed their phylogenic relationship (Figure 2A). Honey bees belong to the order Hymenoptera, which is a large order of insects with over 150,000 species. Orthologues of *bap1* were identified in the major lineages of Hymenoptera, including Apocrita (wasps and Anthophila), and Eusymphyta (which include sawflies, horntails, and wood wasps). Whereas orthologous genes were not identified in Diptera

(including *Drosophila melanogaster,* as well as mosquitos in genera *Culex, Aedes,* and *Anopheles*) or most other insect sequences in NCBI databases with the exception of one protein in *Blatella germanica* (the German cockroach).

Orthologues of *bap1* were identified via the Position-Specific Iterated Basic Local Alignment (PSI-BLAST) tool on the website of the National Center for Biotechnology Information (NCBI) (Supplemental Table S2) (Altschul et al., 1990; Altschul et al., 1997; Gertz, 2005). We identified 73 orthologues with percent amino acid identities ranging from 17% to 82% (Supplemental Figure S1 and Table S3). The protein sequences were aligned using MEGA X (MUSCLE with default parameters) (Kumar et al., 2018) and analyzed by Bayesian inference phylogenetic analysis using MrBayes v3.2.7a (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with *Blatella germanica* as the out group since it is the only non-Hymenopteran taxon on the tree (Figure 2A). Unfortunately, Euymphyta and Apocrita (aside from Anthophila) remain very under sampled on this tree due to a lack of genome assemblies or low-quality genome assemblies. It is interesting that *bap1* orthologues were not identified in model insects such as *Drosophila melanogaster (*Diptera*), Spodoptera frugiperda* (Lepidoptera)*,* or *Tribolium castaneum* (Coleoptera). Phylogenomic analysis using 1,478 orthologue groups and published transcriptome data sets suggests the ancestors of Hymenoptera and Blattodea split roughly 400 million years ago, compared to the ancestors of Diptera, Lepidoptera, and Coleoptera, which are phylogenetically more similar to Hymenoptera, having diverged about 350 million years ago (Misof et al., 2014). Even though bees and cockroaches diverged long before bees and flies, a

bap1 orthologue was identified in *Blatella germanica*, the German cockroach. Interestingly, experiments aimed at investigating gene function in this species using RNAi-mediated gene knockdown, revealed that *dicer2* expression was increased in response to ns-dsRNA (Lozano et al., 2012). Although complete transcriptome profiling of dsRNA-treated cockroaches has yet to be evaluated, we hypothesize that *bap1* orthologues were retained in several phylogenetic lineages that have a transcriptional response to dsRNA, and that this response may be antiviral– as it is in honey bees and bumble bees. In line with our hypothesis, fruit flies and mosquitos, which do not have *bap1* orthologues, also do not exhibit virus-limiting responses to ns-dsRNA. However, there are organisms that limit virus infection in response to ns-dsRNA that lack a *bap1* orthologue (e.g., the sand fly, *Lutzomyia longipalpis*) (Pitaluga et al., 2008). It not clear whether this phenotype in *L. longipalpis* is a retained (from an ancestor) or derived phenotype.

To confirm that some insects lack a *bap1* orthologue, PSI-BLAST searches directed to several genomes (i.e., *Drosophila melanogaster, Lutzomyia longipalpis,* and *Aedes albopictus*) were performed, and a Hidden Markov Model approach to identify deep homologues (JackHMMER) was utilized; all failed to identify additional orthologues (Johnson et al., 2010). This indicates that *bap1* is a taxonomically restricted gene either due to lineage-specific losses or high divergence (i.e., high average amino acid substitution at each position) in other lineages thereby making orthologues unidentifiable.

The phylogenetic relationships based on the Bap1 protein sequence, grouped wasps and sawflies together, which was likely an artifact of long branch lengths, and not an accurate representation of lineage (Figure 2A). If the Bap1 protein tree reflected the true phylogenetic relationships within Hymenoptera, you would expect Eusymphyta to form the basal clade of Hymenoptera (Peters et al., 2017). Instead, in this tree, Anthophila appears to have split from the rest of Apocrita and Formicoidea before they diverged, when in fact Anthophila is the most derived clade within Hymenoptera (Peters et al., 2017).

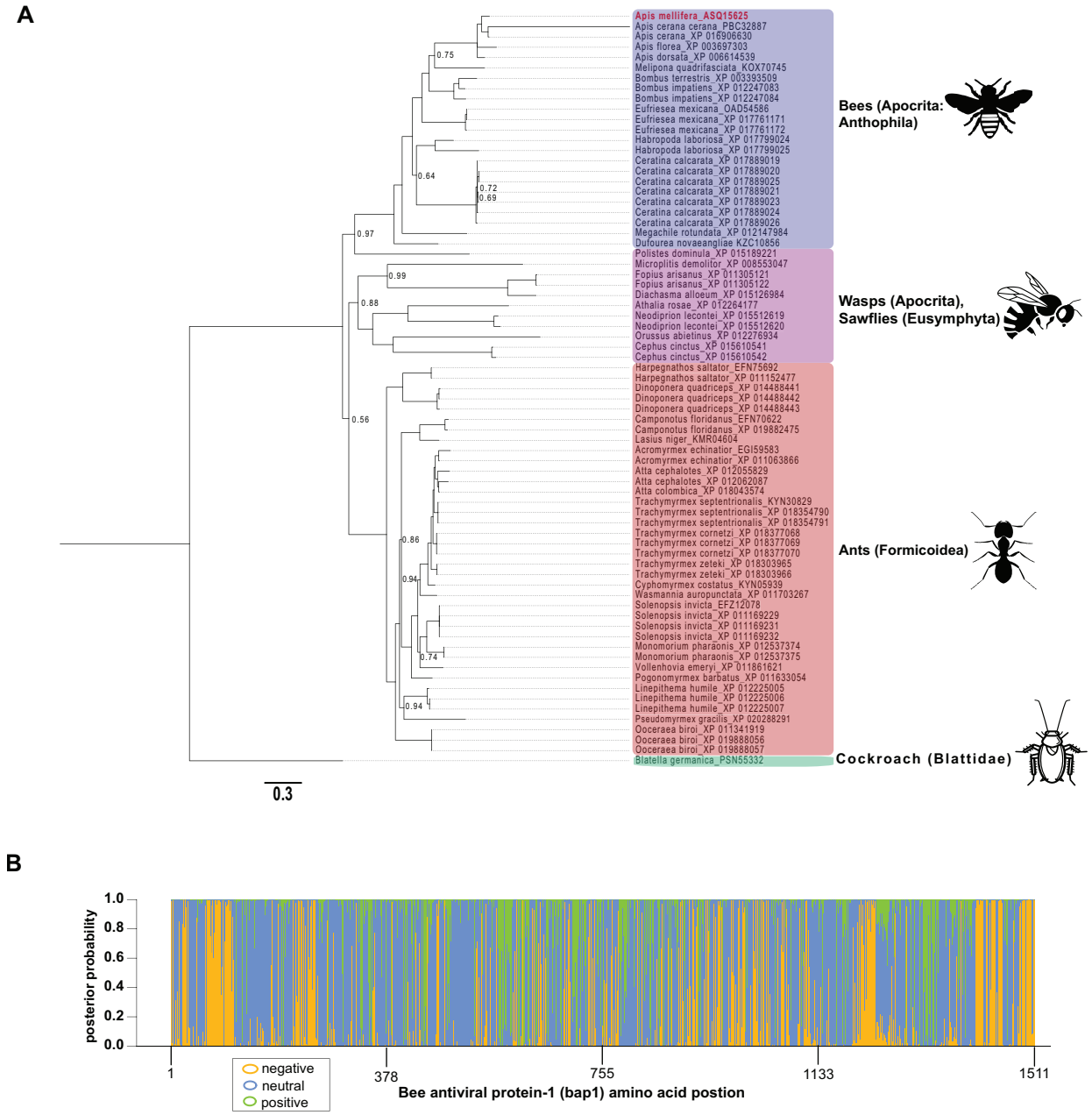


Figure 2. Bee antiviral protein-1 phylogenic relationship to other Hymenopteran orthologues inferred from amino acid sequence.

(A) Majority rule Bayesian consensus tree of Bap1 homologues derived from Bayesian analysis of amino acid alignment implemented in Mr. Bayes v3.2 using a Jones substitution model. Numbers on branches and nodes are posterior probabilities (0-1), though posterior probabilities values of 1 are not shown to improve clarity. The scale bar corresponds to proportion of amino acid change. Accession numbers are included on the branch tips and in Supplemental Table S2.

(B) A corresponding majority Rule Bayesian consensus tree derived from Bayesian analysis of a codon alignment was used in a selection analysis in the CODEML package in Phylogenetic Analysis by Maximum Likelihood (PAML) 4.9. The Bayes Empirical Bayes method under model 2, which assumes 3 site classes (negative, neutral, and positive selection), was used to calculate the posterior probability that each amino acid position belonged to each site class. The posterior probabilities were then plotted as a stacked bar chart along the length of Bap1 amino acids. This method shows clear diffuse neutral selection with regions under strong positive or negative selection.

The high rate of amino acid substitution in Bap1 orthologues (0.3 amino acid substitutions per position) is indicative of rapid evolution (Figure 2A). To contextualize the seemingly high amino acid substitution rate on the Bap1 tree (Figure 2A), a parallel analysis was carried out on RPB1, the largest subunit of eukaryotic DNA-directed RNA polymerase II, which showed a more than 10-fold lower substitution rate (0.02, Supplemental Figure S2, Supplemental Table S4) (Koleske and Young, 1995). Given the high substitution rate in Bap1 we hypothesized that Bap1 was under positive selection causing divergence between lineages. A gene is under positive selection when the ratio of non-synonymous mutations (a change in amino acid; dN) to synonymous mutations (no amino acid change, dS) as greater than 1. This indicates some selective pressure is driving the fixation of advantageous mutations in lineages. A gene is under negative selection when the dN/dS ratio is less than 1, which indicates selection pressure is purging amino acid-changing mutations because they are deleterious. One might expect that an antiviral gene is under positive selection due to the arms-race between a pathogen an its host, which may favor non-synonymous mutations (Paterson et al., 2010). Alternatively, genes that are important to numerous important biological processes, including some immune genes, could be under negative selection, in order to ensure structural and functional preservation. Furthermore,

individual amino acids can experience unique selective pressure and, therefore, whole-gene dN/dS ratios are unlikely to be reflective of evolution at individual amino acids. A maximum likelihood analysis of site-wise dN/dS ratios using PAML revealed that *bap1* has codons evolving under negative (260 sites, dN/dS = 0.16) and positive selection (123 sites, dN/dS = 1.94) but most sites are evolving under neutral selection (797 sites, dN/dS = 1.00) (Figure 2B, Table 1, Supplemental Figure S3 Supplemental Tables S5-S6 and S10) (Yang, 2007). This is in stark contrast to RPB1, the largest subunit of eukaryotic DNA-directed RNA polymerase II, which also has sites under neutral (191 sites, dN/dS = 1) and positive (98 sites, dN/dS= 14) selection, but most sites are under very strong negative selection (1,314 sites, dN/dS = 0.0006), which is characteristic of highly conserved proteins involved in fundamental biological processes, including transcription (Supplemental Figures S2B and S4, Supplemental Tables S7-S10). An Empirical Bayes approach was used to calculate the posterior probability that each amino acid was evolving under negative, neutral, or positive selection (Figure 2B, Supplemental Figure S2B). Overall, the pattern of selection and phylogenetic distribution indicate that the *bap1* is a taxonomically restricted orthologue group evolving primarily under neutral selection causing rapid divergence, as compared to a control gene (RPB1) (Figure 2 Supplemental Figure S2).

Table 1. Bee antiviral protein-1 (*bap1*) orthologues are primarily under neutral selection. Measures of selection (dN/dS) were calculated for *bap1* (GenBank: MF116383) orthologues under three models. M0 assumes one dN/dS ratio for the whole protein. M1 assumes the protein is evolving under near-neutral selection calculates dN/dS for two site classes (dN/dS = 1 and dN/dS < 1). Lastly, M2 assumes there are three codon site classes in the protein (dN/dS < 1, dN/dS = 1 and dN/dS >1). The tests show M2 is the best model and that *bap1* orthologues have sites under negative, neutral, and positive selection.

	M0	M1	M2
Description	Assumes one dN/dS ratio for whole gene	Assumes near-neutral selection	Assumes 3 classes with varying selection (negative, neutral or positive)
-lnL	-160,366.55	-154,730.907	-154,513.0653
Test Statistic (null is M0)		11,271.286	M2-M0: 11,706.97 M2-M1: 434
p-value		$<1x10^{-15}$	M2-M0: $<1x10^{-15}$ M2-M1: $p<0.001$
dN/dS	0.628	negative = 0.146, neutral= 1.000	negative = 0.16 neutral = 1.00 positive = 1.94

2.3 Bee antiviral protein-1 (Bap1) is a disordered protein that is trafficked to the endoplasmic reticulum

Relaxed evolution is a hallmark of disordered proteins due to relaxed constraints on their structure (Lin et al., 2007; Brown et al., 2010; Brown et al., 2011). To ascertain whether the largely neutral selection on *bap1* orthologues (Figure 2B) was due to relaxed structural constraints, PrDOS (protein disorder prediction system) was used to calculate disorder prediction at each amino acid position with the default 5% false positive rate (Figure 3A) (Ishida and Kinoshita, 2007). This analysis indicated that the majority (i.e., 67.2%) of the Bap1 amino acid sequence is disordered or lacks structure (i.e., above 0.5 probability, Figure 3A). Intrinsic

disorder in protein structure allows the function of a protein to be highly modular since it allows the binding of multiple partners, including proteins and nucleic acids (Wright and Dyson, 2014). In fact the relaxed structural constraint, leading to decreased packing density, is associated with more rapid evolution (higher substitution rates) of disordered proteins, which is in-line with the bioinformatic analyses of *bap1,* described herein (Figure 2A) (Lin et al., 2007; Xia et al., 2009; Brown et al., 2010; Brown et al., 2011). Due to their modularity, intrinsically disordered proteins (IDPs) often act as hubs in protein interaction networks (Dunker et al., 2005; Higurashi et al., 2008; Kim et al., 2008). When bound to their target, many IDPs adopt a more structured conformation (Spolar and Record, 1994; Dyson and Wright, 2002). Future analyses, including the use of nuclear magnetic resonance (NMR) and X-ray scattering techniques, are required to definitively characterize the structure of the Bap1 protein in bound or unbound states.

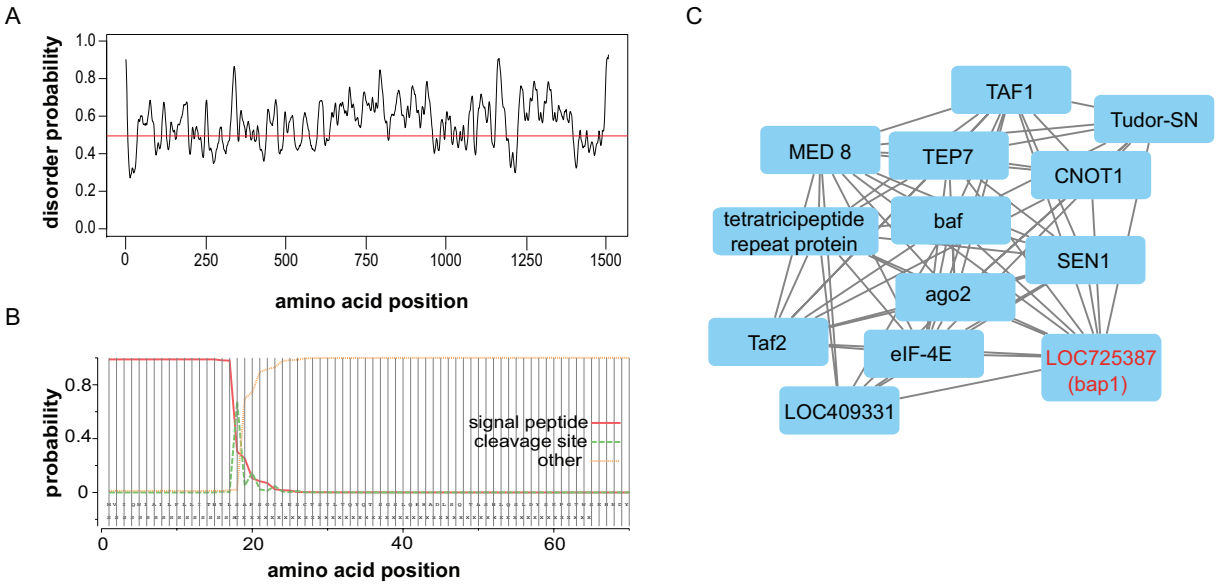


Figure 3. Bee antiviral protein-1 (Bap1) is a disordered protein coexpressed with several other immune genes.
(A) The PrDOS web tool was used to predict disorder probability at each amino acid position in Bap1. PrDOS predicted that 67.2% of Bap1 has a more than 50% probability of being disordered. **(B)** SignalP-5.0 was used to predict the presence of a signal peptide on the N-terminus. It is highly likely that bap1 has a signal peptide (probability of 0.983) that is cleaved off between amino acids 21 and 22. **(C)** Weighted gene correlation network analysis (WGCNA) was used to identify genes that are highly coexpressed in various contexts (i.e., virus infection or dsRNA administration). WGCNA identified that module (a highly coexpressed cluster of genes) #38 was associated with virus infection with a correlation of 0.54 ($p = 1x10^{-4}$, Supplemental Figure S5) and contained *bap1*. This module also contained several immune genes including *ago1*, *Tudor-SN,* and *TEP7*. For full subnetwork see Supplemental Figure S6.

To examine the putative subcellular localization of the protein encoded by *bap1*, we used SignalP-5.0 to predict the presence of a signal peptide on the N-terminus (Almagro Armenteros et al., 2019). Signal-P predicted the presence of a signal peptide with a probability of 0.983 with a cleavage site between amino acids 21 and 22 (Figure 3B). Signal peptides are N-terminal

sequences on proteins that promotes targeting to the endoplasmic reticulum (ER), via recognition by the signal recognition particle (reviewed in (Nothwehr and Gordon, 1990; von Heijne, 1990)). Once translocated across the membrane into the ER lumen, the protein can either be released from the membrane via cleavage of the signal peptide by signal peptidases or it can remain associated with the membrane (Coleman et al., 1985; von Heijne, 1990; Kapp et al., 2009). This process can happen co-translationally for larger proteins or post-translationally for smaller proteins (Meyer, 1991). Once in the ER, however, the protein may be secreted, stay in the ER, or be sorted into intracellular compartments like the Golgi, endosomes or lysosomes (von Heijne, 1990; Bonifacino and Traub, 2003; Tokarev et al., 2009). We were unable to identify any further localization signals that might indicate subcellular localization. Therefore, our data indicate that Bap1 likely resides in a membrane-bound compartment (or may be secreted) and may serve as a hub of molecular interactions (i.e., nucleic acid-protein or protein-protein) in the honey bee antiviral response. Further work to identify how Bap1 is limiting virus infection is an exciting avenue of research.

2.4 Bee antiviral protein-1 (*bap1*) is co-expressed with several honey bee immune genes

Genes with a coordinated function (e.g., act in antiviral defense) are often co-expressed. The honey bee antiviral gene *bap1* was first described and annotated in a transcriptome level study of honey bees infected with a model virus in the presence and absence of dsRNA species (Brutscher et al., 2017). This study assessed gene expression in individual bees over a time course (i.e., 6, 48, and 72 hours post-injection) as compared to mock-infected (i.e., buffer-injected) control bees and presented fold-change and statistical significance data independently for each annotated gene

at all time-points (Brutscher et al., 2017). To further evaluate the genes highly coexpressed with *bap1* we constructed a signed weighted gene correlation network from our previously published transcriptome dataset using the R package Weighted Gene Correlation Network Analysis (WGCNA) (Langfelder and Horvath, 2008; 2012; Brutscher et al., 2017). After filtering, WGCNA identified 39 modules, which are clusters of highly co-expressed genes. Select modules are discussed below and pairwise correlations between genes in the identified modules are presented in Supplemental Tables S13-S20. There were three gene modules that most correlated with virus (SINV) infection (i.e., #8, #15, and #38), and one module that best correlated with dsRNA treatment (i.e., #20) (Supplemental Figure S5). These modules are described below, and the genes and gene-gene correlations of additional modules are included in Supplementary Tables S17-S20.

Gene module #8 and SINV infection had a correlation coefficient of 0.56 ($p = 4x10^{-5}$) (Supplemental Figure S5). This is a small module with only 38 genes, therefore gene ontology analysis of this group was not possible, and most of the genes in this module have no known or predicted function. However, interestingly, several predicted non-coding RNAs (ncRNAs) belong to this module (e.g., LOC100578712, LOC102654940, LOC102655797, and mir3764) (Supplementary Table S13). Noncoding RNAs perform a wide range of functions, but most notably pre- and post-transcriptional regulation of gene expression through methylation or RNAi, respectively (Mattick, 2001; Jackson and Standart, 2007; Fernandes et al., 2019; Gil and Ulitsky, 2020). Non-coding RNAs may have either a pro- or anti-viral role (reviewed in (Bruscella et al.,

2017)). One gene in this module was IGFn3-6 (also known as DSCAM2-like), a gene in the immunoglobulin-like superfamily. DSCAM2 is a gene that undergoes extensive mutually exclusive alternative splicing to create a wide range of somatic diversity. It is involved in neuronal development in *Drosophila melanogaster* and DSCAMs have also been implicated in immunity, including potential roles in antiviral immunity (Millard et al., 2007; Lah et al., 2014; Armitage et al., 2015; Armitage et al., 2017). In honey bees (*A. mellifera syriaca)* DSCAM2 SNPs were also associated with *Varroa destructor* mite resistance phenotypes, and DSCAM exhibited higher expression in virus-infected bees than in control bees (Flenniken and Andino, 2013; Haddad et al., 2016).

Gene module #15 and SINV infection had a correlation coefficient of 0.54 ($p = 8x10^{-5}$)(Supplemental Figure S5). This module contains 122 genes, but since many of them lacked functional assignments, no significant gene ontology terms were detected (Supplemental Table S14). Like gene module #8, gene module #15 also included several non-coding RNAs (LOC100577428, LOC102655361, and LOC102654929), as well as genes involved in pathogen recognition (*pgrp-s2*), transcriptional repression (*snail*), cell morphogenesis (*trbl*), cell migration regulation (*PVF1*), and a gene involved in the anti-parasitoid response (*Flo2*). *Pgrp-s2* also exhibited increased expression in this data set in response to SINV infection. Pgrp-s2 is a member of a class of pathogen recognition receptors that activate the Toll or Imd pathway in response to pathogens, and it has also been implicated in antiviral immunity in *Bombyx mori* where it activates the Imd pathway (Zhao et al., 2018). The Toll and Imd pathways, which are

mediated by NF-kB-like transcription factors are both important for antiviral defense against certain viruses in *Drosophila melanogaster* and other insects (Zambon et al., 2005; Avadhanula et al., 2009; Costa et al., 2009) (reviewed in (Kingsolver et al., 2013)). The mechanism of activation of these pathways by virus infection is unclear and their precise role is not fully characterized, though Imd may contribute through regulation of apoptosis (Costa et al., 2009).

Gene module #38 and SINV infection had a correlation coefficient of 0.54 ($p = 1x10^{-4}$, Supplementary Figure S5). This module contains 481 genes, similar to other modules, gene ontology could not be assessed since numerous genes in this cluster lacked functional annotation. However, this module included genes involved in metabolism, transcription, translation, and immunity (Supplemental Table S15). The immune genes in this module included *bap1* (LOC725387), which was co-expressed with additional immune genes *tep7*, *tudor-SN*, and *ago-2* (Rand et al., 2004; Tomari et al., 2004; van Rij et al., 2006; Wang et al., 2006; Kim et al., 2007)(reviewed in (Kingsolver et al., 2013)). Thioester containing protein 7 (TEP7) is a member of the thioester containing protein family which includes vertebrate complement proteins (reviewed in (Blandin and Levashina, 2004)). In fruit flies and mosquitoes, *TEP* expression is likely regulated by JAK in response to bacterial infection and they function in bacterial recognition, opsonization and phagocytosis (reviewed in (Blandin and Levashina, 2004; Shokal and Eleftherianos, 2017). However, in *Aedes aegypti* TEP1 and TEP2 limit West Nile virus infection and TEP is indirectly involved in combatting Dengue virus (Cheng et al., 2011; Xiao et al., 2014). Lastly, Tudor-SN is a nuclease and a core component of RISC of Drosophila that is

also involved in cleavage of hyper-ADAR-edited dsRNAs (Caudy et al., 2003; Schwarz et al., 2004; Scadden, 2005). In summary, *bap1* is co-expressed with several genes that are implicated in antiviral immunity in several species (Figure 3C).

Gene module #20 and dsRNA-treatment were moderately associated with a correlation coefficient of 0.35 ($p = 0.02$) (Supplemental Figure S5, Supplemental Table S16). This module contained 806 genes and had significant enrichment for genes involved in nucleic acid binding ($p = 0.016$), metal ion binding ($p = 0.016$), and genes with a zinc finger C2H2-like domain ($p = 0.002$). These groups comprise proteins with a wide range of functions including protein, DNA, ssRNA, and dsRNA binding functions (reviewed in (Iuchi, 2001), sometimes interacting with both nucleic acids and proteins. The zinc finger C2H2 domain is by far the most common domain in metazoan transcription factors (reviewed in (Stubbs et al., 2011)), but their wide range of binding activities suggests functional diversity. Indeed, there are antiviral mammalian zinc finger nucleases which can either directly bind to viral nucleic acids and promote their degradation, or otherwise regulate the immune response (reviewed in (Wang and Zheng, 2021)).

2.5 Confirmation of *bap1* and a*go2* coexpression in honey bees

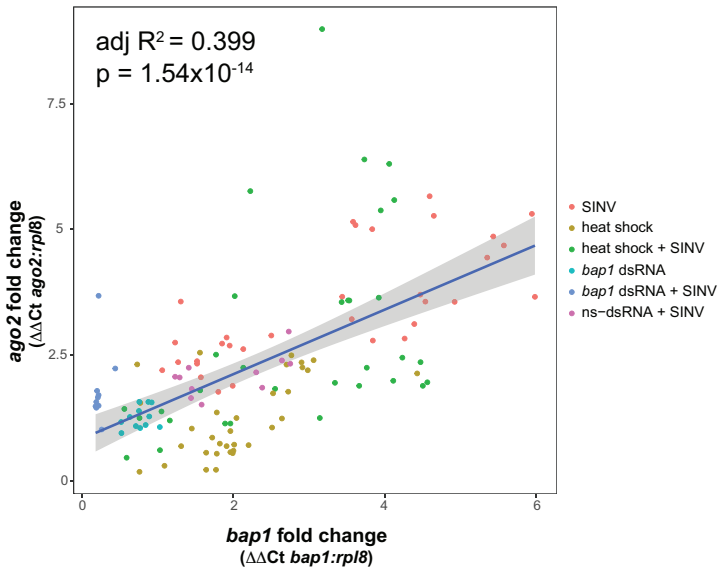
To confirm the co-expression of *bap1* and *ago2*, a post hoc analysis was performed on a dataset, composed of previously published data and data generated in his study (Figure 1B) (McMenamin et al., 2020). The previously published dataset was from a study that determined that the heat shock response in honey bees is antiviral (McMenamin et al., 2020). Specifically, expression data from individual bees that were (a) SINV-infected, (b) exposed to only heat shock (42°C for

4 hrs) only, or (c) both virus-infected and heat shocked (McMenamin et al., 2020). The association between *bap1* and *ago2* expression was analyzed using the following linear model:

$$ago2\ fold\ change \sim \beta_0 + \beta_1 * MF116383\ fold\ change * \beta_2 * SINV\ RNA\ copies$$

This model structure was selected because SINV and *ago2* expression are also weakly associated (adjusted $R^2 = 0.06$, $p = 0.003$, log likelihood = -233.4), and therefore including the interaction term significantly improved the model. The model indicates that *bap1* expression (fold change relative to control buffer-injected bees) is a good predictor of *ago2* expression (Adjusted $R^2 = 0.399$, $p = 1.55 \times 10^{-14}$, log likelihood = -203.3, Figure 4A). This model also indicates that SINV remains a weak predictor of *ago2* expression, which was expected since *ago2* expression is induced by SINV infection (Brutscher et al., 2017). See Table 2 for full model output.

A



B

Partial Pearson correlation coeficients	*ago2* fold change	*bap1* fold change	log_{10}(SINV)
ago2 fold change	1		
bap1 fold change	0.58	1	
log_{10}(SINV)	0.42	-0.12	1

Partial Pearson correlation p-values	*ago2* fold change	*bap1* fold change	log_{10}(SINV)
Ago2 fold change	0		
bap1 fold change	$8.42x10^{-12}$	0	
log_{10}(SINV)	$1.87x10^{-6}$	0.18	0

Figure 4. Expression of *bap1* and *ago2* is associated after accounting for the effect of virus abundance.
The relative expression of *bap1* and *ago2* in bees that received a variety of treatments was assessed by qPCR relative to mock infected bees. **(A)** *Bap1* and *ago2* fold changes are associated in bees that received a variety of treatments (adjusted $R^2 = 0.399$, $p = 1.54x10^{-14}$). **(B)** A partial Pearson's correlation coefficient was calculated to test for a correlation between *bap1* and *ago2* expression after accounting for the effect of virus (SINV) abundance. While there is a correlation between *ago2* and SINV level ($r = 0.42$, $p = 1.87x10^{-6}$) there was no correlation between *bap1* and SINV. After accounting for the effect of SINV on *ago2* expression, there is a strong correlation between *ago2* and *bap1* ($r = 0.58$, $p = 8.42x10^{-12}$).

Because there is multicollinearity in the model, partial Pearson's correlation coefficients were calculated to isolate the association between *bap1* and *ago2* expression from the effect of SINV RNA copies on either variable (Figure 4B). After accounting for the partial effect of log_{10}(SINV RNA copies) on *ago2* expression ($r = 0.42$, $p = 1.87x10^{-6}$) *bap1* expression has a high partial correlation with *ago2* expression ($r = 0.58$, $p = 8.42x10^{-12}$, Figure 4B). Therefore, *bap1* and *ago2* are likely co-regulated. It is intriguing to speculate that this co-expression could be due to

functions in a common pathway. Alternatively, it could simply be case of co-expression for a similar function (i.e., antiviral).

Table 2. Linear model output for the association between bap1 and ago2 expression. Ago2 expression was modeled as a function of bap1 expression coupled with SINV RNA copy number. The model indicates that, after accounting for the interaction between SINV RNA copies and bap1 expression, the expression of bap1 and ago2 are highly associated, and corroborated WGCNA analysis.

Term	**Estimate**	**Standard error**	**t-value**	**p-value**
Intercept	0.444	0.25	1.77	0.079
bap1 fold change	0.733	0.092	7.99	7.26×10^{-13}
SINV RNA copies	3.92×10^{-9}	1.39×10^{-9}	2.82	0.0056
*bap1**SINV	-7.23×10^{-10}	3.28×10^{-10}	-2.213	0.0287
multiple $R^2 = 0.413$, adjusted $R^2 = 0.399$, $F = 29.56_{df1=3, df2=126}$, $p = 1.54 \times 10^{-14}$				

3 Conclusions

Honey bee antiviral defense mechanisms include the Toll and Imd (NFkB) signaling pathways and dsRNA-triggered mechanisms, including sequence-specific RNAi and a non-sequence specific triggered pathway that is less well-characterized (Nazzi et al., 2012; Di Prisco et al., 2013; Flenniken and Andino, 2013; Chen et al., 2014; Ryabov et al., 2014; Galbraith et al., 2015; Niu et al., 2016a; Ryabov et al., 2016; Brutscher et al., 2017; Wang et al., 2017) (reviewed in (Brutscher et al., 2015; McMenamin et al., 2018). Transcriptome level analysis and in vivo experiments determined that *bee antiviral protein-1* (*bap1*, GenBank: MF116383) plays an important role in honey bee antiviral defense. Herein, we further characterized *bap1* using bioinformatic and phylogenetic approaches that revealed that *bap1* is taxonomically restricted to Hymenoptera and *B. germanica* and that it is evolving primarily under neutral selection, although some sites were under positive or negative selection. In line with those analyses,

structural analysis predicted that Bap1 is highly disordered and neutral evolution is common for disordered proteins. We hypothesize that, like other disordered proteins, Bap1 may have the capability to bind many different protein or nucleic acid targets and thus serve as a molecular interaction hub in the honey bee antiviral defense. Future work will determine whether regions of positive selection are particularly important for interfacing with binding partners (i.e., nucleic acids or protein), and identifying those binding partners. To begin to identify the pathways *bap1* may interact with, a weighted gene correlation network analysis was performed and determined *bap1* expression is highly correlated with several immune genes, including *ago2* which was confirmed in an independent dataset. Whether coexpression of *bap1* and *ago2* is due to coregulation by a shared pathway or simply due to their shared antiviral function remains to be elucidated. Overall, these data indicated that *bap1* is an exciting avenue of research as a novel antiviral gene. Identifying new genes and pathways involved in combatting virus infections in bees presents opportunities to develop strategies that mitigate future colony honey bee colony deaths due to virus infection.

4 Materials and Methods

4.1 Age-matched live honey bees

Honey bee (*Apis mellifera)* colonies were established from packages (~1.5 kg of worker bees and a naturally-mated queen) of primarily *Apis mellifera carnica* stock purchased from a commercial producer in Montana in April 2018. Honey bees were kept in Langstroth hives located on Montana State University's Horticulture Farm in Bozeman, MT, US. Colonies were maintained using standard apicultural practices, including bi-monthly evaluation of *Varroa destructor* mite

infestation levels using the powdered sugar roll method (Milbrath, 2018). Colonies were treated with formic acid polysaccharide gel strips (Mite Away Quick Strips®, Nature's Own Design Apiary Products) when mite infestation was greater than 3% (3 mites per 100 bees (Milbrath, 2018).

Honey bees for laboratory-based experiments were obtained from brood containing frames with newly emerging bees, which were collected one day prior to each experiment and maintained at 32˚C in a laboratory incubator overnight. Young, age-matched (~ 24 hours post-emergence), female adult bees were utilized for all experiments. For the duration of the experiment, honey bees were housed in modified deli-containers at 32˚C and fed bee candy (powdered sugar mixed with corn syrup until pliable) and water *ad libitum* (Evans et al., 2009; Flenniken and Andino, 2013; Brutscher et al., 2017; McMenamin et al., 2020).

4.2 Double stranded RNA preparation

Double stranded RNA was generated by *in vitro* transcription with T7 RNA polymerase (Saleh et al., 2009; Brutscher et al., 2017). T7 promoter-containing PCR-products were amplified using primers listed in Supplemental Table S1, with the following thermocycler program: pre-incubation of 95°C (5 min), 35 cycles of 95°C (30 s), 60°C (30 s), and 72°C (1 min) followed by a final incubation at 72°C (5 min). PCR products were used as template for T7 polymerase transcription (100 µl reactions: NTPs (each 7.5 mM final), RNase OUT (40 units) (Invitrogen),

buffer (400 mM HEPES pH 7.5, 120 mM $MgCl_2$, 10 mM spermidine, 200 mM DTT); reactions were carried out at 37°C overnight (8–10 hours). DNA was removed by adding 1 unit of RQ1 DNAse (Promega) and incubating for 15 minutes at 37°C. dsRNA products were ethanol precipitated with 1:10 volume 3M sodium acetate (pH 5.5), suspended in 200 µL RNase-free water, the RNA secondary structure was denatured via incubation at 100°C for 5 minutes, and then complementary RNA strands were annealed by slow cooling to room temperature. The dsRNA products were purified by phenol:chloroform extraction and subsequent ethanol precipitation with a 1:10 volume of 5M ammonium acetate. The precipitated dsRNAs were dissolved in 60-100 µL 10mM Tris HCl (pH 7.5). The dsRNA quality was assessed by agarose gel electrophoresis and spectrophotometry. The dsRNA was quantified based on the band intensity relative to a standard with ImageJ version 1.50i (Schneider et al., 2012).

4.3 Virus infection and heat shock protocol

Honey bee virus infections were established via intra-thoracic injections using glass needles made by pulling borosilicate glass capillary tubes (100 mm long, 1 mL capacity, Kimble-Chase) with a coil temperature of 61°C using a PC-10 Dual-Stage Glass Micropipette Puller (Narishige). Prior to injection, age-matched honey bees (~ 24-hours post-emergence) were cold anesthetized for 10 minutes at 4°C. Honey bees were infected with recombinant Sindbis virus expressing green fluorescent protein (SINV-GFP; 3,750 plaque forming units (pfu) in 2 µl 10 mM Tris HCl buffer pH 7.5) (Hahn et al., 1992; Flenniken and Andino, 2013; Brutscher et al., 2017) via intra-

thoracic injection using a Harbo syringe (Honey bee Insemination Service) and microcapillary glass needles; mock-infected bees were injected with 2 µL buffer (10 mM Tris HCl, pH 7.5).

Experimental treatment groups that were subjected to temperature stress were intrathoracically injected with either buffer or virus, allowed to recover for 6 hours at 32˚C, exposed to heat shock (i.e., 42°C for 4 hours), and then transferred back to 32˚C for the remainder of the study. The heat shock experiments were carried out using three independent honey bee cohorts obtained from three different colonies on distinct dates (i.e., replicate 1 in June 2018, replicate 2 in August 2018, and replicate 3 in July 2019). The data from these sampled are analyzed *post hoc* from a previously published manuscript (McMenamin et al., 2020).

4.4 RNA extraction

Honey bee samples were dissected into head, thorax, and abdomen. The abdomen was chosen for further analysis as it is the primary site of immune cell generating fat bodies and it is distal from the site of injection, and thus virus infection naturally spread to that tissue. Honey bee abdomens were homogenized in 300 µL of deionized water with a sterile steel ball (5 mm) using a Tissue Lyser II (Qiagen) at 30 Hz for 2 minutes. Then 300 µL of TRIzol reagent (Invitrogen) was added to the homogenate, vortexed for 15 seconds and incubated at room temperature for 5 minutes. Next, 60 µL of chloroform was added, samples were shaken by hand for 15 seconds and incubated on the benchtop for another 2 minutes. Samples were then centrifuged at 12,000 *xg* at 4°C for 15 minutes and the aqueous phase was transferred to a clean centrifuge tube. One

volume of isopropanol was added to the aqueous phase, mixed by inversion, and nucleic acid was precipitated by incubation at room temperature for 10 minutes. The precipitate was pelleted by centrifugation at 12,000 xg at 4°C for 10 minutes. Pellets were then washed with 500 µL of 75% ethanol and centrifuged at 7,500 *xg* at 4°C for 5 minutes, then air dried for 10 minutes at room temperature and dissolved in 30 µL of deionized water. RNA concentrations and quality were assessed on a Nanodrop 2000 spectrophotometer (Thermo Fisher). When quality was low, RNA was precipitated a second time by addition of four volumes of cold ethanol and 1:10 of a volume 3M sodium acetate (pH 5.5) and incubation at -20°C overnight. Nucleic acids were pelleted by centrifugation at 12,000 *xg* at 4°C for 10 minutes and pellets were washed one time with 500 µL 70% ethanol and centrifuged at 12,000 *xg* at 4°C for 5 minutes. Pellets were air dried and suspended in 30 µL dH_2O. Samples were stored at -80˚C until analysis.

4.5 Reverse transcription/cDNA synthesis

Reverse transcription reactions were performed by incubating 2,000 ng total RNA, 200 units M-MLV reverse-transcriptase (Promega) and 500 ng random hexamer primers (IDT) for 1 hour at 37°C, according to the manufacturer's instructions. cDNA was diluted 1:2 and 2 µL was used for PCR or qPCR analysis.

4.6 Quantitative polymerase chain reaction

Quantitative PCR (qPCR) was used to analyze the abundance of virus (i.e., SINV-GFP) and the relative abundances of honey bee immune gene and heat shock protein transcripts. All qPCR

reactions were performed in triplicate with 2 μL of cDNA template. Each 20 μL reaction contained 1× ChoiceTaq Mastermix (Denville), 0.4 μM each forward and reverse primer, 1× SYBR Green (Life Technologies), and 3 mM $MgCl_2$. A CFX Connect Real Time instrument (BioRad) was used for the following thermo-profile: pre-incubation 95°C for one minute followed by 40 cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 15 s, with a final melt curve analysis at 65°C for 5 s to 95°C.

To quantify viral RNA copy numbers in the samples, SINV-GFP plasmid standards were used as templates, with concentrations ranging from 10^3 to 10^9 copies per reaction to create a linear standard curve. The detection limit was 10^3 copies of the SINV cDNA using primers qSindbisFW4495 and qSindbisREV4635. The host gene *Am rpl8, Am mf116393* or *Am ago2* were amplified in triplicate for sample for comparison, using primers *Amrpl8*-Fw1 and *Am rpl8*-Rev1 (Supplementary Table S1). Reactions without template were carried out as negative controls. The qPCR specificity was verified through melt point analysis and via gel electrophoresis, and all products were previously verified by sequencing. The linear equation for the plasmid standard for SINV was: $Ct = -3.348x + 40.25$ ($R^2 = 0.996$, efficiency = 98.9%) where 'x' is the log(SINV genome equivalents). The relative expression of host genes was determined by a ranked ΔΔCt method in which the ΔCt was calculated by subtracting the *rpl8* Ct value from the Ct of the gene of interest. Then the ΔCt values were ranked to control for natural inter-individual variation in gene expression and the matching mock-infected ΔCt was subtracted from

the treatment group ΔCt to obtain the ΔΔCt. The fold-change in cDNA abundance was calculated by the equation $2^{-\Delta\Delta Ct}$.

4.7 Phylogenetic Analysis

Bee antiviral protein-1 (*bap1*) (GenBank: MF116383) encodes an antiviral protein. The precise function of Bap1 has yet to be elucidated. Phylogenetic analyses of *bap1* were performed to gain functional insight. To perform a phylogenetic analysis, amino acid and nucleic acid sequences were acquired from NCBI nonredundant protein sequence database by searching using NCBI PSI BLAST function and exporting any sequence with an e-value below 0.001. An amino acid alignment was generated in MEGAX (Kumar et al., 2018) using MUSCLE with default parameters.

A majority rule Bayesian consensus tree of Bap1 orthologues was derived from Bayesian analysis of amino acid alignment implemented in Mr. Bayes v3.2 using a Jones substitution model. Metropolis-coupled Markov Chain Monte Carlo (MCMC) permutation of parameters were initiated with a random tree and involved two runs each with 16 chains set at default temperatures (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Markov chains were run for 10,000,000 generations and sampled every 10,000 generations. MrBayes identified this sampling rate and a 25% burn-in as sufficient because of the non-autocorrelation of adjacently sampled trees and the complete convergence of the two separate MCMC runs at likelihood stationarity. Numbers on nodes are posterior probabilities (0-1), though posterior

probabilities of 1 were not shown to improve figure clarity. The scale bar corresponds to proportion of amino acid change per site.

To perform PAML analysis, nucleic acid accession numbers were retrieved by creating a local BLAST database containing all of the taxa on the amino acid tree and performing BLAST analyses of all the amino acid sequences against the BLASTn database (Altschul et al., 1990; Altschul et al., 1997). Nucleic acid sequences were retrieved by submitting the accession numbers to NCBI's batch ENTREZ (2006). Sequences were again aligned by codon in MEGAX using MUSCLE with default parameters. Majority rule Bayesian consensus trees of *bap1* and *RPB1* orthologues were derived from a Bayesian analysis of a nucleic acid codon alignments implemented in Mr. Bayes v3.2 using the protein nucleic acid model. In this model, MrBayes first translates codons into protein and then infers relationships. Metropolis-coupled Markov Chain Monte Carlo (MCMC) permutation of parameters were initiated with a random tree and involved two runs each with 16 chains set at default temperatures (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Markov chains were run for 5,000,000 generations and sampled every 10,000 generations. MrBayes identified this sampling rate and a 25% burn-in as sufficient because of the non-autocorrelation of adjacently sampled trees and the complete convergence of the two separate MCMC runs at likelihood stationarity. Numbers on nodes are posterior probabilities (0-1), though posterior probabilities of 1 were left off to improve clarity. Generated trees were then partially edited in FigTree v 1.4.3 (Rambaut and Drummond, 2021) and Adobe Illustrator to further improve clarity. The scale bar corresponds to proportional

changes per codon site. Accession numbers are included on the branch tips and in Supplemental Tables S4-S5.

The codon alignments and the majority rule Bayesian consensus trees generated with this method were then used for selection analysis using the CODEML package in Phylogenetic Analysis by Maximum Likelihood (PAML) 4.9 (Yang, 2007). Relative rates of nonsynonymous substitution (parameter ω) were estimated using fixed branch lengths for models M0, M1, and M2a. All models were run twice to check convergence. The loglikelihood ratio test (LRT) statistic was calculated as twice the log likelihood difference between a test and null models (2ΔlnL) which was then compared against the χ^2 distribution with critical values of 3.841 (M1-M0), 5.99 (M2-M1) and 7.814 (M2a-M0) at an α level of 0.05. PAML's Bayes Empirical Bayes (BEB) method was used on M2a to calculate the posterior probability that each amino site along Bap1 belonged to each site class ($\omega<1$, $\omega=1$, $\omega>1$). The dN/dS ratios calculated by the BEB method at each site containing an amino acid were then plotted as a stacked bar chart (Figure 2B and Supplemental Figure S2B). Site-specific dN/dS ratios were plotted and those with posterior probability of belonging to a given site class greater than an arbitrary cutoff of 0.8 were color coded as evolving under positive (red), neutral (blue) or negative (green) selection (Supplemental Figures S3 and S4). Raw BEB probabilities and dN/dS ratios listed in Supplemental Tables S5 and S8.

4.8 Honey bee RNAseq data and analysis

Honey bee transcriptome sequence data were generated and described in Brutscher et al 2017 (Brutscher et al., 2017). Sequence data were deposited into NCBI Sequence Read Archive under

accession number SRP101337 and is linked with NCBI BioProject #PRJNA377749. In brief, RNA samples obtained from individual honey bees (n= 47) from bees that were mock-infected, SINV-infected, dsRNA-treated, SINV+non-specific dsRNA, treated and SINV+ specific dsRNA treated. Samples were processed and the normalized number of Fragments Per Kilobase of transcript per Million mapped reads (FPKM) was determined using CuffDiff as described in Brutscher et al (Trapnell et al., 2012; Trapnell et al., 2013; Brutscher et al., 2017) (Supplementary Table S10). FPKM values were $\log_2$ transformed and used for Weighted Gene Correlation Network Analysis (WGCNA).

4.9 Weighted gene correlation network analysis

To identify genes are that co-expressed with *bap1* a weighted gene correlation network was constructed using the package WGCNA (Langfelder and Horvath, 2008; 2012) in R 4.0.2 from data analyzed *post hoc* (Brutscher et al., 2017). WGCNA uses an unsupervised hierarchical clustering algorithm that groups genes into modules based on similarity of expression. In this case WGCNA analysis was performed on $\log_2$ transformed transcript FPKM values from three individuals in several treatment groups at several time points as described above. Recommended settings were used for the analysis (Langfelder and Horvath, 2008; 2012). However, briefly, minimum module size was set to 20 and deep split was set to 2. A signed correlation matrix was generated using default parameters, and a signed gene correlation network was constructed using a soft power threshold of 10. Modules were merged based on a branch cut height of 0.25. Trait data were used to identify module eigengenes differentially expressed in response to various traits (e.g., SINV RNA copies or whether or not the individual was injected with dsRNA) and

plotted as a heat map (Supplemental Figure S5; Supplemental Tables S13-S20). The module that MF116383 belonged to was manually identified and pared down to include only the strongest connections with correlations above 0.5 or below -0.5. Then this network was imported into Cytoscape v 3.8.2 (Shannon et al., 2003) for visualization and the *bap1* subnetwork was isolated manually (Supplemental Figure S6). For clarity, genes with no predicted function were removed from the network (Figure 3C). All remaining connections in this subnetwork are positive correlations above 0.5.

4.10 Statistical analysis

All statistical analyses were carried out in R 4.0.2, except the likelihood-ratio test statistics to compare models of selection which were calculated manually by subtracting the null model from the test model and multiplying by 2 ($2\Delta lnL$). ANOVAs and linear models were constructed using the base R package (Team, 2016; Team, 2019). Partial Pearson's Correlation coefficients were calculated using the *ppcor* package in R (Kim, 2015) Because our dataset contains zero values in our SINV measurement (untreated bees), SINV levels were $\log_{10}$ transformed prior to calculating correlation coefficients to improve homoscedasticity and improve normality.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

Conceptualization, A.J.M., and M.L.F.; methodology, A.J.M., K.F.D and M.L.F.; formal analysis A.J.M. and M.L.F.; investigation, A.J.M., L.B., K.F.D and M.L.F.; resources, M.L.F.; data curation, A.J.M., L.B., and M.L.F.; writing—original draft preparation, A.J.M., and M.L.F.; writing—review and editing, A.J.M. and M.L.F.; visualization, A.J.M. and M.L.F.; supervision, M.L.F.; project administration, M.L.F.; funding acquisition, M.L.F. All authors have read and agreed to the published version of the manuscript.

7 Funding

The Flenniken laboratory is supported by the National Science Foundation CAREER Program (Award Number 1651561), Montana Department of Agriculture Specialty Crop Block Grant Program, Hatch Multistate Funding (NC-1173), and Project Apis m.-Costco Scholar Fellowship Program for Honey Bee Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of this manuscript.

8 Data availability statement

The majority of the data generated or analyzed during this study are included in this published article and its Supplementary Materials files (available online). Additional data are available from the corresponding author upon request.

9 Acknowledgments

We are grateful to Dr. Matt Lavin for discussions throughout the experiments. We would also like to thank the members of Flenniken laboratory (Fenali Parekh, Brian Ross and Naomi Kaku) for reviewing this manuscript prior to publication.

References

(2006). "Batch Entrez," in Encyclopedic Reference of Genomics and Proteomics in Molecular Medicine. (Berlin, Heidelberg: Springer Berlin Heidelberg), 131-131.

Almagro Armenteros, J.J., Tsirigos, K.D., Sønderby, C.K., Petersen, T.N., Winther, O., Brunak, S., et al. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol 37(4), 420-423. doi: 10.1038/s41587-019-0036-z.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J Mol Biol 215(3), 403-410. doi: 10.1016/s0022-2836(05)80360-2.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25(17), 3389-3402. doi: 10.1093/nar/25.17.3389.

Armitage, S.A., Peuss, R., and Kurtz, J. (2015). Dscam and pancrustacean immune memory - a review of the evidence. Dev Comp Immunol 48(2), 315-323. doi: 10.1016/j.dci.2014.03.004.

Armitage, S.A.O., Kurtz, J., Brites, D., Dong, Y., Du Pasquier, L., and Wang, H.C. (2017). Dscam1 in Pancrustacean Immunity: Current Status and a Look to the Future. Front Immunol 8, 662. doi: 10.3389/fimmu.2017.00662.

Avadhanula, V., Weasner, B.P., Hardy, G.G., Kumar, J.P., and Hardy, R.W. (2009). A Novel System for the Launch of Alphavirus RNA Synthesis Reveals a Role for the Imd Pathway in Arthropod Antiviral Response. PLOS Pathogens 5(9), e1000582. doi: 10.1371/journal.ppat.1000582.

Blandin, S., and Levashina, E.A. (2004). Thioester-containing proteins and insect immunity. Molecular Immunology 40(12), 903-908. doi: https://doi.org/10.1016/j.molimm.2003.10.010.

Bonifacino, J.S., and Traub, L.M. (2003). Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 72**,** 395-447. doi: 10.1146/annurev.biochem.72.121801.161800.

Brennan, C.A., and Anderson, K.V. (2004). Drosophila: The Genetics of Innate Immune Recognition and Response. Annual Review of Immunology 22**,** 457-483. doi: 10.1146/annurev.immunol.22.012703.104626.

Bronkhorst, A.W., and van Rij, R.P. (2014). The long and short of antiviral defense: small RNA-based immunity in insects. Current Opinion in Virology 7**,** 19-28. doi: https://doi.org/10.1016/j.coviro.2014.03.010.

Brown, C.J., Johnson, A.K., and Daughdrill, G.W. (2010). Comparing models of evolution for ordered and disordered proteins. Mol Biol Evol 27(3)**,** 609-621. doi: 10.1093/molbev/msp277.

Brown, C.J., Johnson, A.K., Dunker, A.K., and Daughdrill, G.W. (2011). Evolution and disorder. Curr Opin Struct Biol 21(3)**,** 441-446. doi: 10.1016/j.sbi.2011.02.005.

Bruscella, P., Bottini, S., Baudesson, C., Pawlotsky, J.M., Feray, C., and Trabucchi, M. (2017). Viruses and miRNAs: More Friends than Foes. Front Microbiol 8**,** 824. doi: 10.3389/fmicb.2017.00824.

Brutscher, L.M., Daughenbaugh, K.F., and Flenniken, M.L. (2015). Antiviral defense mechanisms in honey bees. Current Opinion in Insect Science 2**,** 1-12. doi: 10.1016/j.cois.2015.04.016.

Brutscher, L.M., Daughenbaugh, K.F., and Flenniken, M.L. (2017). Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. Scientific Reports 7**,** 6448. doi: 10.1038/s41598-017-06623-z.

Brutscher, L.M., and Flenniken, M.L. (2015). RNAi and antiviral defense in the honey bee. Journal of Immunology Research 2015. doi: 10.1155/2015/941897.

Buchon, N., Silverman, N., and Cherry, S. (2014). Immunity in Drosophila melanogaster-from microbial recognition to whole-organism physiology. Nature Reviews Immunology 14**,** 796-810. doi: 10.1038/nri3763.

Cappelle, K., Smagghe, G., Dhaenens, M., and Meeus, I. (2016). Israeli Acute Paralysis Virus Infection Leads to an Enhanced RNA Interference Response and Not Its Suppression in the Bumblebee Bombus terrestris. Viruses 8(12). doi: 10.3390/v8120334.

Caudy, A.A., Ketting, R.F., Hammond, S.M., Denli, A.M., Bathoorn, A.M.P., Tops, B.B.J., et al. (2003). A micrococcal nuclease homologue in RNAi effector complexes. Nature 425(6956)**,** 411-414. doi: 10.1038/nature01956.

Chejanovsky, N., Ophir, R., Schwager, M.S., Slabezki, Y., Grossman, S., and Cox-Foster, D. (2014). Characterization of viral siRNA populations in honey bee colony collapse disorder. Virology 454-455**,** 176-183. doi: 10.1016/j.virol.2014.02.012.

Chen, Y.P., Pettis, J.S., Corona, M., Chen, W.P., Li, C.J., Spivak, M., et al. (2014). Israeli Acute Paralysis Virus: Epidemiology, Pathogenesis and Implications for Honey Bee Health. PLOS Pathogens 10(7)**,** e1004261. doi: 10.1371/journal.ppat.1004261.

Cheng, G., Liu, L., Wang, P., Zhang, Y., Zhao, Y.O., Colpitts, T.M., et al. (2011). An In Vivo Transfection Approach Elucidates a Role for Aedes aegypti Thioester-Containing Proteins in Flaviviral Infection. PLOS ONE 6(7)**,** e22786. doi: 10.1371/journal.pone.0022786.

Coleman, J., Inukai, M., and Inouye, M. (1985). Dual functions of the signal peptide in protein transfer across the membrane. Cell 43(1)**,** 351-360. doi: https://doi.org/10.1016/0092-8674(85)90040-6.

Cooper, M.D., and Alder, M.N. (2006). The evolution of adaptive immune systems. Cell 124**,** 815-822. doi: 10.1016/j.cell.2006.02.001.

Costa, A., Jan, E., Sarnow, P., and Schneider, D. (2009). The Imd pathway is involved in antiviral immune responses in Drosophila. PLoS ONE 4. doi: 10.1371/journal.pone.0007436.

Daugherty, M.D., and Malik, H.S. (2012). Rules of Engagement: Molecular Insights from Host-Virus Arms Races. Annual Review of Genetics 46(1)**,** 677-700. doi: 10.1146/annurev-genet-110711-155522.

Deddouche, S., Matt, N., Budd, A., Mueller, S., Kemp, C., Galiana-Arnoux, D., et al. (2008). The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in drosophila. Nature Immunology 9(12)**,** 1425-1432. doi: 10.1038/ni.1664.

Desai, S.D., Eu, Y.J., Whyard, S., and Currie, R.W. (2012). Reduction in deformed wing virus infection in larval and adult honey bees (Apis mellifera L.) by double-stranded RNA ingestion. Insect Molecular Biology 21**,** 446-455. doi: 10.1111/j.1365-2583.2012.01150.x.

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., et al. (2013). Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. Proceedings of the National Academy of Sciences of the United States of America 110**,** 18466-18471. doi: 10.1073/pnas.1314923110.

Dunker, A.K., Cortese, M.S., Romero, P., Iakoucheva, L.M., and Uversky, V.N. (2005). Flexible nets. The roles of intrinsic disorder in protein interaction networks. Febs j 272(20)**,** 5129-5148. doi: 10.1111/j.1742-4658.2005.04948.x.

Dyson, H.J., and Wright, P.E. (2002). Coupling of folding and binding for unstructured proteins. Curr Opin Struct Biol 12(1), 54-60. doi: 10.1016/s0959-440x(02)00289-0.

Eilers, E.J., Kremen, C., Smith Greenleaf, S., Garber, A.K., and Klein, A.-M. (2011). Contribution of Pollinator-Mediated Crops to Nutrients in the Human Food Supply. PLOS ONE 6(6), e21363. doi: 10.1371/journal.pone.0021363.

Elsik, C.G., Worley, K.C., Bennett, A.K., Beye, M., Camara, F., Childers, C.P., et al. (2014). Finding the missing honey bee genes: lessons learned from a genome upgrade. BMC Genomics 15(1), 86. doi: 10.1186/1471-2164-15-86.

Evans, J.D., Chen, Y.P., Prisco, G.d., Pettis, J., and Williams, V. (2009). Bee cups: single-use cages for honey bee experiments. Journal of Apicultural Research 48(4), 300-302. doi: 10.1080/00218839.2009.11101548.

Fernandes, J.C.R., Acuña, S.M., Aoki, J.I., Floeter-Winter, L.M., and Muxel, S.M. (2019). Long Non-Coding RNAs in the Regulation of Gene Expression: Physiology and Disease. Noncoding RNA 5(1). doi: 10.3390/ncrna5010017.

Ferrandon, D., Imler, J.L., Hetru, C., and Hoffmann, J.A. (2007). The Drosophila systemic immune response: Sensing and signalling during bacterial and fungal infections. Nature Reviews Immunology 7, 862-874. doi: 10.1038/nri2194.

Flenniken, M.L., and Andino, R. (2013). Non-Specific dsRNA-Mediated Antiviral Response in the Honey Bee. PLoS ONE 8, 1-16. doi: 10.1371/journal.pone.0077263.

Gabler, F., Nam, S.Z., Till, S., Mirdita, M., Steinegger, M., Söding, J., et al. (2020). Protein Sequence Analysis Using the MPI Bioinformatics Toolkit. Curr Protoc Bioinformatics 72(1), e108. doi: 10.1002/cpbi.108.

Galbraith, D.A., Yang, X., Nino, E.L., Yi, S., and Grozinger, C. (2015). Parallel epigenomic and transcriptomic responses to viral infection in honey bees (Apis mellifera). PLoS Pathog 11(3), e1004713. doi: 10.1371/journal.ppat.1004713.

Galiana-Arnoux, D., Dostert, C., Schneemann, A., Hoffmann, J.A., and Imler, J.-L. (2006). Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. Nature Immunology 7(6), 590-597. doi: 10.1038/ni1335.

Gallai, N., Salles, J.-M., Settele, J., and Vaissière, B.E. (2009). Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological Economics 68, 810-821. doi: 10.1016/j.ecolecon.2008.06.014.

Gertz, E. (2005). BLAST Scoring Parameters.

Gil, N., and Ulitsky, I. (2020). Regulation of gene expression by cis-acting long non-coding RNAs. Nature Reviews Genetics 21(2), 102-117. doi: 10.1038/s41576-019-0184-5.

Haddad, N., Mahmud Batainh, A., Suleiman Migdadi, O., Saini, D., Krishnamurthy, V., Parameswaran, S., et al. (2016). Next generation sequencing of Apis mellifera syriaca identifies genes for Varroa resistance and beneficial bee keeping traits. Insect Science 23(4), 579-590. doi: https://doi.org/10.1111/1744-7917.12205.

Hahn, C.S., Hahn, Y.S., Braciale, T.J., and Rice, C.M. (1992). Infectious Sindbis virus transient expression vectors for studying antigen processing and presentation. Proceedings of the National Academy of Sciences 89(7), 2679. doi: 10.1073/pnas.89.7.2679.

Higurashi, M., Ishida, T., and Kinoshita, K. (2008). Identification of transient hub proteins and the possible structural basis for their multiple interactions. Protein science : a publication of the Protein Society 17(1), 72-78. doi: 10.1110/ps.073196308.

Hoffmann, J.A. (2003). The immune response of Drosophila. Nature 426, 33-38. doi: 10.1038/nature02021.

Huelsenbeck, J.P., and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17(8), 754-755. doi: 10.1093/bioinformatics/17.8.754.

Ishida, T., and Kinoshita, K. (2007). PrDOS: prediction of disordered protein regions from amino acid sequence. Nucleic Acids Res 35(Web Server issue), W460-464. doi: 10.1093/nar/gkm363.

Iuchi, S. (2001). Three classes of C2H2 zinc finger proteins. Cellular and Molecular Life Sciences CMLS 58(4), 625-635. doi: 10.1007/PL00000885.

Jackson, R.J., and Standart, N. (2007). How Do MicroRNAs Regulate Gene Expression? Science's STKE 2007(367), re1. doi: 10.1126/stke.3672007re1.

Johnson, L.S., Eddy, S.R., and Portugaly, E. (2010). Hidden Markov model speed heuristic and iterative HMM search procedure. BMC Bioinformatics 11(1), 431. doi: 10.1186/1471-2105-11-431.

Kapp, K., Schrempf, S., Lemberg, M., and Bernhard, D. (2009). "Post-Targeting Functions of Signal Peptides.," in Protein Transport into the Endoplasmic Reticulum. (Madame Curie Bioscience Database [Internet]: Landes Bioscience).

Kim, K., Lee, Y.S., and Carthew, R.W. (2007). Conversion of pre-RISC to holo-RISC by Ago2 during assembly of RNAi complexes. RNA 13(1), 22-29. doi: 10.1261/rna.283207.

Kim, P.M., Sboner, A., Xia, Y., and Gerstein, M. (2008). The role of disorder in interaction networks: a structural analysis. Molecular systems biology 4**,** 179-179. doi: 10.1038/msb.2008.16.

Kim, S. (2015). ppcor: An R Package for a Fast Calculation to Semi-partial Correlation Coefficients. Communications for statistical applications and methods 22(6)**,** 665-674. doi: 10.5351/CSAM.2015.22.6.665.

Kingsolver, M.B., and Hardy, R.W. (2012). Making connections in insect innate immunity. Proceedings of the National Academy of Sciences of the United States of America 109**,** 18639-18640. doi: 10.1073/pnas.1216736109.

Kingsolver, M.B., Huang, Z., and Hardy, R.W. (2013). Insect antiviral innate immunity: Pathways, effectors, and connections. Journal of Molecular Biology 425**,** 4921-4936. doi: 10.1016/j.jmb.2013.10.006.

Klein, A.-M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.a., Kremen, C., et al. (2007). Importance of pollinators in changing landscapes for world crops. Proceedings. Biological sciences / The Royal Society 274**,** 303-313. doi: 10.1098/rspb.2006.3721.

Koleske, A.J., and Young, R.A. (1995). The RNA polymerase II holoenzyme and its implications for gene regulation. Trends Biochem Sci 20(3)**,** 113-116. doi: 10.1016/s0968-0004(00)88977-x.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 35(6)**,** 1547-1549. doi: 10.1093/molbev/msy096.

Lah, Grace J.-e., Li, Joshua Shing S., and Millard, S.S. (2014). Cell-Specific Alternative Splicing of Drosophila Dscam2 Is Crucial for Proper Neuronal Wiring. Neuron 83(6)**,** 1376-1388. doi: https://doi.org/10.1016/j.neuron.2014.08.002.

Langfelder, P., and Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 9(1)**,** 559. doi: 10.1186/1471-2105-9-559.

Langfelder, P., and Horvath, S. (2012). Fast R Functions for Robust Correlations and Hierarchical Clustering. 2012 46(11)**,** 17. doi: 10.18637/jss.v046.i11.

Leonard, S.P., Powell, J.E., Perutka, J., Geng, P., Heckmann, L.C., Horak, R.D., et al. (2020). Engineered symbionts activate honey bee immunity and limit pathogens. Science 367(6477)**,** 573. doi: 10.1126/science.aax9039.

Lin, Y.S., Hsu, W.L., Hwang, J.K., and Li, W.H. (2007). Proportion of solvent-exposed amino acids in a protein and rate of protein evolution. Mol Biol Evol 24(4), 1005-1011. doi: 10.1093/molbev/msm019.

Liu, X., Zhang, Y., Yan, X., and Han, R. (2010). Prevention of chinese sacbrood virus infection in apis cerana using rna interference. Current Microbiology 61, 422-428. doi: 10.1007/s00284-010-9633-2.

Lozano, J., Gomez-Orte, E., Lee, H.-J., and Belles, X. (2012). Super-induction of Dicer-2 expression by alien double-stranded RNAs: an evolutionary ancient response to viral infection? Development Genes and Evolution 222(4), 229-235. doi: 10.1007/s00427-012-0404-x.

Maori, E., Paldi, N., Shafir, S., Kalev, H., Tsur, E., Glick, E., et al. (2009). IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. Insect Molecular Biology 18, 55-60.

Mattick, J.S. (2001). Non-coding RNAs: the architects of eukaryotic complexity. EMBO reports 2(11), 986-991. doi: 10.1093/embo-reports/kve230.

McMenamin, A.J., Daughenbaugh, K.F., and Flenniken, M.L. (2020). The Heat Shock Response in the Western Honey Bee (Apis mellifera) is Antiviral. Viruses 12(2), 245. doi: 10.3390/v12020245.

McMenamin, A.J., Daughenbaugh, K.F., Parekh, F., Pizzorno, M.C., and Flenniken, M.L. (2018). Honey Bee and Bumble Bee Antiviral Defense. Viruses 10(8), 395. doi: 10.3390/v10080395.

Meyer, D.I. (1991). Protein translocation into the endoplasmic reticulum: a light at the end of the tunnel. Trends in Cell Biology 1(6), 154-159. doi: https://doi.org/10.1016/0962-8924(91)90016-3.

Milbrath, M. (2018). Varroa Mite Monitoring: Using a sugar roll to identify populations of Varroa destructor in honey bee colonies [Online]. pollinators.msu.edu. Available: https://pollinators.msu.edu/resources/beekeepers/varroa-mite-monitoring1/ [Accessed 2 January 2020].

Millard, S.S., Flanagan, J.J., Pappu, K.S., Wu, W., and Zipursky, S.L. (2007). Dscam2 mediates axonal tiling in the Drosophila visual system. Nature 447(7145), 720-724. doi: 10.1038/nature05855.

Misof, B., Liu, S., Meusemann, K., Peters, R.S., Donath, A., Mayer, C., et al. (2014). Phylogenomics resolves the timing and pattern of insect evolution. Science 346(6210), 763. doi: 10.1126/science.1257570.

Müller, W.E.G., and Müller, I.M. (2003). Origin of the metazoan immune system: identification of the molecules and their functions in sponges. Integrative and comparative biology 43**,** 281-292. doi: 10.1093/icb/43.2.281.

Myles, K.M., Wiley, M.R., Morazzani, E.M., and Adelman, Z.N. (2008). Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. Proceedings of the National Academy of Sciences 105(50)**,** 19938. doi: 10.1073/pnas.0803408105.

Nazzi, F., Brown, S.P., Annoscia, D., Del Piccolo, F., Di Prisco, G., Varricchio, P., et al. (2012). Synergistic Parasite-Pathogen Interactions Mediated by Host Immunity Can Drive the Collapse of Honeybee Colonies. PLOS Pathogens 8(6)**,** e1002735. doi: 10.1371/journal.ppat.1002735.

Niu, J., Meeus, I., Cappelle, K., Piot, N., and Smagghe, G. (2014). The immune response of the small interfering RNA pathway in the defense against bee viruses. Current Opinion in Insect Science 6**,** 22-27. doi: 10.1016/j.cois.2014.09.014.

Niu, J., Meeus, I., Coninck, D.I.M.D., Deforce, D., Etebari, K., Asgari, S., et al. (2017). Infections of virulent and avirulent viruses differentially influenced the microRNAs in Bombus terrestris. Nature Publishing Group**,** 1-11. doi: 10.1038/srep45620.

Niu, J., Meeus, I., and Smagghe, G. (2016a). Differential expression pattern of Vago in bumblebee (Bombus terrestris), induced by virulent and avirulent virus infections. Scientific Reports 6**,** 34200. doi: 10.1038/srep34200.

Niu, J., Smagghe, G., De Coninck, D.I., Van Nieuwerburgh, F., Deforce, D., and Meeus, I. (2016b). In vivo study of Dicer-2-mediated immune response of the small interfering RNA pathway upon systemic infections of virulent and avirulent viruses in Bombus terrestris. Insect Biochem Mol Biol 70**,** 127-137. doi: 10.1016/j.ibmb.2015.12.006.

Nothwehr, S.F., and Gordon, J.I. (1990). Targeting of proteins into the eukaryotic secretory pathway: signal peptide structure/function relationships. Bioessays 12(10)**,** 479-484. doi: 10.1002/bies.950121005.

Obbard, D.J., Jiggins, F.M., Halligan, D.L., and Little, T.J. (2006). Natural Selection Drives Extremely Rapid Evolution in Antiviral RNAi Genes. Current Biology 16(6)**,** 580-585. doi: https://doi.org/10.1016/j.cub.2006.01.065.

Palmer, W.H., Hadfield, J.D., and Obbard, D.J. (2018). RNA-Interference Pathways Display High Rates of Adaptive Protein Evolution in Multiple Invertebrates. Genetics 208(4)**,** 1585-1599. doi: 10.1534/genetics.117.300567.

Paterson, S., Vogwill, T., Buckling, A., Benmayor, R., Spiers, A.J., Thomson, N.R., et al. (2010). Antagonistic coevolution accelerates molecular evolution. Nature 464(7286)**,** 275-278. doi: 10.1038/nature08798.

Peters, R.S., Krogmann, L., Mayer, C., Donath, A., Gunkel, S., Meusemann, K., et al. (2017). Evolutionary History of the Hymenoptera. Current Biology 27(7), 1013-1018. doi: https://doi.org/10.1016/j.cub.2017.01.027.

Piot, N., Snoeck, S., Vanlede, M., Smagghe, G., and Meeus, I. (2015). The effect of oral administration of dsRNA on viral replication and mortality in Bombus terrestris. Viruses 7, 3172-3185. doi: 10.3390/v7062765.

Pitaluga, A.N., Mason, P.W., and Traub-Cseko, Y.M. (2008). Non-specific antiviral response detected in RNA-treated cultured cells of the sandfly, Lutzomyia longipalpis. Developmental & Comparative Immunology 32(3), 191-197. doi: https://doi.org/10.1016/j.dci.2007.06.008.

Poirier, E.Z., Goic, B., Tomé-Poderti, L., Frangeul, L., Boussier, J., Gausson, V., et al. (2018). Dicer-2-Dependent Generation of Viral DNA from Defective Genomes of RNA Viruses Modulates Antiviral Immunity in Insects. Cell Host Microbe 23(3), 353-365.e358. doi: 10.1016/j.chom.2018.02.001.

Rambaut, A., and Drummond, A. (2021). "FigTree version 1.4.0".).

Rand, T.A., Ginalski, K., Grishin, N.V., and Wang, X. (2004). Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity. Proc Natl Acad Sci U S A 101(40), 14385-14389. doi: 10.1073/pnas.0405913101.

Ronald, P.C., and Beutler, B. (2010). Plant and Animal Sensors of Conserved Microbial Signatures. Science 330, 1061-1064. doi: 10.1126/science.1189468.

Ronquist, F., and Huelsenbeck, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19(12), 1572-1574. doi: 10.1093/bioinformatics/btg180.

Ryabov, E.V., Fannon, J.M., Moore, J.D., Wood, G.R., and Evans, D.J. (2016). The Iflaviruses Sacbrood virus and Deformed wing virus evoke different transcriptional responses in the honeybee which may facilitate their horizontal or vertical transmission. PeerJ 4, e1591. doi: 10.7717/peerj.1591.

Ryabov, E.V., Wood, G.R., Fannon, J.M., Moore, J.D., Bull, J.C., Chandler, D., et al. (2014). A Virulent Strain of Deformed Wing Virus (DWV) of Honeybees (Apis mellifera) Prevails after Varroa destructor-Mediated, or In Vitro, Transmission. PLoS Pathogens 10. doi: 10.1371/journal.ppat.1004230.

Saleh, M.C., Tassetto, M., van Rij, R.P., Goic, B., Gausson, V., Berry, B., et al. (2009). Antiviral immunity in Drosophila requires systemic RNA interference spread. Nature 458(7236), 346-350. doi: 10.1038/nature07712.

Scadden, A.D.J. (2005). The RISC subunit Tudor-SN binds to hyper-edited double-stranded RNA and promotes its cleavage. Nature Structural & Molecular Biology 12(6)**,** 489-496. doi: 10.1038/nsmb936.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9(7)**,** 671-675. doi: 10.1038/nmeth.2089.

Schwarz, D.S., Tomari, Y., and Zamore, P.D. (2004). The RNA-induced silencing complex is a Mg2+-dependent endonuclease. Curr Biol 14(9)**,** 787-791. doi: 10.1016/j.cub.2004.03.008.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome research 13(11)**,** 2498-2504. doi: 10.1101/gr.1239303.

Shokal, U., and Eleftherianos, I. (2017). Evolution and Function of Thioester-Containing Proteins and the Complement System in the Innate Immune Response. Frontiers in immunology 8**,** 759-759. doi: 10.3389/fimmu.2017.00759.

Spolar, R.S., and Record, M.T., Jr. (1994). Coupling of local folding to site-specific binding of proteins to DNA. Science 263(5148)**,** 777-784. doi: 10.1126/science.8303294.

Stubbs, L., Sun, Y., and Caetano-Anolles, D. (2011). "Function and Evolution of C2H2 Zinc Finger Arrays," in A Handbook of Transcription Factors.: Springer, Dordrecht).

Suzuki, Y., Baidaliuk, A., Miesen, P., Frangeul, L., Crist, A.B., Merkling, S.H., et al. (2020). Non-retroviral Endogenous Viral Element Limits Cognate Virus Replication in Aedes aegypti Ovaries. Current Biology 30(18)**,** 3495-3506.e3496. doi: https://doi.org/10.1016/j.cub.2020.06.057.

Tassetto, M., Kunitomi, M., and Andino, R. (2017). Circulating Immune Cells Mediate a Systemic RNAi-Based Adaptive Antiviral Response in Drosophila. Cell 169**,** 314-325.e313. doi: 10.1016/j.cell.2017.03.033.

Team, R. (2016). "RStudio: Integrated Development Environment for R". 1.1.456 ed. (Vienna, Austria: RStudio, Inc.).

Team, R.C. (2019). "R: A language and environment for statistical computing.". R Foundation for Statistical Computing).

Tokarev, A.A., Alfonso, A., and Segev, N. (2009). "Intracellular Compartments and Trafficking Pathways," in Trafficking Inside Cells: Pathways, Mechanisms and Regulation. (Madame Curie Bioscience Database [Internet]).

Tomari, Y., Matranga, C., Haley, B., Martinez, N., and Zamore, P.D. (2004). A Protein Sensor for siRNA Asymmetry. Science 306(5700), 1377. doi: 10.1126/science.1102755.

Trapnell, C., Hendrickson, D.G., Sauvageau, M., Goff, L., Rinn, J.L., and Pachter, L. (2013). Differential analysis of gene regulation at transcript resolution with RNA-seq. Nature Biotechnology 31(1), 46-53. doi: 10.1038/nbt.2450.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., et al. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature Protocols 7, 562-578. doi: 10.1038/nprot.2012.016.

van Rij, R.P., Saleh, M.-C., Berry, B., Foo, C., Houk, A., Antoniewski, C., et al. (2006). The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes & Development 20(21), 2985-2995. doi: 10.1101/gad.1482006.

von Heijne, G. (1990). The signal peptide. J Membr Biol 115(3), 195-201. doi: 10.1007/bf01868635.

Wang, G., and Zheng, C. (2021). Zinc finger proteins in the host-virus interplay: multifaceted functions based on their nucleic acid-binding property. FEMS Microbiol Rev 45(3). doi: 10.1093/femsre/fuaa059.

Wang, H., Meeus, I., and Smagghe, G. (2016). Israeli acute paralysis virus associated paralysis symptoms, viral tissue distribution and Dicer-2 induction in bumblebee workers (Bombus terrestris). Journal of General Virology 97(8), 1981-1989. doi: https://doi.org/10.1099/jgv.0.000516.

Wang, H., Smagghe, G., and Meeus, I. (2017). The role of a single gene encoding the Single von Willebrand factor C-domain protein (SVC) in bumblebee immunity extends beyond antiviral defense. Insect Biochemistry and Molecular Biology 91, 10-20. doi: https://doi.org/10.1016/j.ibmb.2017.10.002.

Wang, X.-H., Aliyari, R., Li, W.-X., Li, H.-W., Kim, K., Carthew, R., et al. (2006). RNA Interference Directs Innate Immunity Against Viruses in Adult Drosophila. Science 312, 452-454. doi: 10.1126/science.1125694.

Wright, P.E., and Dyson, H.J. (2014). Intrinsically disordered proteins in cellular signalling and regulation. Nature Reviews Molecular Cell Biology 16, 18. doi: 10.1038/nrm3920.

Xia, Y., Franzosa, E.A., and Gerstein, M.B. (2009). Integrated Assessment of Genomic Correlates of Protein Evolutionary Rate. PLOS Computational Biology 5(6), e1000413. doi: 10.1371/journal.pcbi.1000413.

Xiao, X., Liu, Y., Zhang, X., Wang, J., Li, Z., Pang, X., et al. (2014). Complement-Related Proteins Control the Flavivirus Infection of Aedes aegypti by Inducing Antimicrobial Peptides. PLOS Pathogens 10(4), e1004027. doi: 10.1371/journal.ppat.1004027.

Yang, Z. (2007). PAML 4: Phylogenetic Analysis by Maximum Likelihood. Molecular Biology and Evolution 24(8), 1586-1591. doi: 10.1093/molbev/msm088.

Zambon, R.A., Nandakumar, M., Vakharia, V.N., and Wu, L.P. (2005). The Toll pathway is important for an antiviral response in Drosophila. Proc Natl Acad Sci U S A 102(20), 7257-7262. doi: 10.1073/pnas.0409181102.

Zambon, R.A., Vakharia, V.N., and Wu, L.P. (2006). RNAi is an antiviral immune response against a dsRNA virus in Drosophila melanogaster. Cellular Microbiology 8(5), 880-889. doi: 10.1111/j.1462-5822.2006.00688.x.

Zhao, P., Xia, F., Jiang, L., Guo, H., Xu, G., Sun, Q., et al. (2018). Enhanced antiviral immunity against Bombyx mori cytoplasmic polyhedrosis virus via overexpression of peptidoglycan recognition protein S2 in transgenic silkworms. Developmental & Comparative Immunology 87, 84-89. doi: https://doi.org/10.1016/j.dci.2018.05.021.

Zimmermann, L., Stephens, A., Nam, S.Z., Rau, D., Kübler, J., Lozajic, M., et al. (2018). A Completely Reimplemented MPI Bioinformatics Toolkit with a New HHpred Server at its Core. J Mol Biol 430(15), 2237-2243. doi: 10.1016/j.jmb.2017.12.007.

CHAPTER SIX

INVESTIGATING VIRUS-HOST INTERACTIONS IN CULTURES PRIMARY HONEY BEE CELLS

Contributions of authors and Co-authors

Manuscript in Chapter Six

Author: Alexander J. McMenamin

Contributions: Acquired funding, designed the study, collected and analyzed data, interpreted the results, wrote and revised the manuscript

Author: Fenali Parekh

Contributions: Designed the study, collected and analyzed data, interpreted the results, wrote and revised the manuscript

Co-author: Verana Lawrence

Contributions: Collected and analyzed data, interpreted results, revised manuscript

Co-author: Michelle L. Flenniken

Contributions: Supervised the study, acquired funding, designed the study, interpreted the results, wrote and revised the manuscript

Manuscript Information

Alexander J. McMenamin*, Fenali Parekh*, Verena Lawrence, Michelle L. Flenniken

*These authors contributed equally to this work

Investigating virus-host interactions in cultured primary honey bee cells

Insects

Status of Manuscript
___ Prepared for submission to a peer-reviewed journal
___ Officially submitted to a peer-reviewed journal
___ Accepted by a peer-reviewed journal
_X__ Published in a peer-reviewed journal

29 June 2021. 12(7), 653; https://doi.org/10.3390/insects12070653

Investigating Virus–Host Interactions in Cultured Primary Honey Bee Cells

Alexander J. McMenamin [1,2,3,†], Fenali Parekh [1,2,3,†], Verena Lawrence [1,3] and Michelle L. Flenniken [1,2,3,*]

[1] Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717, USA; alexmcmenamin2@gmail.com (A.J.M.); fenali10@gmail.com (F.P.); verena.cml@gmail.com (V.L.)

[2] Department of Microbiology and Cell Biology, Montana State University, Bozeman, MT 59717, USA

[3] Pollinator Health Center, Montana State University, Bozeman, MT 59717, USA

* Correspondence: michelle.flenniken@montana.edu; Tel.: +1-406-994-7229

† These authors contributed equally to this work.

Simple Summary: Honey bees are eusocial insects that live in colonies comprised of ~30,000 individuals. They are the primary pollinators of plants that produce fruits, nuts, and vegetables. High annual losses of honey bee colonies have made it challenging for beekeepers to provide enough colonies to meet the demand for pollination services. Multiple stressors, including viruses, contribute to colony deaths. To better understand viral pathogenesis and bee antiviral defense mechanisms, we obtained primary cells from honey bee larvae and pupae, maintained them in culture, and infected them with a panel of viruses. We determined that larval hemocytes, which are primary immune cells, support replication of a honey bee virus (sacbrood virus) and a model virus (Flock House virus). Similarly, we determined that mixed cell populations derived from honey bee pupae support replication of sacbrood virus, Flock House virus, and another honey bee virus (deformed wing virus). Evaluation of the host cellular level response to infections with each of these viruses revealed unique expression profiles of three immune genes. In summary, this study demonstrates the utility of honey bee

primary cell cultures to investigate the impacts of virus infection on honey bees at the cellular level, which in turn affects individual bee and colony health.

Abstract:

Honey bee (*Apis mellifera*) health is impacted by viral infections at the colony, individual bee, and cellular levels. To investigate honey bee antiviral defense mechanisms at the cellular level we further developed the use of cultured primary cells, derived from either larvae or pupae, and demonstrated that these cells could be infected with a panel of viruses, including common honey bee infecting viruses (i.e., sacbrood virus (SBV) and deformed wing virus (DWV)) and an insect model virus, Flock House virus (FHV). Virus abundances were quantified over the course of infection. The production of infectious virions in cultured honey bee pupal cells was demonstrated by determining that naïve cells became infected after the transfer of deformed wing virus or Flock House virus from infected cell cultures. Initial characterization of the honey bee antiviral immune responses at the cellular level indicated that there were virus-specific responses, which included increased expression of *bee antiviral protein-1* (GenBank: MF116383) in SBV-infected pupal cells and increased expression of *argonaute-2* and *dicer-like* in FHV-infected hemocytes and pupal cells. Additional studies are required to further elucidate virus-specific honey bee antiviral defense mechanisms. The continued use of cultured primary honey bee cells for studies that involve multiple viruses will address this knowledge gap.

Keywords: honey bee; *Apis mellifera*; primary cell culture; insect antiviral defense; honey bee viruses; RNA viruses; insect viruses

---

1. Introduction

All organisms are infected by viruses, which are obligate intracellular pathogens that rely on host cell machinery to replicate. In turn, hosts evolve mechanisms to prevent and/or limit virus infections. To investigate host–virus interactions at the cellular and molecular levels, scientists rely extensively on cell cultures, including immortalized cell lines and primary cells [1–3]. Cultured cells are amenable to techniques that augment or silence gene expression (e.g., transfection of plasmids and nucleic acids) and are thus invaluable to studies aimed at elucidating biological mechanisms. Furthermore, cell cultures are also utilized to produce pure virus stocks, which are important for experimentation and vaccine production [4–7]. In spite of their utility and importance, robust cell culture systems are not available for many non-model organisms, including honey bees (*Apis mellifera*). While approximately 1,000 cell lines have been derived from insect tissues, including 19 hymenopteran cell lines (the insect order that includes bees, ants, and wasps), only one immortalized honey bee cell line, AmE-711, has been established [8–10].

The development of additional tools to understand honey bee host–virus interactions at the cellular level is critical for determining the impact of virus infections at the individual bee and colony levels and may ultimately result in the development of strategies that reduce the number of virus-associated honey bee colony deaths. Honey bee colony losses averaged 38% annually from 2010

to 2018, in part due to viral infections [11–19]. Honey bees are cosmopolitan agricultural pollinators that are impacted by numerous stressors, including agrochemicals, lack of quality forage and habitat, colony and habitat management, the *Varroa destructor* mite, and pathogens including bacteria, fungi, trypanosomatids, and viruses [20–31]. The largest group of honey-bee-infecting pathogens are small RNA viruses (reviewed in [23,32]). The list of honey bee-infecting and honey-bee-associated viruses is ever growing, in part due to virus discovery efforts aided by the increased use of high throughput sequencing of either total RNA or virally enriched RNA sample sequencing [33–41] (reviewed in [23,24]). The most ubiquitous and well-studied honey-bee-associated viruses include acute bee paralysis virus [42], black queen cell virus [43], chronic bee paralysis virus [44,45], deformed wing virus [46,47], Israeli acute paralysis virus [48], Kashmir bee virus [49], sacbrood virus [50], and the Lake Sinai virus group [40,51]. Viral infections can result in various symptoms, from paralysis to deformity or shortened lifespan, but individual bees can also harbor high viral loads (~100 billion copies per bee) and show no apparent symptoms [40,52–54].

The *Iflaviridae* family comprises several arthropod-associated viruses, including two honey-bee-infecting viruses (i.e., sacbrood virus and deformed wing virus) [55–58]. Sacbrood virus (SBV) infects both larvae and adults and is likely spread throughout a colony by adult nurse bees that ingest infected larvae, develop SBV infections, including in their hypopharyngeal glands, and transmit the virus to new larvae by feeding them contaminated brood food [59,60]. While SBV may shorten the lifespan of adult bees, it is most pathogenic to young larvae, which become discolored and yellow as the infection progresses. As the infected larva develops, its cuticle

becomes leathery, which prevents molting, and fluid builds up, creating a saclike appearance [58]. Concurrent with larvae production, SBV prevalence typically peaks in honey bee colonies in the spring and summer months [40,61–64]. Deformed wing virus (DWV) infects both developing and adult bees. Infection of larvae or pupae can cause wing deformities, likely due to infection of nascent wing buds [65–68]. DWV infection of adults is associated with short, bloated abdomens, shortened life span, and precocious foraging, while reducing total foraging time [69–72]. Infection of the brain is sometimes associated with increased aggression, reduced associative learning, and downregulation of genes associated with neuronal signaling, suggesting a dysregulation of normal brain function [73–75]. Deformed wing virus can be acquired vertically through contamination of eggs via infected ovarian tissue or DWV-positive semen or horizontally via mating, contaminated food (i.e., brood food or pollen), trophallaxis, pupal cannibalism, or the bite of a *Varroa destructor* mite [76–81] (reviewed in [82]). Varroa mites, which primarily feed on the honey bee fat body, serve as passive and/or replication-competent viral vectors [66,83–87]. DWV strains include DWV-A, DWV-B/VDV-1, which share approximately 84% nucleotide identity, and DWV-C, as well as recombinant viruses [88–93]. DWV is one of the most well-studied honey bee viruses, and high DWV levels, coupled with mite infestation, are associated with honey bee colony losses [94–98]. Recent development of DWV infectious clones will further our understanding of this virus and its interactions with bee and mite hosts [65,78,99]. In the absence of infectious clones, scientists have utilized crude virus preparations obtained from infected honey bees, including at the larval and pupal stages [10,68,86,100–104].

Model viruses, including Sindbis virus and Flock House virus, have also been utilized to investigate honey bee antiviral defense mechanisms [4,105–109]. These viruses are relatively easily to propagate and purify and are important tools for investigating antiviral response across a range of different species, including honey bees, fruit flies, and mosquitos [4,105–112]. Flock House virus (FHV) is a small, nonenveloped, icosahedral insect virus in the *Nodaviridae* family. FHV is genetically and structurally well-characterized and has been extensively utilized in studies aimed at understanding basic viral processes, including genome replication and virion assembly [113–120]. FHV is also a valuable model virus for investigating insect antiviral immunity. Although FHV was originally isolated from grass grubs (*Costelytra Zealandica*), it infects a range of insects, including mosquitoes, fruit flies, tsetse flies, and honey bees [105,106,110,111,121,122]. To counteract the host immune defense systems, several invertebrate viruses have evolved viral suppressors of RNAi (VSR), and RNA-based strategies (i.e., decoy molecules or miRNAs) (reviewed in [123]). Suppressors of RNAi are virus-encoded proteins that either inhibit the RNAi machinery (e.g., Dicer, Argonaute) or bind viral RNAs and protect them from Dicer-mediated cleavage or loading onto the RNA-induced silencing complex (RISC) (reviewed in [124–126]). The FHV-encoded B2 protein is a VSR that suppresses RNAi-mediated virus silencing by binding both long double-stranded RNAs (dsRNAs) and short interfering RNAs (siRNAs). Binding of FHV-B2 to long dsRNA, including viral replicative intermediates, protects them from cleavage, whereas high affinity binding of B2 to siRNAs prevents their incorporation into RISC, both of which result in suppression of the host RNAi response [113,127,128].

Similar to other insects, honey bee antiviral defense involves numerous processes and pathways, including autophagy, endocytosis, apoptosis, eicosanoid signaling, melanization, and the Toll and Imd (Immune Deficiency) pathways involving NF-κB (Nuclear Factor κB) homologues Dorsal and Relish, JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription), JNK (c-Jun N-terminal kinase), MAPK (Mitogen-Activated Protein Kinases), and RNA interference (RNAi) pathways (reviewed in [23,129–131]). RNA interference is a sequence-specific post-transcriptional gene silencing mechanism that is induced upon recognition of long double-stranded RNA (dsRNA) (endogenous or virally derived) by the endonuclease Dicer. Dicer cleaves dsRNA into 21–22 bp double-stranded short interfering RNAs (siRNAs). Then, in a process mediated by heat shock proteins, a single strand, called the guide strand is retained by Argonaute-2, within the RNA-induced silencing complex (RISC) [132,133]. RISC surveils the cell, binds RNA complementary to the guide strand, and cleaves it [108,112,124,130,131,134–139]. In honey bees and bumble bees, dsRNA, a virus-associated molecular pattern, also induces a non-sequence-specific antiviral immune response that limits virus infections [107–109,140–144]. To date, investigations of bee antiviral defense have been performed primarily in pupae and adult bees [95,96,101,105–109,138–143,145–150] (reviewed in [23,129]). Further development and use of honey bee cell cultures will enable more mechanistic studies of bee virology and immunology.

There is only one immortalized honey bee cell line, AmE-711, which was established from honey bee embryonic tissue [9] (reviewed in [8]). This cell line was used to study the dynamics of several co-infecting honey-bee-infecting viruses, including sacbrood virus (SBV), deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), black queen cell virus (BQCV), and Kashmir bee

virus (KBV) [10]. In that study, AmE-711 cells were inoculated with a mixed virus preparation that included varying levels of SBV, DWV, IAPV, and BQCV. In inoculated cells, IAPV outcompeted SBV, DWV, and BQCV; however, when cells were infected with a different inoculum that contained KBV, SBV, IAPV, and BQCV, KBV outcompeted all other viruses, including IAPV [10]. While an important step towards the development of immortalized honey bee cell lines, AmE-711 has a long doubling time, is difficult to maintain in other labs, and it is persistently infected with DWV [10]. Therefore, the use of non-honey-bee cells lines, including the Lepidopteran hemocytic cell line (P1), to study honey-bee-infecting viruses, including DWV, have been explored and will likely serve as an important tool for investigating bee viruses at a cellular level [8,151]. While immortalized cell cultures provide a convenient and consistent tool, they often do not fully recapitulate in situ biology. For example, the immune response that AmE-711, a honey bee fibroblast-type cell line, mounts against viruses may not represent the immune response in other honey bee tissues or cell types (i.e., fat body, endothelium, or hemocytes).

Primary honey bee cell cultures derived from different tissues provide a useful alternative to immortalized cell lines for the study of basic honey bee virology and immunology (reviewed in [152]). There are several advantages and disadvantages associated with the use of either immortalized cell lines or primary cells. For example, primary cultures may harbor preexisting infections, while immortalized cells may be persistently infected; therefore, it is best to carry out pathogen testing to ensure that the results obtained are not impacted by other infections [153–160]. Beneficial features of tissue-derived primary cell cultures include that they can contain diverse cell types or be enriched for one cell type. In addition, they can be generated from pooled tissue

samples to control for the inter-individual variability associated with whole-animal experiments. Primary cell cultures that represent multiple individuals may be particularly important for generating robust data that is representative of outbred honey bee colonies comprising individuals from numerous unique patrilines. Primary cell cultures established from whole pupae, with differentiated head, thorax, and abdomen body segments, likely include numerous adipocytes and epithelial cells as well as more specialized cells including neurons and muscle cells. These mixed cell cultures may be beneficial for investigating virus-tropism and intercellular signaling in response to virus infection. Similarly, the establishment and use of primary cell cultures from specific tissues or cell types facilitates a more precise mechanistic-level understanding of host–virus interactions [3,161,162]. Hemocytes are the primary insect immune cells that phagocytose or encapsulate pathogens. There are several subtypes of hemocytes, which are characterized morphologically, histochemically, and/or functionally. These include prohemocytes, granulocytes, plasmatocytes, spherulocytes, and oenocytoids, which may be further distinguished based on functional diversity [163]. Honey bee hemocytes also exhibit functional and morphological heterogeneity. The majority of circulating phagocytic cells in bee larvae are granulocytes, whereas plasmatocytes are more prevalent in adults [164,165]. There may be additional infection-induced cell types not yet described, like lamellocytes in *Drosophila melanogaster*, which are induced upon infestation by a parasitoid wasp [166]. In addition to their heterogeneity in phagocytic ability, honey bee hemocytes differ in their expression of hemolectin, which is associated with an encapsulation response of foreign bodies [167]. While recent studies in *Drosophila melanogaster* demonstrate that hemocytes play a role in systemic antiviral immune response, the potential for

hemocytes to be infected by viruses or the extent to which they may contribute to honey bee antiviral immunity is not known [96,163,168–171].

To investigate honey bee antiviral defense mechanisms at the cellular level, we further developed the use of cultured primary cells, derived from either larvae or pupae, and demonstrated that these cells could be infected with a panel of viruses, including common honey bee infecting viruses (i.e., SBV and DWV) and a model virus (FHV). Specifically, hemocytes obtained from stage L4-L5 honey bee larvae supported infection and replication of SBV and FHV. Virus replication was verified via detection of the replicative intermediate of both viruses using negative-strand-specific reverse transcription (RT), followed by polymerase chain reaction (PCR), and using quantitative PCR (qPCR) to demonstrate increased virus abundance over an infection time course. Mixed cell populations derived from purple-eyed honey bee pupae supported replication of SBV, DWV, and FHV. Virus replication was verified via negative-strand-specific RT-PCR and qPCR. Peak virus abundance was observed at 96 h post-infection (hpi) for SBV and DWV, while virus abundance peaked at 72 hpi for FHV. Furthermore, the production of infectious virions in cultured honey bee pupal cells was demonstrated by determining that naïve cells became infected after the transfer DWV or FHV infected cell culture material. Initial characterization of the honey bee antiviral immune responses mounted against SBV, DWV, and FHV at the cellular level indicate varying virus-specific responses, including increased expression of *bee antiviral protein-1* (GenBank: MF116383) in SBV-infected pupal cells and increased expression of *argonaute-2* and *dicer-like* in FHV-infected hemocytes and pupal cells [107,172]. Additional studies utilizing primary honey

bee cell cultures and multiple viruses will help elucidate virus-specific antiviral defense mechanisms.

2. Materials and Methods

2.1. Honey Bee Colonies

Packages (~1.5 kg of worker bees and a mated queen) of Carniolan honey bees (*Apis mellifera carnica*) were purchased from a commercial producer in Montana and installed in Langstroth hives at Montana State University, Bozeman, MT, USA. Colonies were maintained using standard apicultural practices, including bi-monthly *Varroa destructor* mite checks by the sugar roll method and treatment for mites with Mite Away Quick Strips (Nature's Own Design Apiary Products, Frankford, ON, Canada) when mite infestation was evaluated to be greater than 3% (3 mites per 100 bees [173,174]).

2.2. Honey Bee Cell Cultures

2.2.1. Hemocytes

Three days before experiments using primary honey bee hemocytes were performed, frames of brood with a lot of 4th and 5th instar larvae (L4–L5) were brought to the laboratory and maintained in a humidified incubator at 28 °C. Larvae that fell out of their cells were caught in a glass dish and collected in batch the next morning. After collection, larvae were surface-sterilized for 3 min in 0.6% hypochlorite (dilute bleach), followed by 3 min in 70% ethanol, and then briefly washed in sterile water for injection (Gibco) in a biological safety cabinet (Class II type A/B3, Nuaire). Larvae were placed into 200 µL of WH2 media (pH 6.4, 150 mL Schneider's insect medium,

SIGMA cat# S0146; 200 mL 0.06M L-Histidine, SIGMA cat# H5659; 50 mL heat-inactivated fetal bovine serum, Life Technologies cat# 16000044; 15 mL CMRL medium, Gibco cat# 11530-037; 5 mL Hank's balanced salts, SIGMA cat# H9394; 2 mL insect media supplement, SIGMA cat# I7267), supplemented with 1× penicillin/streptomycin (ThermoFisher, cat# 15070063), 10 at a time [33,175]. Sterile 18-guage needles were used to wound larvae on the lateral cuticle of the abdomen. Each larva was wounded once on each side and exsanguinated directly into the media. Care was taken to minimize fat body release from the carcass to produce cultures with minimal adipocyte contamination (Supplemental Figure S1). Hemocytes were pooled in 15 mL conical tubes (Falcon, Corning) until enough were collected for each experiment. Hemocytes were then seeded in vacuum gas plasma treated polystyrene 48-well plates (Falcon, Corning). On average, six larvae provided enough hemocytes (~1×10^5 cells per well) for five wells on a 48-well plate.

To test for virus association with carcasses versus hemocytes, an independent set of larvae was obtained from two different colonies, and sterile 18-guage needles were used to wound individual larva ($n = 37$) on the lateral cuticle of the abdomen. Each larva was wounded one on each side and exsanguinated. Hemolymph was directly collected by pipetting (approximately 40 µL). Hemolymph was diluted with 100 µL WH2 media; then, hemocytes were pelleted by centrifuging at 1000× *g* for 10 min. Supernatant was removed, and hemocytes were suspended in 100 µL WH2 media. Then, 800 µL of TRIzol® reagent (Invitrogen) was added to the hemocytes, and they were stored at −80 °C until RNA extraction.

2.2.2. Pupal Cells

Two days prior to honey bee pupal cell experiments, a brood frame containing purple-eyed pupae was brought into the lab. Fine-point, curved-tip forceps (Bioquip) were used to carefully remove the wax capping from pupae. Wide-tip, featherweight forceps (Bioquip) were used to gently grasp pupae between the eyes to remove them from the wax comb cells in which they develop. Pupae were incubated at 28 °C overnight in 12-well plates, and wounded (i.e., melanized) pupae were discarded. Primary cells were harvested from surface-sterilized honey bee pupae in a biosafety cabinet (Class II type A/B3, Nuaire). Surface sterilization was carried out in a sterile polystyrene petri dish (100 mm × 15 mm, Fisher), in which pupae were swirled in 0.6% hypochlorite solution (diluted bleach) for 3 min, 70% ethanol for 3 min, and briefly in sterile water for injection (Gibco). In groups of two, pupae were dissected into head, thorax, and abdomen segments in 2 mL WH2 medium in a 47 mm dish using sterile 18-gauge needles to vigorously disturb tissues and release cells into the medium [175]. The media containing the cells was then transferred to a 50 mL conical tube, and cells from individual pupa (~24 × 10^6 cells) were pooled. Cell suspensions with approximately 10^6 cells per 300 μL were plated into each well of a 48-well plate and incubated at room temperature overnight before infection.

2.3. Virus Preparation and Infection

2.3.1. Sacbrood Virus

A stock of sacbrood virus was prepared from five symptomatic larvae, which were homogenized in 10 mM Tris (pH 7.5) using a sterile steel ball (5 mm) in a 2 mL microcentrifuge tube, using a

Tissue Lyser II (Qiagen) at 30 Hz for 2 min. Cell debris was pelleted by centrifugation at 10,000× *g* for 10 min at 4 °C, and the clarified lysate was sequentially filtered through a 0.45 μm filter and then a 0.2 μm filter to remove small debris and microbes. To characterize this virus preparation, RNA was isolated from 100 μL using TRIzol® reagent (as described below), and cDNA template was used for pathogen-specific PCR and qPCR. PCR confirmed the presence of SBV and the absence of other common honey bee viruses, including ABPV, BQCV, CBPV, DWV, IAPV, KBV, and LSVs (Supplemental Figure S11). Quantitative PCR was used to determine the relative abundance of SBV, using SBV RNA copy number as a proxy for virus abundance, while recognizing that in this crude virus preparation, RNA copies may represent both RNA genomes and transcripts. The SBV inoculum contained 5900 copies/ng RNA. Each well of a 48-well plate was inoculated with 2.5×10^6 SBV RNA copies.

2.3.2. Deformed Wing Virus

DWV inoculum was prepared according to standard methods [176]. In brief, white-eyed pupae were carefully removed from the brood comb and collected into a petri dish lined with filter paper dampened with sterile water. Microcapillary glass needles for injections were made by pulling borosilicate glass capillary tubes (100 mm long, 1 mL capacity, Kimble-Chase) with a coil temperature of 61 °C on the PC-10 Dual-Stage Glass Micropipette Puller (Narishige). Pupae were injected with DWV (3.41×10^7 DWV RNA copies in 2 μL) between the 2nd and 3rd integuments of the abdomen using the borosilicate glass needle and a Harbo syringe (Honey bee Insemination Service). Pupae were incubated at 30 °C in a humid incubator over the course of infection and examined daily to ensure they were alive. Live pupae were harvested at 10 days post-injection,

and individual pupae were homogenized in 1 mL PBS (pH 7.4) using a sterile steel ball (5 mm) and Tissue Lyser II (Qiagen) at 30 Hz for 2 min. Cell debris was pelleted by centrifugation at 10,000× *g* for 10 min at 4 °C. To characterize this virus preparation, RNA was isolated from 100 µL using TRIzol® reagent (Invitrogen) (as described below), and cDNA template was used for pathogen-specific PCR and qPCR. PCR confirmed the presence of DWV and the absence of other common honey bee viruses, including ABPV, BQCV, CBPV, IAPV, KBV, LSVs, and SBV (Supplemental Figure S12). Quantitative PCR was used to determine the relative abundance of DWV, using virus RNA copy number as a proxy for virus abundance, while recognizing that in this crude virus preparation, RNA copies may represent both RNA genomes and transcripts. The DWV inoculum contained 5.48×10^7 DWV RNA copies/µL. For DWV infection studies, approximately 10^6 pupal cells in 300 µL (per well in a 48-well plate) were inoculated with 4.3×10^6 DWV RNA copies.

2.3.3. Flock House Virus

FHV was propagated in *Drosophila melanogaster* Schneider 2 (S2) cells (Invitrogen), an immortalized cell line originally derived from *D. melanogaster* embryos. S2 cells were grown in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and 1% penicillin-streptomycin. A sterile T75 flask was seeded with 4×10^7 S2 cells/mL and infected with FHV at a multiplicity of infection of 1 pfu/cell, as provided by Dr. Anette Schneemann (The Scripps Research Institute, La Jolla, CA, USA). The virus stock was quantified via plaque assay by the Schneemann lab using previously described protocols [177]. The S2 cells infected using this aliquot were incubated at 28 °C for 48 h, lysed by addition of 10%

(*v*/*v*) Nonidet P-40, and incubated on ice for 10 min with periodic swirling [178]. Cell debris was pelleted at 13,800× *g* for 10 min at 4 °C, and the clarified supernatant was transferred to a fresh tube. Using an ultracentrifuge (Beckman Coulter), virus was pelleted through 1 mL volume of 30% (*wt*/*wt*) sucrose in 50 mM HEPES (pH 7) at 40,000 rpm for 2.5 h at 11 °C in a SW41 Ti swinging-bucket rotor. The resulting pellets were resuspended in 0.5 mL buffer (HEPES 50 mM, pH 7), and any remaining insoluble material was removed by centrifugation at 10,000 rpm for 10 min. The clarified supernatant was layered on a continuous sucrose gradient (40%, 35%, 30%, 25%, 20%, 15%, and 10% (*wt*/*wt*)) and centrifuged at 40,000 rpm for 1.5 h at 11 °C to sediment the virus between 25–35% sucrose gradients. RNA was isolated from 100 µL virus preparations (as described below), and FHV abundance was quantified by qPCR, with the copy number based on a standard curve. For the infection experiments described herein, hemocytes were infected with 1×10^6 FHV RNA copies, and pupal cells were infected with 2×10^8 FHV RNA copies. FHV infection experiments were monitored over a time course from 0 h to 96 h post-infection.

2.4. Transfer of Virus Infection from Infected to Naïve Primary Honey Bee Pupal Cells

Small amounts of virus-infected cell culture, including cells and culture supernatant, from initial infections were transferred to naïve pupal cells, seeded in 48-well plates as described above. Primary purple-eyed honey bee pupal cells were seeded at approximately 10^6 cells per 300 µL media and incubated at room temperature overnight before infection. Each well was infected with 10 µL of mock- or virus-infected pupal cell culture. The resulting infected cells were incubated at room temperature for 0 h, 48 h, and 72 h post-infection.

2.5. RNA Isolation

RNA was isolated from cultured adherent hemocytes or non-adherent pupal cells/cell culture media at indicated time points using TRIzol® reagent (Invitrogen) according to manufacturer's instructions. In brief, cell culture medium from each well (300 µL) was collected into a microcentrifuge tube. Then, 300 µL of TRIzol® reagent (Invitrogen) was added to each well, incubated for 1 min, and then combined with the corresponding cell culture media sample. Samples were stored at −80 °C until RNA extraction. To complete RNA isolation, an additional 450 µL of TRIzol was added to each sample, which was vortexed and incubated at room temperature for 5 min. Then, 160 µL of chloroform was added, and samples were shaken by hand (15 s) and incubated at room temperature (2 min). Samples were centrifuged at 12,000× *g* for 15 min, and the aqueous phase was transferred to a clean microcentrifuge tube. An equal volume of isopropanol (~550 µL) and 20 µg of glycogen (Thermo Scientific) were added to the aqueous phase, which was then mixed by inverting. RNA was precipitated after overnight incubation (~18 h) at −20 °C by centrifugation at 4 °C for 15 min at 14,000× *g*. Supernatants were carefully removed by pipetting, and each pellet was washed twice with 75% ethanol. Final pellets were air-dried at room temperature until visually dry (~20 min). The pellet was suspended in 30 µL nuclease-free water; then, 3 µL 3M sodium acetate (pH 5.5) and 120 µL ethanol were added, and RNA was precipitated overnight at −20 °C. Next, samples were centrifuged at 14,000× *g* for 15 min, and supernatants were carefully removed by pipetting. Each pellet was washed twice with 75% ethanol. Finally, RNA pellets were air-dried at room temperature until visually dry (~20 min) and then dissolved in

20 µL nuclease-free water. RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher).

To extract RNA from larval carcasses, 300 µL of deionized H_2O was added to the exsanguinated carcass. The carcass was homogenized with a sterile steel BB using a TissueLyzer (Qiagen) at 30 Hz for 2 min. Then, 800 µL TRIzol® reagent (Invitrogen) was added to the lysate and vortexed and incubated at room temperature for 5 min; 200 µL of chloroform was then added. The tubes were shaken by hand for 15 s and incubated at room temperature for 2 min. The samples were then centrifuged for 15 min at 4 °C at 12,000× *g*. The aqueous phase was transferred to a clean microcentrifuge tube (approximately 800 µL), one volume of isopropanol was added, and tubes were mixed by inverting. Samples were incubated at −20 °C for one hour and then centrifuged for 10 min at 4 °C at 12,000× *g.* The supernatant was decanted, and the pellet was washed by adding 500 µL 75% ethanol and centrifuging for 5 min (7500× *g*) at 4 °C. The supernatant was decanted, and pellets were air-dried at room temperature. Pellets were suspended in 100 µL of dH_2O.

2.6. Reverse Transcription/cDNA Synthesis

Reverse transcription (RT) reactions to produce complementary DNA (cDNA) were performed by incubating 100–250 ng total RNA for cultured cell experiments, 200 units M-MLV reverse-transcriptase (Promega), and 500 ng random hexamer primers (IDT) for 2 h at 37 °C, according to the manufacturer's instructions. For larvae/hemocyte assays, 2000 and 1000 ng total RNA, respectively, were used for RT reactions.

2.7. Negative Strand-Specific RT-PCR

Virus-treated samples (with SBV, DWV, or FHV) were analyzed for the presence of the negative-strand RNA of the viral genome (i.e., the replicative intermediate form) using strand-specific PCR [88,179,180]. Reverse transcription/cDNA synthesis reactions were performed with M-MLV (Promega) according to the manufacturer's instructions using negative strand-specific primers tagged with an additional 21 nt of sequence at their 5′ end (Supplemental Table S1). The tag sequence (5′GGCCGTCATGGTGGCGAATAA3′) shares no homology with the viruses used in this study nor with the honey bee genome. Reactions were carried out by incubating 100–250 ng RNA and primers (10 pmol specific or 500 ng hexamers) with 200 units M-MLV reverse-transcriptase (Promega) according to the manufacturer's instructions. Reverse transcription reactions were incubated for 2 at 37 °C. Unincorporated primers present in the RT reactions were digested with 2 units exonuclease I (Life Technologies) per reaction at 37 °C for 30 min, followed by heat inactivation at 85 °C for 5 min. To detect negative-strand-derived cDNA products, 5 µL of cDNA template in 12.5 µL aqueous buffer containing 10 pmol of each of a tag-specific forward primer (TAGS) and virus-specific reverse primer (Table S1) was amplified with 0.5 µL (5 U/µL) ChoiceTaq polymerase (Thomas Scientific) using the following cycling conditions: 95 °C for 5 min; 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, 35 cycles; final elongation 72 °C for 5 min, hold at 4 °C. Negative control PCR reactions were performed using template generated from RNA-containing RT reactions that were incubated in the absence of RT enzyme or using cDNA template that was generated using random-hexamer primers and only including the reverse primer in the PCR. Positive controls included PCR using virus-specific qPCR primers (Supplemental Table S1).

Viral and other long single-stranded RNA molecules often exhibit secondary structures, which serve to prime reverse transcription reactions in the absence of exogenous primers (i.e., self-priming). To test for self-priming, RT reactions were carried out in the absence of primers, followed by PCR with virus-specific qPCR primers (See Supplemental Table S1 for full list of controls).

2.8. Polymerase Chain Reaction (PCR)

PCR was performed according to standard methods [33,63,64,97]. In brief, 2 μL cDNA template in 12.5 μL aqueous buffer containing 10 pmol of each forward and reverse primer (Table S1) was amplified with 0.5 μL (5 U/μL) ChoiceTaq polymerase (Thomas Scientific) according to the manufacturer's instructions using the following cycling conditions: 95 °C for 5 min; 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, 35 cycles; final elongation 72 °C for 5 min, hold at 4 °C. PCR products were visualized by 1.5% agarose gel electrophoresis and stained with SYBRsafe (Invitrogen). Positive and negative control reactions were included for all analyses and exhibited the expected results. Select products were purified with the Qiaquick PCR Purification Kit (Qiagen), quantified using a NanoDrop spectrophotometer (Fisher), and Sanger sequenced.

2.9. Quantitative PCR

Quantitative PCR (qPCR) was used to quantify the viral RNA (i.e., genome and transcript) abundance in the samples and assess the relative abundances of honey bee encoded transcripts (i.e., a housekeeping gene, *rpl8*), and three immune genes (i.e., *ago2, dcr-like,* and *bap1/mf116383*) using the primer sets listed in Supplemental Table S1. All qPCR reactions were

performed in triplicate using 2 µL of cDNA template and a no-template negative control was run in triplicate for each qPCR reaction. Each 20 µL reaction contained 1× Denville ChoiceTaq Mastermix, 0.4 µM each forward and reverse primer, 1× SYBR Green (Life Technologies), and 3 mM $MgCl_2$. Reactions were carried out in 96-well plates using a CFX Connect Real-Time instrument using Maestro software (Bio-Rad) with the following thermo-profile: initial denaturation at 95 °C for one minute; followed by 40 cycles of 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 15 s, with a final melt curve analysis at 65 °C for 5 s to 95 °C. Positive and negative control reactions were included for all qPCR analyses and exhibited the expected results. Viral RNA copies were estimated using a plasmid DNA standard for SBV, DWV, and FHV, with a detection range of 10^3 to 10^9 to create a linear standard curve used for copy number interpolation. The linear equation for the plasmid standard for FHV was $Ct = -3.240x + 40.138$ ($R^2 = 0.980$, efficiency = 103%), determined by a previously described method [181]. Similarly, the linear equation for DWV was $Ct = -3.5627x + 39.091$ ($R^2 = 0.9973$, efficiency = 90.5%). The linear equation for the SBV standard curve was $Ct = -3.39x + 37.42$ ($R^2 = 0.994$, efficiency = 97.2%). The relative expression of host genes was determined by a ranked ΔΔCt method in which the ΔCt was calculated by subtracting the *rpl8* Ct value from the Ct of the gene of interest. Then, the within-group ΔCt values were ranked from highest to lowest, and the relevant corresponding control ΔCt value was subtracted from the treatment group ΔCt to obtain the ΔΔCt. The fold-change in cDNA abundance was calculated by the equation $2^{-\Delta\Delta Ct}$. See Supplemental Tables S2–S12 for qPCR data presented in the figures.

2.10. Microscopy

To prepare hemocytes for imaging, they were isolated as above and seeded into #1.5 German cover glass chamber slides (Thermo Fisher Scientific) in WH2 media. Live-cell and fixed-cell images were taken on a Nikon Ti-Eclipse (Nikon Instruments) inverted microscope equipped with a SpectraX LED (Lumencor) excitation module and fast-switching emission filter wheels (Prior Scientific). Brightfield images were captured using a Plan Fluor 20 phase contrast (Ph) objective and an iXon 896 EM-CCD (Andor Technology Ltd.) camera using NIS Elements software. To visualize cells with an intact nucleus, the cell culture media was removed, and the cells were washed with PBS and then incubated in 4% paraformaldehyde in PBS for 10 min at room temperature. The paraformaldehyde containing PBS was removed, and cells were washed two times with PBS. Hoechst stain (Hoechst 33258, Invitrogen) prepared to 30 mg/mL in PBS was further diluted 1:5000 in PBS to create a working solution, which was then overlaid on the cells. Then, fluorescent images were captured using paired excitation/emission filters and dichroic mirrors for DAPI (Chroma Technology Corp).

2.11. Statistical Analysis

All data were analyzed using R v4.0.2 in RStudio V1.3.1073. Unless otherwise stated, comparisons for virus abundance were evaluated using the *DunnettTest* function in the DescTools R stats package to perform the post hoc pairwise multiple comparisons to a control [182]. Relative gene expression differences were evaluated using a one-sided Wilcoxon [183]. For each analysis, comparisons were considered significant if $p < 0.05$.

3. Results and Discussion

3.1. Sacbrood Virus (SBV) Replicates in Primary Honey Bee Hemocytes

To determine whether sacbrood virus (SBV) infects hemocytes, the primary immune cell in honey bees, approximately 1×10^5 larval (L4–L5) hemocytes were seeded into wells of a 48-well tissue culture plate with relatively few contaminating adipocytes and treated with either phosphate buffered saline (PBS) or SBV (i.e., bee larval lysate containing 2.5×10^6 SBV RNA copies) (Supplemental Figure S1). Cells were harvested at distinct time points post-infection (i.e., from 0 hours post-infection (hpi) to 96 hpi) and virus abundance, using RNA copy number as a proxy, was determined by qPCR. In one experimental replicate, the primary honey bee hemocyte culture had a pre-existing SBV infection, which exhibited a 501x increase in SBV by 96 h post-treatment ($p = 0.042$) (Figure 1A). This result indicates that hemocytes are a natural site of SBV infection and that SBV virus infections can persist in cells grown in culture. The addition of SBV increased the infection level by 13× over the initial levels by 24 hpi ($p < 0.001$), and it rose to 69.2× by 48 hpi ($p < 0.001$), to 147.9× by 72 hpi ($p < 0.0001$), and peaked at 354.8× at 96 hpi ($p < 0.0001$). In a second biological replicate of this experiment, confounding SBV infection was not detected (Figure 1 B). In inoculated hemocytes, there was a 24× increase from 0 hpi to 24 hpi ($p = 0.0001$), and by 48 hpi SBV had increased 182× ($p < 0.0001$) and reached peak infection. There was a decrease in SBV relative to 48 hpi at 72 hpi (i.e., 49× greater virus levels than at 0 hpi, $p < 0.0001$), with a subsequent rise at 96 hpi (i.e., 132× increase over 0 hpi, $p < 0.0001$). Although it is unclear why there was a reduction in viral abundance at 72 hpi, it could have been due to a decrease in the

number of permissive hemocytes in the culture; by 96 hpi, cell division and/or differentiation may have occurred and resulted in additional susceptible host cells.

To further validate that the increase in SBV over the infection time course was due to active virus replication in honey bee hemocytes, we carried out strand-specific RT-PCR. The SBV genome is positive-sense single-stranded RNA (+ssRNA). During virus replication, a negative strand template is produced [88,179,180,184]. The negative strand of SBV was not detected in the inoculum (i.e., at 0 hpi), but was detected at all other time points (Figure 1 C). Together these data indicate that not only does SBV actively replicate in hemocytes, but hemocytes also serve as a natural site of virus infection. Furthermore, virus testing of honey bee larvae (i.e., corresponding carcass and hemocyte samples) indicate that hemocytes are natural sites of infection for black queen cell virus (BQCV) (Supplemental Table S24). While, in general, hemocytes had low incidence of infection, isolated larval hemocytes were positive for BQCV in 9.1% of cases where the larval carcass was also BQCV-positive (Supplemental Table S24). Together, these data suggest hemocytes might be a site of infection for multiple viruses. These results are in line with reports of virus infections in other invertebrates, including persistent infection of hemocytes by hepatitis A virus (HAV) and murine norovirus (MNV) in oysters (*Crassostrea virginica*) [185]. In addition, white spot syndrome virus (WSSV) infects subpopulations of shrimp hemocytes [186]. Results from honey bees, oysters, and shrimp differ from the observation that hemocytes of Sindbis-virus-infected *Drosophila melanogaster* remain uninfected [170]. As described above, hemocytes consist of several subpopulations. Future studies aimed at identifying the hemocyte subpopulations that are permissive to specific viruses will provide insight into the role of these important immune

cells in virus dissemination and antiviral defense. Understanding how viruses interact with hemocytes and how they might frustrate the cellular immune function may aid in the long-term goal of developing therapeutics and technologies that mitigate the negative impacts of viruses on bees.

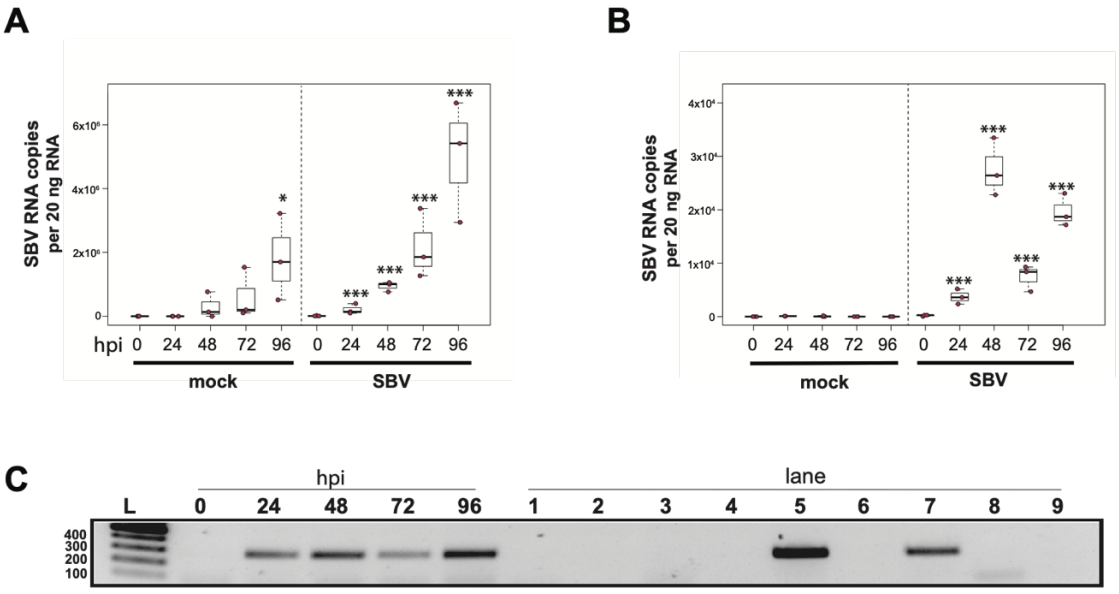


Figure 1. Sacbrood virus (SBV) naturally infects hemocytes and replicates in primary honey bee hemocyte cell cultures. SBV abundance was assessed in mock- or SBV-treated larval hemocytes by qPCR over a time course (i.e., 0 hpi, 24 hpi, 48 hpi, 72 hpi, and 96 hpi). (**A**) Cells isolated from asymptomatic larvae and treated with PBS (pH 7.4) had a 501× increase in mean SBV abundance by 96 hpi relative to 0 hpi (p = 0.042), indicating that the hemocytes in this experimental replicate had a pre-existing SBV infection and that hemocytes are a natural site of infection for SBV. The addition of filtered larval lysate containing 2.5 × 10^6 SBV RNA copies resulted in a 13× increase by 24 hpi relative to 0 hpi ($p < 0.001$) and a peak fold increase of 355× by 96 hpi ($p <$ 0.0001). (**B**) In a second replicate of the experiment, mock-infected cells remained uninfected over the course of the experiment. The addition of filtered larval lysate containing 2.5 × 10^6 SBV RNA copies resulted in a peak fold increase of 182× ($p <$ 0.0001) at 48 hpi, with a decrease at 72 hpi (49× relative to 0 hpi, $p < 0.0001$) and a

subsequent rise again at 96 hpi (132× relative to 0 hpi, $p < 0.0001$). Raw data are included in Supplemental Table S2. Significance levels: * $p < 0.05$; *** $p < 0.0005$. (**C**) To confirm that SBV was productive in infecting larval hemocytes, the presence of the negative strand (a replicative intermediate) was assessed by negative-strand-specific reverse transcription (RT) followed by PCR. Negative strand was detected at all time points after 0 hpi. Additional control reactions were performed using pooled RNA from time points 24–96 hpi (Lanes labeled 1–9). Specifically, RNA isolated from SBV containing cell lysate was reverse transcribed with the primer listed below, treated with Exonuclease I to remove excess primer, and amplified using the PCR primers listed for each lane. (L) Molecular weight ladder. (0, 24, 48, 96 hpi)—RT with random hexamers, PCR with SBV-221-240-For and SBV-478-497-Rev. (1) Negative control: No RT in the presence of (SBV-TAGS-F), PCR with TAGS and SBV-478-497-Rev. (2) Negative control: RT in the presence of (SBV-TAGS-F), PCR with only the SBV-478-497-Rev primer. (3) Negative control: RT with random hexamer primer, PCR with TAGS and SBV-478-497-Rev. (4) Negative control: No RT enzyme in the presence of random hexamers, PCR with SBV-221-240-For and SBV-478-497-Rev. (5) Positive control: RT with random hexamers, PCR with SBV-221-240-For and SBV-478-497-Rev. (6) Negative control: RT with random hexamers, PCR with only SBV-478-497-Rev primer. (7) Self-priming: RT without a primer, PCR with SBV-221-240-For and SBV-478-497-Rev. (8) Negative control: No template, no RT, PCR with TAGS and SBV-478-497-Rev. (9)Negative control: No template, no RT, PCR with SBV-221-240-For and SBV-478-497-Rev. Additional details provided in Supplemental Table S25. When reverse transcription was performed without primer, SBV cDNA was detectable (lane 7), indicating that the secondary structure in the RNA genome may serve as a primer for cDNA synthesis. All differences in means relative to 0 hpi were assessed by a Dunnett's test.

To our knowledge, this is the first quantitative evidence that SBV productively infects honey bee hemocytes. A previous study used transmission electron microscopy to visualize SBV virions within larval and adult hemocytes [168]. While those images indicated that SBV may replicate in hemocytes, they could have also been explained by phagocytosis of virions [168]. The observation that honey bee hemocytes are naturally infected by SBV has important implications for viral pathogenesis. While we did not observe any cytopathic effects (CPEs), it is possible the cells would have shown CPEs if the experiments were carried out for a longer time period. Additionally, the immune function of hemocytes may be dysregulated by viral infection, since viruses subvert

cellular processes and disrupt cellular homeostasis [187,188]. For example, DWV suppresses larval melanization in response to wounding, suggesting an interaction between DWV and hemocytes [96]. This is further supported by gene expression studies of DWV-infected larvae, which exhibited reduced expression of *Amel*\102, a gene involved in melanization and encapsulation [96].

In addition to hosting viral infection, honey bee hemocytes are likely instrumental in antiviral immune responses, as they are in *Drosophila melanogaster*. Direct evidence for an antiviral role for hemocytes in fruit flies includes the results of in vivo experiments in which hemocytes were either saturated via injection of latex beads or genetically depleted [189]. Flies with reduced hemocyte function that were infected with either Cricket paralysis virus (CrPV), FHV, or vesicular stomatitis virus (VSV) had increased viral abundance and higher mortality than flies with phagocytic hemocytes. In contrast, reduced hemocyte function had no impact on Drosophila C virus (DCV), Sindbis virus (SINV), and Invertebrate iridescent virus 6 (IIV6) infections in flies [189]. These results also indicated that the relative importance of hemocytes in host antiviral defense will vary for specific host–virus pairs and may also depend on additional factors (e.g., co-infection, nutritional status, age). Hemocytes are required for systemic adaptive RNAi response in flies, which limits virus infection [170,171]. This finding provides strong support for an antiviral role of hemocytes against some viruses in fruit flies, which suggests that hemocytes likely serve an antiviral role in insects in general.

While the function of hemocytes in honey bee antiviral response requires further investigation, there is limited indirect evidence of their antiviral role. Specifically, genes involved in migration,

phagocytosis, and expression immunoglobulin domain encoding, were expressed at greater levels in honey bees infected with a model virus, SINV, relative to mock-infected bees [107]. Additionally, IAPV infection resulted in increased expression of genes involved in cell projection, cellular organization, autophagic cell death, microtubule-based movement, and phagocytosis [101,190]. Our observation that viruses replicate in cultured hemocytes will facilitate future studies aimed at elucidating their role in antiviral defense, including further investigation of the genes and processes highlighted by transcriptional level studies.

3.2. Sacbrood Virus (SBV) Replicates in Primary Honey Bee Pupal Cell Cultures

To determine whether a mixed cell culture derived from purple-eyed pupae supported SBV infection, approximately 1×10^6 cells were treated with either PBS or SBV (i.e., bee larval lysate containing 2.5×10^6 SBV RNA copies). Cells were harvested at distinct time points post-infection, and virus abundance was assessed by qPCR. Three independent experiments were performed (Supplemental Figure S2). In replicate one, SBV abundance was 7.8× higher by 48 hpi than the levels after 0 hpi ($p < 0.0001$). SBV abundance remained high at 72 hpi (12×, $p < 0.0001$) and 96 hpi (11.4×, $p < 0.0001$) (Supplemental Figure S2A). Likewise, in the second experimental replicate, SBV abundance was higher by 48 hpi (59×, $p < 0.0001$), remained high at 72 hpi (126×, $p < 0.001$), and peaked at 96 hpi (1047×, $p < 0.0001$) (Figure 2 A, Supplemental Figure S2 B). While SBV levels at 24 hpi were close to the levels of the 0 hpi time points in the first two biological replicates, they were significantly lower in replicate three (i.e., 0.017×, $p < 0.0001$). Like the other two replicates, SBV levels increased by 48 hpi (2.2×, $p = 0.0012$), remained high at 72 hpi (14×, $p = 0.00013$), and peaked at 96 hpi (72×, $p < 0.0001$) (Supplemental Figure S2C).

The overall increase in SBV levels for replicate three was 1,817× (i.e., from 24 hpi to 96 hpi ($p < 0.0001$)), which was similar to replicate two, which had an overall 1047× increase, whereas the replication and relative increase in abundance for replicate one was lower (i.e., only 72×) (Supplemental Figure S2A). To further verify that SBV productively infected cultured honey bee pupal cells, we used strand-specific RT-PCR to assay for the negative strand (i.e., replicative intermediate form of SBV). The SBV-negative strand was detected at three time points post-infection, which corresponded to peak virus abundances (i.e., 48, 72, and 96 hpi) (Figure 2B). Together, these data demonstrate that primary honey bee pupal cell cultures are permissive to SBV infection. Furthermore, since the fold increase of SBV in honey bee pupal cells was greater than in hemocytes, we hypothesize that cell types present in pupal cultures, including endothelial cells and fibroblasts, may be more suitable hosts for SBV compared to immune cells.

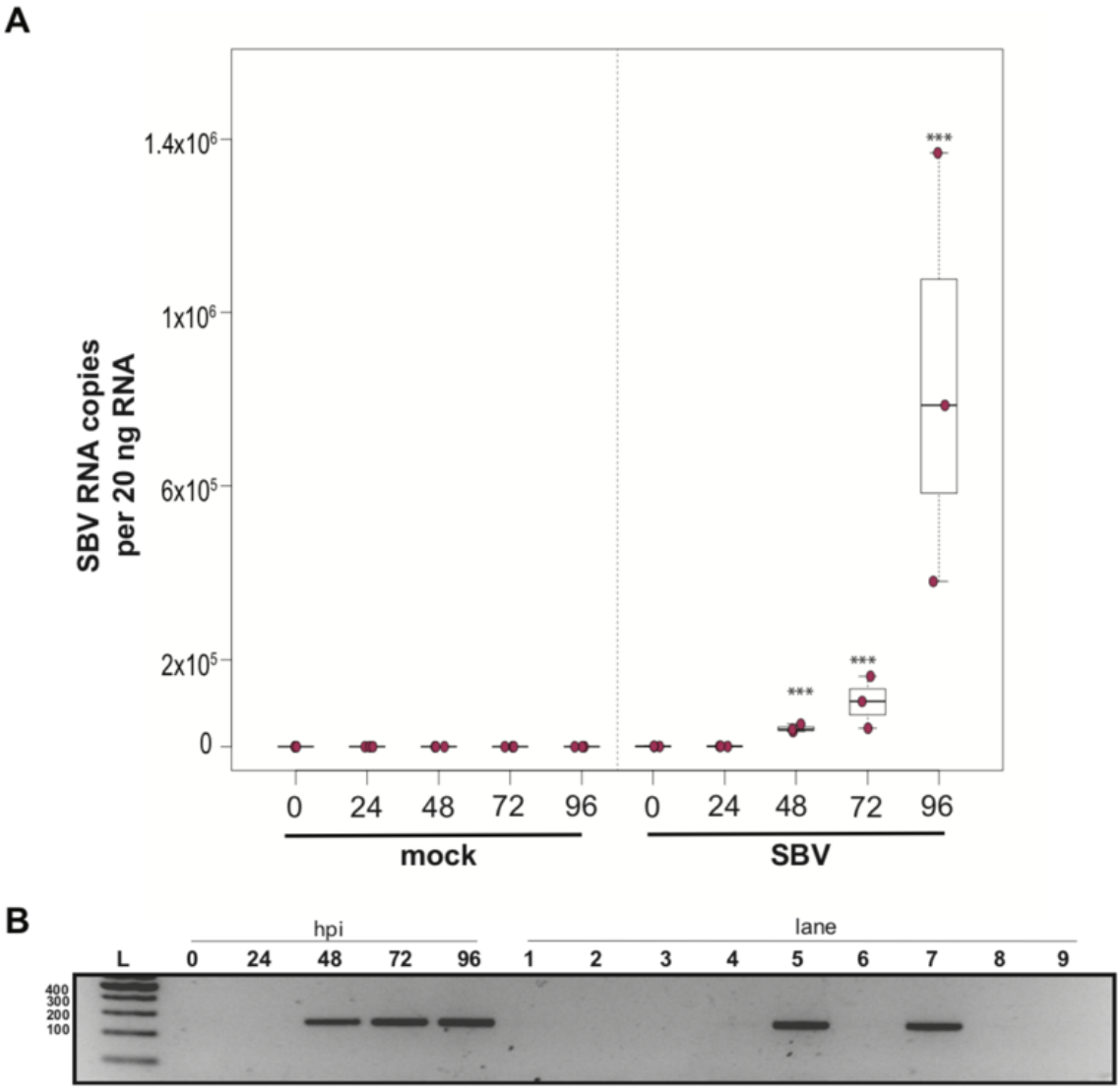


Figure 2. Sacbrood virus (SBV) replicates in primary honey bee pupal cell cultures. (**A**) SBV abundance was assessed in mock- or SBV-treated pupal cells by qPCR over a time course (i.e., 0 hpi, 24 hpi, 48 hpi, 72 hpi, and 96 hpi). A representative replicate of SBV-infection in pupal cells is plotted. The addition of larval lysate to pupal cells resulted in a 59× fold increase relative to 0 hpi by 48 hpi ($p < 0.0001$), with subsequent increases at 72 hpi (126×, $p < 0.0001$) and 96 hpi (1,047×, $p < 0.0001$). Raw data are included in Supplemental Table S3. See Supplemental Figure S2 for additional replicates. All differences in means relative to 0 hpi were assessed by a Dunnett's test. Significance levels: *** $p < 0.0005$. (**B**) To confirm that SBV was productive in infecting larval hemocytes, the presence of the negative strand (a replicative intermediate) was assessed by negative strand-specific reverse transcription (RT) followed by PCR. Negative strand was detected at all time points after 24 hpi. Additional control reactions

were performed using pooled RNA from time points 24–96 hpi (Lanes labeled 1–9). Specifically, RNA isolated from SBV containing cell lysate was reverse-transcribed with the primer listed below, treated with Exonuclease I to remove excess primer, and amplified using the PCR primers listed for each lane. (L) Molecular weight ladder. (0, 24, 48, 96 hpi)—RT with random hexamers, PCR with SBV-221-240-For and SBV-478-497-Rev. (1) Negative control: No RT in the presence of (SBV-TAGS-F), PCR with TAGS and SBV-478-497-Rev. (2) Negative control: RT in the presence of (SBV-TAGS-F), PCR with only the SBV-478-497-Rev primer. (3) Negative control: RT with random hexamer primer, PCR with TAGS and SBV-478-497-Rev. (4) Negative control: No RT enzyme in the presence of random hexamers, PCR with SBV-221-240-For and SBV-478-497-Rev. (5) Positive control: RT with random hexamers, PCR with SBV-221-240-For and SBV-478-497-Rev. (6) Negative control: RT with random hexamers, PCR with only SBV-478-497-Rev primer. (7) Self-priming: RT without a primer, PCR with SBV-221-240-For and SBV-478-497-Rev. (8) Negative control: No template, no RT, PCR with TAGS and SBV-478-497-Rev. (9) Negative control: No template, no RT, PCR with SBV-221-240-For and SBV-478-497-Rev. Additional details provided in Supplemental Table S25.
..

While the primary concern of SBV infection of honey bee colonies is larval mortality, SBV also reduces adult lifespan. SBV infections in pupae may inhibit the melanization pathway by causing reduced expression of *prophenoloxidase activating enzyme* (*PPAE*) and increased expression of a putative serpin protein encoding gene (GB48820) [59,145]. Pupae with a suppressed melanization response may be more susceptible to infection after wounding by *Varroa destructor* mites. It is not known whether SBV-infected pupae can clear infections prior to eclosion, and in turn if SBV-infected pupae can develop into healthy adults. Therefore, tools to study the impact of SBV and the molecular mechanisms of antiviral defense at all life stages are needed and include the SBV-permissive cultured primary cells described herein.

3.3. Deformed Wing Virus Replicates in Primary Honey Bee Pupal Cells

To investigate the utility of primary honey bee pupal cells to support DWV infection, approximately $1x10^6$ purple-eyed pupal cells were incubated with 4.3×10^6 DWV RNA copies.

Cells were harvested at distinct time points post-infection (i.e., from 0 hpi to 96 hpi), and virus abundance, using RNA copy number as a proxy, was determined by qPCR. Data from two independent experiments demonstrated that DWV abundance increased from 0 hpi to 96 hpi (Supplemental Figure S3). In replicate one, DWV levels remained unchanged for the first 24 h and were 2.7× greater at 48 hpi ($p = 0.002$). DWV abundance increased 3.5× and 4× at 72 hpi ($p < 0.001$) and 96 hpi ($p < 0.001$), respectively, with peak DWV levels at 96 hpi relative to 0 hpi (Figure 3A). In replicate two, DWV levels remained similar to inoculum levels through 48 hpi but were 3.2× higher at 72 hpi ($p = 0.001$) and 6.6× higher at 96 hpi, relative to 0 hpi ($p = 0.003$) (Supplemental Figure S3). To verify DWV replication in primary cultures of purple-eyed honey bee pupal cells, negative-strand specific PCR was performed, and the replicative intermediate form (i.e., negative strand) of DWV was detected at 72 hpi (Figure 3B). Together, DWV abundance and negative-strand data confirm that primary cultures of honey bee pupal cells support DWV replication.

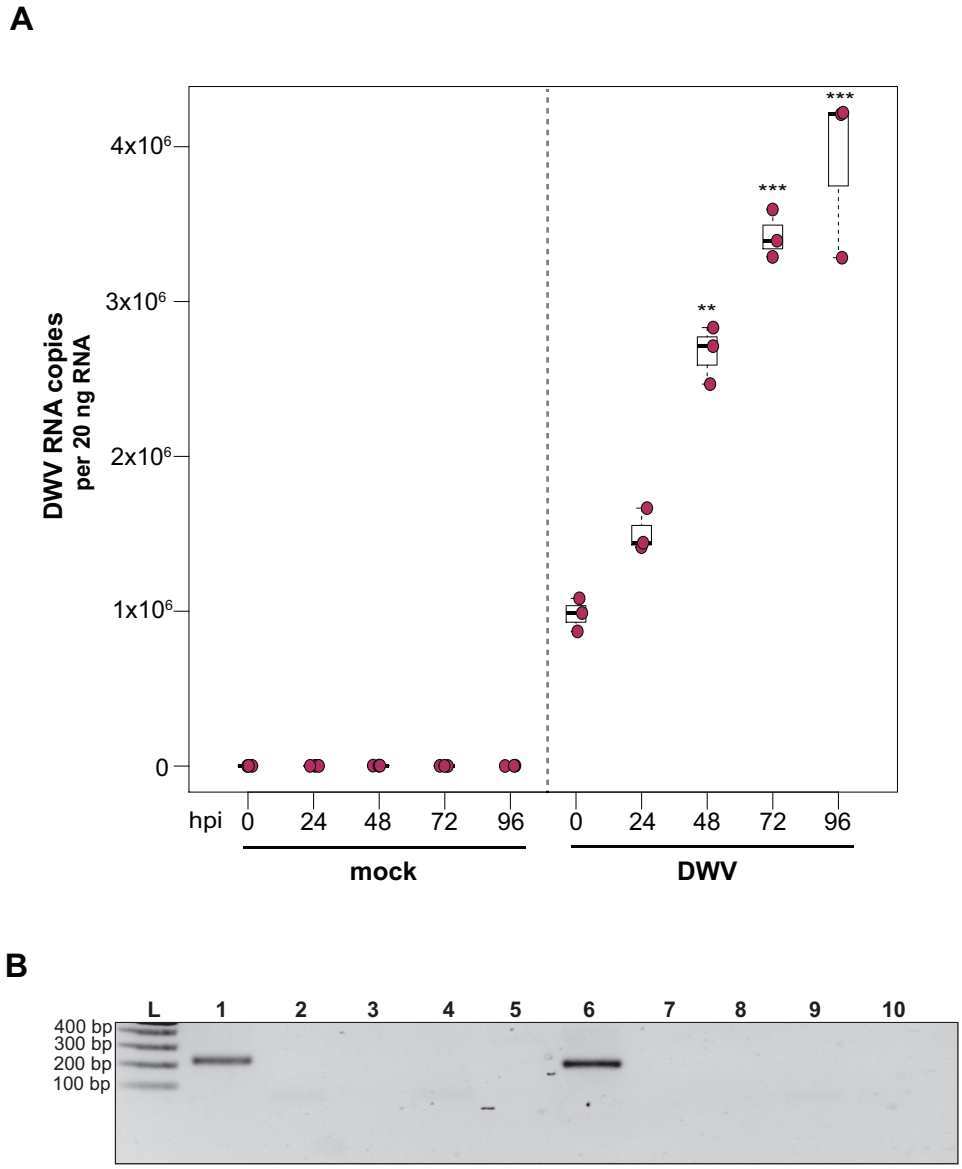


Figure 3. Deformed wing virus (DWV) replicates in primary honey bee pupal cell cultures. DWV abundance was assessed in mock- or DWV-infected pupal cells via qPCR over a time course (i.e., 0 hpi, 24 hpi, 48 hpi, 72 hpi, and 96 hpi). (**A**) Pupal cells infected with 4.3×10^6 DWV RNA copies had a 2.7× ($p < 0.001$) and 3.5× ($p < 0.001$) increase in mean DWV abundance at 48 hpi and 72 hpi, respectively, relative to 0 hpi. At 96 hpi, mean DWV abundance was 4× higher relative to 0 hpi ($p < 0.001$). Figure 3 includes results from one representative biological replicate. The data for additional replicates are presented in Supplemental Figure S3. All differences in means relative to 0 hpi were assessed by a Dunnett's test. Raw data are included in Supplemental Table S4. Significance levels: ** $p < 0.005$; *** $p < 0.0005$. (**B**) To verify DWV replication in purple-eyed pupal cells, negative-strand-specific RT-PCR was

performed, and the replicative intermediate form (i.e., negative strand) of DWV was detected at 72 hpi. Additional control reactions were performed using pooled RNA from time points 24–96 hpi (Lanes labeled 2–9). Specifically, RNA isolated DWV containing cell lysate was reverse-transcribed with the primer listed below, treated with Exonuclease I to remove excess primer, and amplified using the PCR primers listed for each lane. (L) Molecular weight ladder. (1) 72 hpi RNA template—RT with DWV-TAGS-F, PCR with TAGS and DWV-Rev. (2) Negative control: No RT in the presence of (DWV-TAGS-F), PCR with TAGS and DWV-Rev. (3) Negative control: RT in the presence of (DWV-TAGS-F), PCR with only the DWV-Rev primer. (4) Negative control: RT with random hexamer primer, PCR with TAGS and DWV-Rev. (5) Negative control: No RT enzyme in the presence of random hexamers, PCR with DWV-F and DWV-Rev. (6) Positive control: RT with random hexamers, PCR with DWV-F and DWV-Rev. (7) Negative control: RT with random hexamers, PCR with only DWV-Rev primer. (8) Self-priming: RT without a primer, PCR with DWV-F and DWV-Rev (9) Negative control: No template, no RT, PCR with TAGS and DWV-Rev. (10) Negative control: No template, no RT, PCR with DWV-F and DWV-Rev. Additional details provided in Supplemental Table S25.
.

The ability to infect primary honey bee cells with DWV opens new avenues for studying a pathogen that has co-evolved with honey bees. Primary cell cultures may also facilitate the propagation of virus stocks that are free of confounding viruses. Experiments in cultured honey bee cells also provide an opportunity to study the mechanistic details of DWV infections and virus–host dynamics in a system that complements experiments carried out in larvae, pupae, and adult honey bees. Recent efforts have focused on exploring the ability of alternative host cell lines to support honey bee virus infections; for example, the Lepidopteran hemocyte-derived cell line (P1) has been shown to support DWV infection [151]. While exploring the ability of alternative cell lines to support honey bee virus infections is an attractive approach, the immune response elicited by these alternative host cells may or may not completely recapitulate the immune response elicited by honey bees. Therefore, the potential to infect primary honey bee cells with naturally infecting honey bee viruses, including DWV, will be a powerful tool to explore the natural host–virus interactions at the cellular level.

3.4. Flock House Virus Replicates in Primary Honey Bee Hemocytes

To investigate the ability of honey bee immune cells to support the replication of a model virus, approximately 1×10^5 larval-derived hemocytes maintained in culture were incubated with PBS or FHV (1×10^6 FHV RNA copies). Cells were harvested at distinct time points post-infection (i.e., from 0 hpi to 96 hpi), and virus abundance was determined by qPCR. Data from two independent experiments demonstrated that FHV abundance increased from 0 hpi to 72 hpi and decreased from 72 hpi to 96 hpi (Supplemental Figure S4). In replicate one, FHV levels in hemocytes increased 3.7× by 24 hpi ($p = 0.026$) and 10.7× by 48 hpi, relative to 0 hpi ($p < 0.001$) (Figure 4A). FHV abundance continued to increase from 48 hpi to 72 hpi, which had 37× ($p < 0.001$) the initial FHV level (i.e., compared to 0 hpi) (Figure 4A). By 96 hpi, FHV abundance was less than levels quantified at 72 hpi ($p = 0.034$) but remained significantly higher relative to 0 hpi ($p < 0.001$) (Figure 4A). One possible explanation for reduced FHV abundance at 96 hpi is that, by that time most hemocytes were FHV-infected, there may have been a lack of naïve host cells to support further virus replication. In replicate two of this experiment, FHV abundance was constant at 24 hpi but was 16× higher at 48 hpi ($p < 0.01$) relative to 0 hpi. At 72 hpi, FHV levels peaked, with 39.8× higher FHV levels compared to 0 hpi ($p < 0.001$). FHV abundance was reduced at 96 hpi relative to 72 hpi but was still 12.6× higher that levels at 0 hpi ($p < 0.001$) (Supplemental Figure S4B). To further validate that FHV, which has a +ssRNA genome, productively infected cultured honey bee hemocytes, negative-strand-specific PCR was utilized to detect the replicative intermediate of FHV. Since FHV abundance peaked at 72 hpi, this time point was selected to assay for the presence of negative strand, and a negative-strand-specific FHV PCR product was detected

(Figure 4B). Together, FHV abundance and negative-strand data demonstrate that FHV productively infects primary honey bee hemocytes maintained in culture.

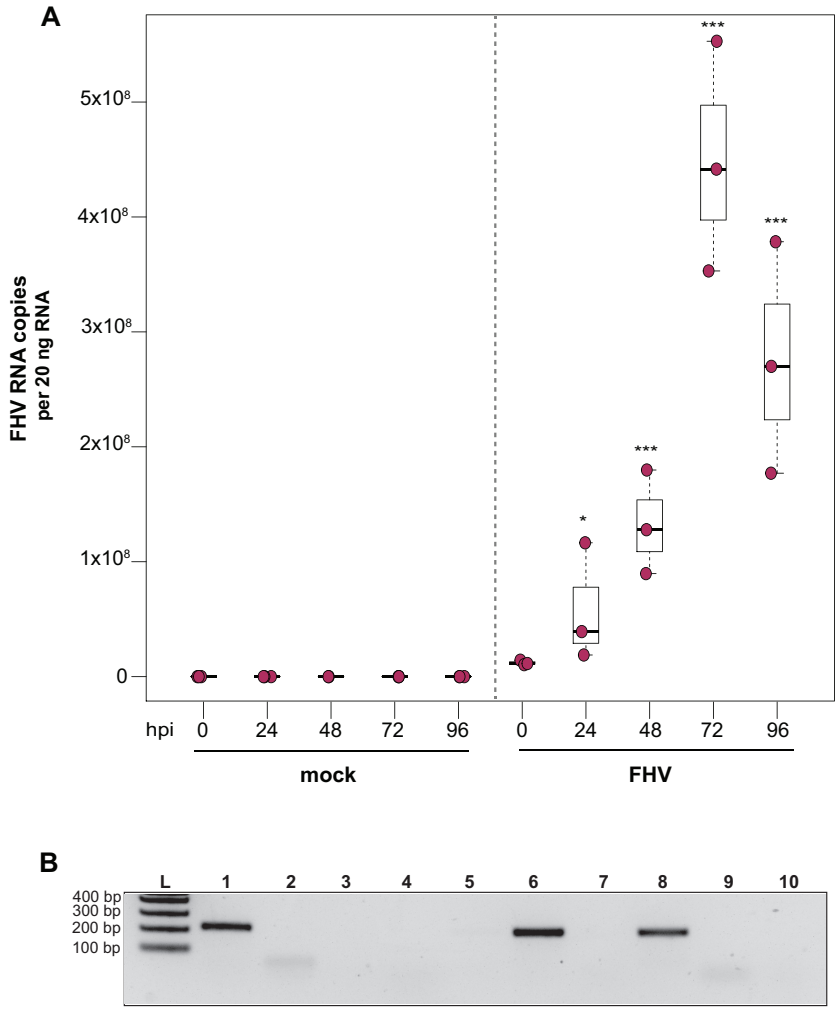


Figure 4. Flock House virus (FHV) replicates in primary honey bee hemocytes. FHV abundance in mock- or FHV-infected (1×10^6 FHV RNA copies) hemocytes was assessed over time (i.e., 0 hpi, 24 hpi, 48 hpi, 72 hpi, and 96 hpi). (**A**) FHV abundance was higher at 24 hpi (3.7×, $p = 0.026$), 48 hpi (10.7×, $p < 0.001$), and 72 hpi (37×, $p < 0.001$), relative to 0 hpi. FHV abundance reduced from 72 hpi to 96 hpi ($p = 0.034$) but was significantly higher ($p < 0.001$) relative to 0 hpi. Raw data are included in Supplemental Table S5. Figure 4 includes results from

one representative biological replicate. The data for additional replicates are presented in Supplemental Figure S4. All differences in means relative to 0 hpi were assessed by a Dunnett's test. Significance levels: * $p < 0.005$; *** $p < 0.0005$. (**B**) FHV-specific negative strand was detected at 72 hpi, which confirms that FHV productively infects primary honey bee hemocytes (Lane 1). Additional control reactions were performed using pooled RNA from time points 24–96 hpi (Lanes labeled 2–9). Specifically, RNA-isolated FHV containing cell lysate was reverse-transcribed with the primer listed below, treated with Exonuclease I to remove excess primer, and amplified using the PCR primers listed for each lane. (L) Molecular weight ladder. (1) 72 hpi RNA template—RT with FHV-TAGS-F, PCR with TAGS and FHV-Rev. (2) Negative control: No RT in the presence of (FHV-TAGS-F), PCR with TAGS and FHV-Rev. (3) Negative control: RT in the presence of (FHV-TAGS-F), PCR with only the FHV-Rev primer. (4) Negative control: RT with random hexamer primer, PCR with TAGS and FHV-Rev. (5) Negative control: No RT enzyme in the presence of random hexamers, PCR with FHV-F and FHV-Rev. (6) Positive control: RT with random hexamers, PCR with FHV-F and FHV-Rev. (7) Negative control: RT with random hexamers, PCR with only FHV-Rev primer. (8) Self-priming: RT without a primer, PCR with FHV-F and FHV-Rev. (9) Negative control: No template, no RT, PCR with TAGS and FHV-Rev. (10) Negative control: No template, no RT, PCR with FHV-F and FHV-Rev. Additional details provided in Supplemental Table S25. .

The ability to infect hemocytes with FHV provides an additional tool for investigating virus–honey bee host interactions at the cellular level. This model virus may prove particularly useful in investigating RNAi in the context of viral suppressors, since FHV-B2 protein suppresses RNAi, as confirmed by studies that utilized deletion mutants (i.e., FHV-□B2) [128,191]. To date there have been relatively few studies on putative bee virus-encoded VSRs, and the results from IAPV-infected honey bees and bumble bees have mixed results regarding the presence of a VSR; therefore, additional investigation is required [150,190]. The proteins produced by other model insect viruses, including cricket paralysis virus and Drosophila C virus, which encode bona fide VSRs, have a conserved amino acid motif (DvExNPGP) downstream of their VSR proteins [192,193]. Sequence analysis indicates that this motif is present in IAPV, KBV, and ABPV, suggesting that these viruses may encode VSRs, although additional studies are needed to identify

and validate putative VSRs. Overall, the use of both model viruses and naturally infecting honey bee viruses, including those that do and do not encode RNAi suppressors, will advance our knowledge of the interactions between viruses and the host antiviral RNAi machinery.

3.5. Flock House Virus Replicates in Primary Honey Bee Pupal Cells

To examine the ability of cells derived from purple-eyed honey bee pupae to support infection and replication of FHV, approximately 1×10^6 primary cells in culture were incubated with FHV (2×10^8 FHV RNA copies), and virus abundance was assessed at distinct time points post-infection. FHV abundance increased from 0 hpi to 72 hpi and was reduced or remained constant at 96 hpi in two experimental replicates. In replicate one, FHV levels increased by 1.7× at 24 hpi ($p = 0.024$) and by 3.4× at 48 hpi, relative to initial levels at 0 hpi ($p < 0.001$). The peak of infection was reached at 72 hpi, with 5× greater FHV levels relative to 0 hpi ($p < 0.001$). FHV abundance in pupal cells did not increase from 72 hpi to 96 hpi but remained 4× higher than at 0 hpi ($p < 0.001$) (Figure 5A). Similarly, in the second biological replicate, FHV abundance was 2.6× higher at 24 hpi ($p = 0.013$) and 7.5× higher at 48 hpi ($p < 0.001$), relative to levels at 0 hpi. FHV abundance increased 20× ($p < 0.001$) at 72 hpi relative to 0 hpi, which was the peak of virus infection. Similar to FHV infection in hemocytes, FHV levels in pupal cells was lower at 96 hpi relative to 72 hpi ($p < 0.01$) but was still 15.8× higher at 96 hpi compared to 0 hpi ($p < 0.001$) (Supplemental Figure S5B). To validate that the increase in FHV abundance was indicative of virus replication, the presence of FHV negative strand was assayed using strand-specific PCR. Active replication of FHV was confirmed by detection of FHV negative-strand PCR product at 72 hpi (Figure 5B). Together, the increase in FHV abundance quantified over the course of infections and the detection

of the replicative intermediate form (i.e., negative strand) of FHV demonstrate that primary honey bee cells derived from purple-eyed pupae support the replication of FHV.

The ability to infect mixed cell types derived from honey bee pupae with a model virus helps address some challenges in studying honey bee host–virus interactions using naturally infecting honey bee viruses. FHV can be easily propagated and purified in the lab, which helps circumvent the problem of mixed virus inoculum produced from infected bees, since many honey bee viruses co-purify due to their similar size and the high prevalence of mixed infections in colonies [10,40,52,63,64,97]. Since FHV does not naturally infect honey bees, the experimental results may not be confounded by pre-existing infections. However, a limitation of using FHV as a model virus to investigate honey bee antiviral defense mechanisms is that it does not naturally infect honey bees and therefore may not induce the same broad-spectrum antiviral or virus-specific immune responses that occur during infection with a co-evolved virus. One benefit to utilizing FHV in studies aimed at elucidating honey bee antiviral defense is that FHV–host interactions have been studied in a broad range of insect hosts, including mosquitoes, fruit flies, honey bees, tsetse flies, and reduviid bugs, thus facilitating comparative immune studies [105,106,110,111].

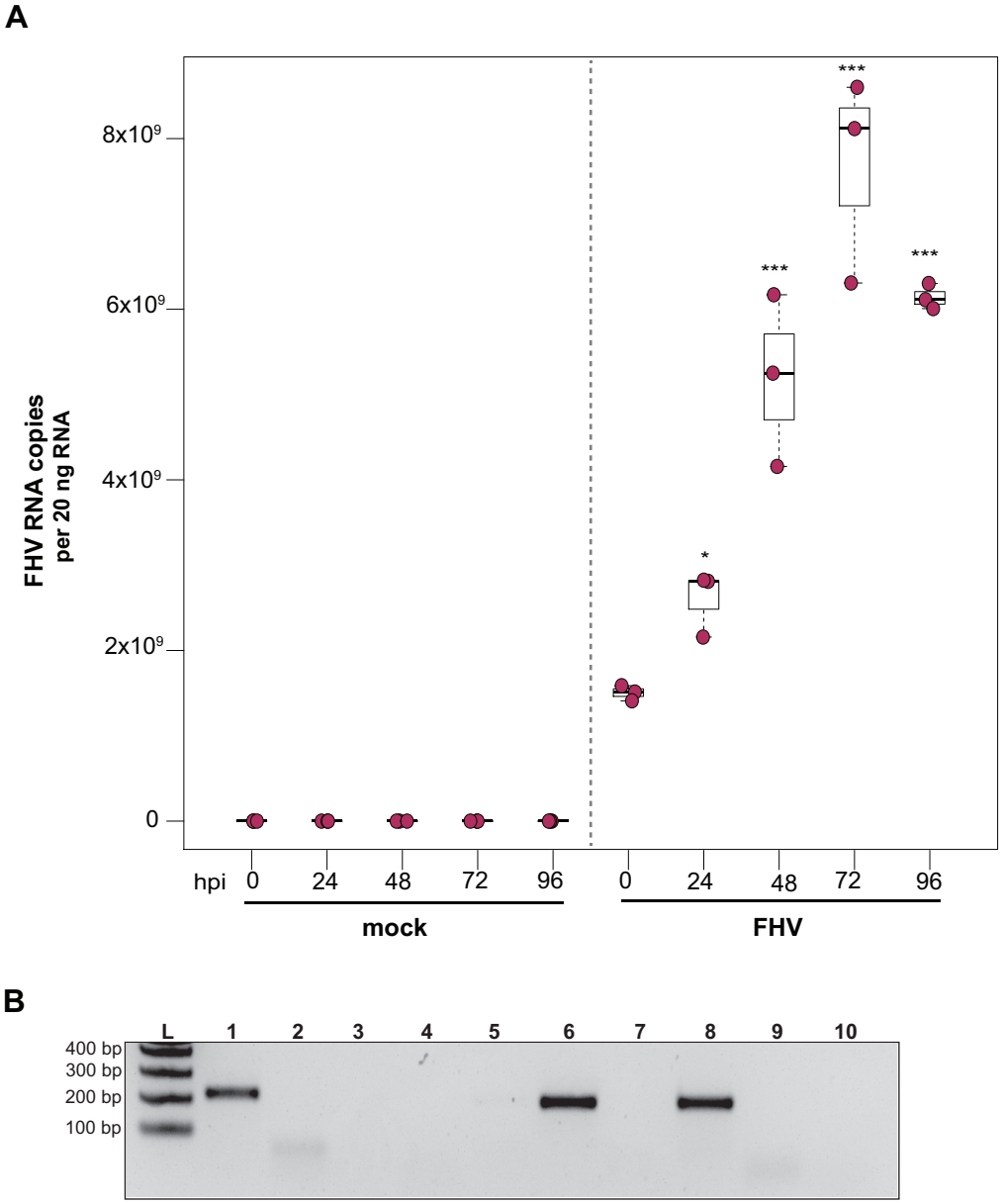


Figure 5. Flock House virus (FHV) replicates in primary honey bee pupal cells. Pupal cells were either mock- or FHV-infected (2×10^8 FHV RNA copies), and FHV abundance was quantified over a course of time (i.e., 0 hpi, 24 hpi, 48 hpi, 72 hpi, and 96 hpi). (**A**) FHV abundance increased at 24 hpi (1.7×, $p = 0.024$) and 48 hpi (3.4× $p < 0.001$), with a peak in FHV infection at 72 hpi (5×, $p < 0.001$), relative to 0 hpi. FHV abundance did not increase from 72 hpi to 96 hpi but remained 4× higher than at 0 hpi ($p < 0.001$). Raw data are included in Supplemental Table S6. Figure 5 includes results from one representative biological replicate. The data for additional replicates are presented in Supplemental Figure S5. All differences in

means relative to 0 hpi were assessed by a Dunnett's test. Significance levels: * $p < 0.05$; *** $p < 0.0005$. (**B**) To validate that the increase in FHV abundance was indicative of virus replication, the presence of the negative strand (replicative intermediate) was assessed by negative-strand-specific RT followed by PCR. The negative strand was detected at 72 hpi (Lane 1)**.** Additional control reactions were performed using pooled RNA from time points 24–96 hpi (Lanes labeled 2–9). Specifically, RNA-isolated FHV containing cell lysate was reverse-transcribed with the primer listed below, treated with Exonuclease I to remove excess primer, and amplified using the PCR primers listed for each lane. (L) Molecular weight ladder. (1) 72 hpi RNA template—RT with FHV-TAGS-F, PCR with TAGS and FHV-Rev. (2) Negative control: No RT in the presence of (FHV-TAGS-F), PCR with TAGS and FHV-Rev. (3) Negative control: RT in the presence of (FHV-TAGS-F), PCR with only the FHV-Rev primer. (4) Negative control: RT with random hexamer primer, PCR with TAGS and FHV-Rev. (5) Negative control: No RT enzyme in the presence of random hexamers, PCR with FHV-F and FHV-Rev. (6) Positive control: RT with random hexamers, PCR with FHV-F and FHV-Rev. (7) Negative control: RT with random hexamers, PCR with only FHV-Rev primer. (8) Self-priming: RT without a primer, PCR with FHV-F and FHV-Rev. (9) Negative control: No template, no RT, PCR with TAGS and FHV-Rev. (10) Negative control: No template, no RT, PCR with FHV-F and FHV-Rev. Additional details provided in Supplemental Table S25.
.

3.6. Flock House Virus Infection Transferred from Infected to Naïve Honey Bee Pupal Cells

To determine if FHV infection can be transferred from FHV-infected honey bee pupal cells to naïve cells, virus-containing cell culture obtained during peak infection (i.e., 72 hpi) was transferred to naïve pupal cells. Virus abundance in the secondary host cells was monitored over time. In replicate one, a small volume of FHV-infected pupal cell culture (i.e., 10 µL with 2.6×10^8 FHV RNA copies) was transferred to freshly seeded pupal cells (Figure 6A). FHV abundance at 48 hpi was 15.8× greater ($p = 0.003$) and at 72 hpi 28× greater than levels at 0 hpi ($p < 0.001$). In the second replicate, FHV-infected pupal cells (i.e., 10 µL infected cell culture with 2.9×10^8 FHV RNA copies) from the first round of infection were transferred to naïve pupal cells, and FHV abundance remained constant from 0 hpi to 48 hpi but was 10.4× higher at 72 hpi ($p < 0.001$) (Supplemental Figure S6B). As expected, FHV was not detected in control experiments, in which

a small volume of mock-infected honey bee pupal cell culture was transferred to naïve cells (Figures 6A and S6B). Together these results confirm that FHV virions produced by primary honey bee pupal cell cultures can be utilized to established new/secondary infections (Figure 6A).

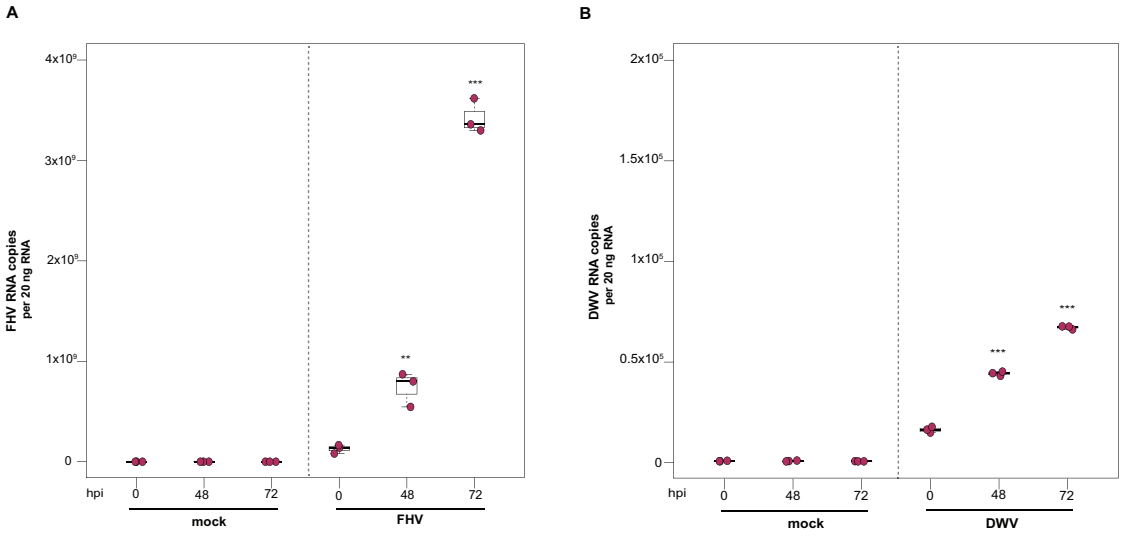


Figure 6. DWV and FHV infection transferred from infected to naïve honey bee pupal cells. To confirm that infectious DWV and FHV virions were produced in infected primary honey bee pupal cells, mock- or virus-infected honey bee pupal cell cultures (10 µL) at 72 hpi were transferred to naïve pupal cells. Virus abundance was assessed over a time course (i.e., 0 hpi, 48 hpi, and 72 hpi) using qPCR. Raw data are included in Supplemental Tables S7 and S8. (**A**) FHV abundance increased 15.8× at 48 hpi ($p = 0.003$), with a subsequent 28× increase at 72 hpi relative to 0 hpi ($p < 0.001$). (**B**) DWV abundance was 2.7× ($p < 0.001$) higher at 48 hpi and 4.1× higher at 72 hpi, relative to 0 hpi ($p < 0.001$). Figure 6 includes results from one representative biological replicate. The data for additional replicates are presented in Supplemental Figure S6. All differences in means relative to 0 hpi were assessed by a Dunnett's test. Significance levels: ** $p < 0.005$; *** $p < 0.0005$.

3.7. Deformed Wing Virus Infection Transferred from Infected to Naïve Honey Bee Pupal Cells

To determine if the DWV-infected cultured primary honey bee pupal cells produced infectious virions, small volumes of DWV-infected cell cultures were transferred to naïve cultured pupal

cells. In replicate one, naïve honey bee pupal cells were treated with cells and cell culture supernatant (10 µL) from either mock-infected or DWV-infected cultures, which contained an equivalent of 1.04×10^5 DWV RNA copies. By 48 hpi, DWV abundance increased 2.7× relative to 0 hpi ($p < 0.001$). DWV levels continued to increase over time, with 4.1× increase at 72 hpi ($p < 0.001$) (Figure 6B). In replicate two, naïve pupal cells were treated with 10 µL DWV-infected pupal cells (i.e., 9×10^4 DWV RNA copies). DWV abundance increased by 2.3× at 48 hpi ($p < 0.01$) and was 10.4× greater at 72 hpi, relative to 0 hpi ($p < 0.001$) (Supplemental Figure S6D). As expected, DWV was not detected in mock-infected cells, in which a small volume of PBS-treated honey bee pupal cell culture was transferred to naïve cells. The increase in DWV abundance over time and detection of negative strand suggest that the DWV infection in honey bee pupal cells results in the production of infectious DWV virions that have the ability to infect naïve host cells.

3.8. Immune Gene Expression in Virus-Infected Primary Honey Bee Cells

Honey bee primary cells will be an important tool to investigate honey bee antiviral defense mechanisms. We hypothesized that differential transcription of immune genes in primary cultures will reflect the transcriptional response in individuals (i.e., pupae or adults) [86,100,101,107,108,145,146,190]. Therefore, to begin to characterize cellular-level honey bee antiviral immune responses, honey bee pupal cells were infected with SBV, DWV, or FHV, and the expression of select antiviral genes, including *dicer-like (dcr-like)*, *argonaute-2 (ago2)*, and *bee antiviral protein-1* (*bap1*), which was formerly designated as GenBank MF116383, were analyzed by qPCR in either two or three experimental replicates (Figure 7 and Supplemental Figures S7–S10).

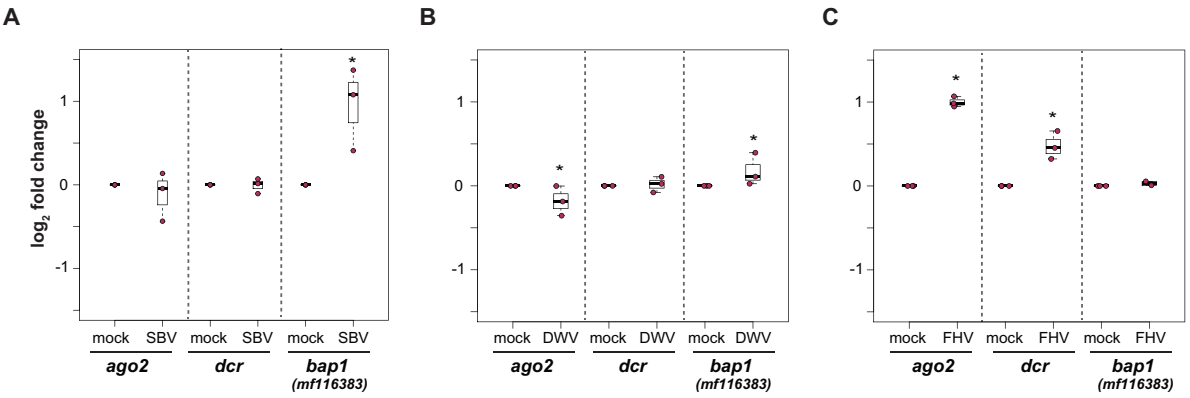


Figure 7. Virus infections of primary honey bee pupal cells differentially impact the expression of select immune genes. The relative expression of three honey bee immune genes, *argonaute-2 (ago2), dicer-like (dcr-like)*, and *bee antiviral protein-1 (bap1)/mf116383*, were assessed by qPCR at 72 hpi. The ΔΔCt method with normalization to *rpl8* was utilized to assess target gene expression in each sample, and then the $\log_2$ fold change values were calculated relative to mock-infected samples. Raw data are included in Supplemental Tables S9–S11. (**A**) SBV infection of pupal cells did not impact *ago2* or *dcr-like* expression, whereas the expression of *bap1* was 0.95 $\log_2$-fold greater at 72 hpi ($p = 0.032$). (**B**) DWV-infected pupal cells exhibited slightly lower *ago2* expression ($p = 0.032$) relative to mock-infected cells at 72 hpi, whereas there was no impact of virus infection on *dcr-like* expression. The expression of *bap1* was 0.18 $\log_2$-fold higher in DWV-infected pupal cells compared to mock-infected cells at 72 hpi ($p = 0.032$). (**C**) In pupal cells infected with FHV, *ago2* and *dcr-like* expression was higher (i.e., 1 and 0.48 $\log_2$-fold change, respectively, $p = 0.032$) in FHV-infected cells relative to mock-infected cells at 72 hpi. The expression of *bap1* was not impacted by FHV infection. The differences in $\log_2$ immune gene relative expression in infected cells relative to mock-infected (PBS-treated) cells at 72 hpi were assessed using a one-sided Wilcoxon Rank Sums test. Significance level: * $p < 0.05$.

Overall, the expression of *bap1 (*GenBank: MF116383*)* was greater in SBV-infected cells than mock-infected pupal cells at 72 hpi. Specifically, in first biological replicate, *bap1* expression was 0.67 $\log_2$-fold greater in SBV-infected pupal cells relative to the expression level in mock-infected cells (Wilcoxon Rank Sums, $p = 0.032$) (Supplemental Figure S7C). Likewise, *bap1* expression was greater in the second biological replication (0.95 $\log_2$-fold, $p = 0.032$, Figure 7A) but not the

third. Since the *bap1* expression was greater two of the three biological replicates of SBV-infected pupal cells, its expression in the third biological replicate was also assessed at 96 hpi, at which time its expression was 0.73 $\log_2$-fold higher in SBV-infected cells relative to mock-infected cells ($p = 0.032$) (Supplemental Figure S7C). Expression of neither *ago2* nor *dcr-like* were appreciably impacted by SBV infection. Specifically, *ago2* was not differentially expressed in the first two replicates but had −0.76 $\log_2$-fold lower expression in the third replicate ($p = 0.032$) (Figures 7A and S7A). Similarly, *dcr-like* was not differentially expressed in the second replicate but exhibited −0.67 $\log_2$-fold lower expression in the third replicate ($p = 0.032$) (Supplemental Figure S7). In DWV-infected pupal cells, *bap1* expression was unchanged in one replicate (Supplemental Figure S8C) and slightly greater in the second experimental replicate relative to mock-infected cells at 72 hpi (0.18 $\log_2$-fold change, $p = 0.032$, Figure 7B). In DWV-infected pupal cells, *ago2* expression was slightly lower compared to mock-infected cells in two biological replicates (−0.28 and −0.18 $\log_2$-fold change, $p = 0.032$, Figures 7B and S8A). In contrast, *dcr-like* expression was slightly lower in DWV-infected pupal cells in one replicate (−0.26 $\log_2$-fold, $p = 0.032$, Figure S8B), while its expression was similar to mock-infected cells in another replicate (Supplemental Figure S7B). The expression of *bap1* was not appreciably impacted by FHV infection of pupal cells (Figures 7C and S9C). Specifically, *bap1* expression was slightly lower in one replicate (−0.4 $\log_2$-fold, $p = 0.032$) and unchanged in the second replicate, compared to mock-infected cells (Figures 7C and S9C). Interestingly, *ago2* expression was consistently higher in FHV-infected cells relative to mock-infected cells in two replicates (i.e., 1 $\log_2$-fold in rep1 and 1 $\log_2$-fold in rep2, $p = 0.032$) (Figures 7C and S9A). Similarly, *dcr-like* expression was greater in FHV-infected cells relative to

mock-infected cells in both replicates (i.e., 0.66 log_2-fold in rep1 and 0.48 log_2-fold in rep2, p = 0.032) (Figures 7C and S9B). The trends in immune gene expression in FHV-infected hemocytes were similar to FHV-infected pupal cells (Supplemental Figure S10). Specifically, *bap1* expression was similar in FHV-infected and mock-infected cells at 72 hpi, whereas FHV-infected hemocytes exhibited greater *ago2* (i.e., 1.1 log_2-fold in rep1 and 0.54 log_2-fold in rep2, p = 0.032) and *dcr-like* expression (i.e., 0.45 log_2-fold in rep1 and 0.63 log_2-fold in rep2, p = 0.032) than mock-infected hemocytes in both biological replicates (Supplemental Figure S10).

Together these experiments indicate that studies in cultured honey bee primary cells recapitulate data obtained from transcriptome level studies in individual honey bees, including those carried out in pupae and adult bees [100], though additional studies are needed to better assess honey bee antiviral immune responses at the cellular level. Further development, including the advancements described herein, and utilization of honey bee primary cells is integral to elucidating the defense pathways important for specific virus infections and, in turn, identifying viral counter defense strategies.

4. Conclusions

In summary, these data indicate that primary honey bee cell cultures of larval hemocytes and explanted pupal tissue serve as a useful tool for studying honey bee–host interactions. In addition to demonstrating that a panel of viruses (SBV, DWV, and FHV) replicates in hemocytes and/or pupal cells, pupal cell cultures showed virus-specific transcriptional regulation of three immune genes (*dcr-like*, *ago2,* and *bap1*). These cultures can be established using rather simple techniques

that are amenable to higher sample size studies. Future work to optimize transfection in these different cell culture types will be paramount for performing more advanced mechanistic studies. This includes utilizing RNAi-mediated gene knockdown, which facilitates the study of honey bee host–virus interactions by directly demonstrating the importance of specific genes in limiting particular viruses. For example, this study indicates that hemocytes are a natural site of infection for SBV and that SBV results in higher *bap1* expression in infected pupal cells. By reducing the expression of *bap1* in cell cultures that are infected with SBV, one could test the hypothesis that this gene is important for cells to combat SBV infection. Additionally, the use of cell culture will facilitate the study of basic virological questions, including virus cellular tropism, entry, and uncoating mechanisms. Additional efforts to establish virus-free cell lines from explanted honey bee tissues will prove useful for the production of pure virus stocks, especially in concert with the development of cDNA clones capable of producing infectious virus. The data presented in this study represent one of many steps toward the development of sophisticated cell culture techniques for the study of honey bee host–virus interactions.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Excel file with Supplementary Tables S1–S24, and Supplemental Figures S1–S12. Supplemental Table and Supplemental Figure titles are listed below: Supplemental Table S1. Primers used in this study. Supplemental Table S2. Raw data for SBV-infected hemocytes (Figure 1). Supplemental Table S3. Raw data for SBV-infected purple-eyed pupal cells (Figures 2 and S2). Supplemental Table S4. Raw data for DWV-infected purple-eyed pupal cells (Figures 3 and S3). Supplemental Table S5. Raw data for FHV-infected hemocytes (Figures 4

and S4). Supplemental Table S6. Raw data for FHV-infected purple-eyed pupal cells (Figures 5 and S5). Supplemental Table S7. Raw data for transfer of DWV infection from DWV-infected purple-eyed pupal cells to naïve purple-eyed pupal cell (Figures 6 and S6). Supplemental Table S8. Raw data for transfer of FHV infection from FHV-infected purple-eyed pupal cells to naïve purple-eyed pupal cells (Figures 6 and S6). Supplemental Table S9. Raw Ct values for immune gene expression in SBV-infected pupal cells (Figures 7 and S7). Supplemental Table S10. Raw data for immune gene expression in DWV-infected honey bee pupal cells (Figures 7 and S8). Supplemental Table S11. Raw data for immune gene expression in FHV-infected honey bee pupal cells (Figures 7 and S9). Supplemental Table S12. Raw data for immune gene expression in FHV-infected hemocytes (Figure S10). Supplemental Table S13. SBV fold change relative to 0 hpi in hemocytes. Supplemental Table S14. SBV fold change relative to 0 hpi in pupal cells. Supplemental Table S15. DWV fold change relative to 0 hpi in purple-eyed pupal cells. Supplemental Table S16. FHV fold change relative to 0 hpi in hemocytes. Supplemental Table S17. FHV fold change relative to 0 hpi in purple-eyed pupal cells. Supplemental Table S18. DWV fold change relative to 0 hpi in naïve pupal cells treated with material from infected cells. Supplemental Table S19. FHV fold change relative to 0 hpi in naïve pupal cells treated with material from infected cells. Supplemental Table S20. Fold change and *p*-values for immune genes in SBV-infected pupae. Supplemental Table S21. Immune gene fold change and *p*-values for DWV-infected honey bee pupal cells. Supplemental Table S22. Immune gene fold change and *p*-values for FHV-infected honey bee pupal cells. Supplemental Table S23. Immune gene fold change and *p*-values for FHV-infected hemocytes. Supplemental Table S24. Virus testing

in corresponding honey bee larval carcass and hemocyte samples indicate that hemocytes are natural sites of infection for black queen cell virus (BQCV). Supplemental Table S25. Negative strand-specific RT-PCR confirms viral replication in hemocytes and pupal cells. Supplemental Figure S1. Larval hemocyte cultures have relatively few contaminating adipocytes. Supplemental Figure S2. Sacbrood virus (SBV) replicates in primary honey bee pupal cell cultures. Supplemental Figure S3. Deformed wing virus (DWV) replicates in primary honey bee pupal cell cultures. Supplemental Figure S4. Flock House virus (FHV) replicates in primary honey bee hemocytes. Supplemental Figure S5. Flock House virus (FHV) replicates in primary honey bee pupal cells. Supplemental Figure S6. DWV and FHV infection transferred from infected to naïve honey bee pupal cells. Supplemental Figure S7. Infection of primary pupal cells with SBV results in higher *bap-1* expression. Supplemental Figure S8. Infection of primary honey bee pupal cells with DWV has modest impact on the expression of select immune genes. Supplemental Figure S9. Infection of primary honey bee pupal cells with FHV results in differential regulation of immune genes. Supplemental Figure S10. Infection of honey bee hemocytes with FHV results in differential regulation of immune genes. Supplemental Figure S11. Sacbrood virus (SBV) inoculum was free of several other common honey bee infecting viruses. Supplemental Figure S12. Deformed wing virus (DWV) inoculum was free of several other common honey bee infecting viruses.

Author Contributions: Conceptualization, A.J.M., F.P. and M.L.F.; methodology, A.J.M., F.P., V.L. and M.L.F.; formal analysis A.J.M., F.P. and M.L.F.; investigation, A.J.M., F.P. and M.L.F.; resources, M.L.F.; data curation, A.J.M., F.P. and M.L.F.; writing—original draft

preparation, A.J.M., F.P. and M.L.F.; writing—review and editing, A.J.M., F.P., V.L. and M.L.F.; visualization, A.J.M., F.P. and M.L.F.; supervision, M.L.F.; project administration, M.L.F.; funding acquisition, M.L.F. All authors have read and agreed to the published version of the manuscript.

Funding: Research in the Flenniken laboratory is supported by the National Science Foundation CAREER Program (Award Number 1651561), Agriculture and Food Research Initiative (USDA-NIFA-AFRI) program, Montana Department of Agriculture Specialty Crop Block Grant Program (Award Number 17SC003108), USDA-NIFA Hatch Multistate Funding (NC-1173), and Project Apis m.-Costco Scholar Fellowship Program for Honey Bee Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of this manuscript.

Institutional Review Board Statement: Ethical review and approval were waived for this study, since the study involved only the use of invertebrate animals (i.e., honey bees), and Biosafety Protocols and Standard Operating Procedures were approved by Montana State University's Biosafety Committee (Approval Date: June 2020; Protocol Number: 2020-5-19).

Data Availability Statement: The majority of the data generated or analyzed during this study are included in this published article and its Supplementary Material files (available online). Additional data are available from the corresponding author upon request.

Acknowledgments: We are grateful to Dr. Marie Pizzorno for help with producing the DWV stock, Dr. Katie F. Daughenbaugh for discussions throughout the experiments, and Dr. Matthew Taylor for assistance with honey bee hemocyte imaging. We would also like to thank the

members of Flenniken laboratory (Brian Ross and Naomi Kaku) for reviewing this manuscript prior to publication.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. van Cleef, K.W.R.; van Mierlo, J.T.; van den Beek, M.; van Rij, R.P. Identification of Viral Suppressors of RNAi by a Reporter Assay in Drosophila S2 Cell Culture. In *Antiviral RNAi: Concepts, Methods, and Applications*; van Rij, R.P., Ed.; Humana Press: Totowa, NJ, USA, 2011; pp. 201–213.

2. Baum, B.; Cherbas, L. Drosophila Cell Lines as Model Systems and as an Experimental Tool. In *Drosophila: Methods and Protocols*; Dahmann, C., Ed.; Humana Press: Totowa, NJ, USA, 2008; pp. 391–424.

3. Harschnitz, O.; Studer, L. Human stem cell models to study host–virus interactions in the central nervous system. *Nat. Rev. Immunol.* **2021**, *21*, 441–453, doi:10.1038/s41577-020-00474-y.

4. Hahn, C.S.; Hahn, Y.S.; Braciale, T.J.; Rice, C.M. Infectious Sindbis virus transient expression vectors for studying antigen processing and presentation. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 2679, doi:10.1073/pnas.89.7.2679.

5. Genzel, Y.; Reichl, U. Vaccine Production. In *Animal Cell Biotechnology: Methods and Protocols*; Pörtner, R., Ed.; Humana Press: Totowa, NJ, USA, 2007; pp. 457–473.

6. Montagnon, B.J.; Fanget, B.; Vincent-Falquet, J.C. Industrial-scale production of inactivated poliovirus vaccine prepared by culture of Vero cells on microcarrier. *Rev. Infect. Dis.* **1984**, *6* (Suppl. 2), S341–S344, doi:10.1093/clinids/6.supplement_2.s341.

7. Hudu, S.A.; Alshrari, A.S.; Syahida, A.; Sekawi, Z. Cell Culture, Technology: Enhancing the Culture of Diagnosing Human Diseases. *J. Clin. Diagn. Res.* **2016**, *10*, DE01–DE05, doi:10.7860/JCDR/2016/15837.7460.

8. Guo, Y.; Goodman, C.L.; Stanley, D.W.; Bonning, B.C. Cell Lines for Honey Bee Virus Research. *Viruses* **2020**, *12*, 236, doi:10.3390/v12020236.

9. Goblirsch, M.J.; Spivak, M.S.; Kurtti, T.J. A Cell Line Resource Derived from Honey Bee (Apis mellifera) Embryonic Tissues. *PLoS ONE* **2013**, *8*, e09831, doi:10.1371/journal.pone.0069831.

10. Carrillo-Tripp, J.; Dolezal, A.G.; Goblirsch, M.J.; Miller, W.A.; Toth, A.L.; Bonning, B.C. In vivo and in vitro infection dynamics of honey bee viruses. *Sci. Rep.* **2016**, *6*, 22265, doi:10.1038/srep22265.

11. vanEngelsdorp, D.; Caron, D.; Hayes, J.; Underwood, R.; Henson, M.; Rennich, K.; Spleen, A.; Andree, M.; Snyder, R.; Lee, K.; et al. A national survey of managed honey bee 2010–11 winter colony losses in the USA: Results from the Bee Informed Partnership. *J. Apic. Res.* **2012**, *51*, 115–124, doi:10.3896/IBRA.1.51.1.14.

12. Spleen, A.M.; Lengerich, E.J.; Rennich, K.; Caron, D.; Rose, R.; Pettis, J.S.; Henson, M.; Wilkes, J.T.; Wilson, M.; Stitzinger, J.; et al. A national survey of managed honey bee 2011–12 winter colony losses in the United States: Results from the Bee Informed Partnership. *J. Apic. Res.* **2013**, *52*, 44–53, doi:10.3896/IBRA.1.52.2.07.

13. Steinhauer, N.A.; Rennich, K.; Wilson, M.E.; Caron, D.M.; Lengerich, E.J.; Pettis, J.S.; Rose, R.; Skinner, J.A.; Tarpy, D.R.; Wilkes, J.T.; et al. A national survey of managed honey bee 2012–2013 annual colony losses in the USA: Results from the Bee Informed Partnership. *J. Apic. Res.* **2014**, *53*, 1–18, doi:10.3896/IBRA.1.53.1.01.

14. Seitz, N.; Traynor, K.S.; Steinhauer, N.; Rennich, K.; Wilson, M.E.; Ellis, J.D.; Rose, R.; Tarpy, D.R.; Sagili, R.R.; Caron, D.M.; et al. A national survey of managed honey bee 2014–2015 annual colony losses in the USA. *J. Apic. Res.* **2015**, *54*, 292–304, doi:10.1080/00218839.2016.1153294.

15. Kulhanek, K.; Steinhauer, N.; Rennich, K.; Caron, D.M.; Sagili, R.R.; Pettis, J.S.; Ellis, J.D.; Wilson, M.E.; Wilkes, J.T.; Tarpy, D.R.; et al. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. *J. Apic. Res.* **2017**, *56*, 328–340, doi:10.1080/00218839.2017.1344496.

16. Traynor, K.S.; Rennich, K.; Forsgren, E.; Rose, R.; Pettis, J.; Kunkel, G.; Madella, S.; Evans, J.; Lopez, D.; vanEngelsdorp, D. Multiyear survey targeting disease incidence in US honey bees. *Apidologie* **2016**, *47*, 325–347, doi:10.1007/s13592-016-0431-0.

17. Steinhauer, N.; Rennich, K.; Caron, D.M.; Ellis, J.D.; Koenig, P.; Kulhanek, K.; Klepps, J.; Lee, K.; Milbrath, M.; Rangel, J.R.; et al. Honey Bee Colony Losses 2016–2017: Preliminary Results; Bee Informed Partnership, 2017. Available online: https://beeinformed.org/2017/05/25/2016-2017-loss-results-thank-you-to-all-survey-participants/ (accessed on 27 June 2021).

18. Bruckner, S.; Steinhauer, N.; Rennich, K.; Aurell, D.D.; Caron, D.M.; Ellis, J.D.; Fauvel, A.M.; Kulhanek, K.; Nelson, K.C.; Rangel, J.; et al. United States Honey Bee Colony Losses 2017–2018: Preliminary Results; Bee Informed Partnership, 2018. Available online: https://beeinformed.org/2018/06/21/preliminary-results-2017-2018-total-and-average-honey-bee-colony-losses-by-state-and-the-district-of-columbia/ (accessed on 27 June 2021).

19. Bruckner, S.; Steinhauer, N.; Aurell, S.D.; Caron, D.M.; Ellis, J.D.; Fauvel, A.M.; Kulhanek, K.; McArt, S.H.; Mullen, E.; Rangel, J.; et al. 2018–2019 Honey Bee Colony Losses in the United States: Preliminary Results; Bee Informed Partnership, 2019. Available online: https://beeinformed.org/citizen-science/loss-and-management-survey/ (accessed on 27 June 2021).

20. Goulson, D.; Nicholls, E.; Botias, C.; Rotheray, E.L. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* **2015**, *347*, 1255957, doi:10.1126/science.1255957.

21. Cornman, R.S.; Tarpy, D.R.; Chen, Y.; Jeffreys, L.; Lopez, D.; Pettis, J.S.; VanEngelsdorp, D.; Evans, J.D. Pathogen webs in collapsing honey bee colonies. *PLoS ONE* **2012**, *7*, e43562, doi:10.1371/journal.pone.0043562.

22. Brutscher, L.M.; McMenamin, A.J.; Flenniken, M.L. The buzz about honey bee viruses. *PLoS Pathog.* **2016**, *12*, e1005757, doi:10.1371/journal.ppat.1005757.

23. Grozinger, C.M.; Flenniken, M.L. Bee Viruses: Ecology, Pathogenicity, and Impacts. *Annu. Rev. Entomol.* **2019**, *64*, 205–226, doi:10.1146/annurev-ento-011118-111942.

24. McMenamin, A.J.; Flenniken, M.L. Recently identified bee viruses and their impact on bee pollinators. *Curr. Opin. Insect Sci.* **2018**, *26*, 120–129, doi:10.1016/j.cois.2018.02.009.

25. Schwarz, R.S.; Bauchan, G.R.; Murphy, C.A.; Ravoet, J.; de Graaf, D.C.; Evans, J.D. Characterization of two species of trypanosomatidae from the honey bee apis mellifera: Crithidia mellificae langridge and mcghee, and lotmaria passim n. gen., n. sp. *J. Eukaryot. Microbiol.* **2015**, *62*, 1–17, doi:10.1111/jeu.12209.

26. Runckel, C.; DeRisi, J.; Flenniken, M.L. A draft genome of the honey bee trypanosomatid parasite crithidia mellificae. *PLoS ONE* **2014**, *9*, e95057, doi:10.1371/journal.pone.0095057.

27. Ravoet, J.; Schwarz, R.S.; Descamps, T.; Yañez, O.; Tozkar, C.O.; Martin-Hernandez, R.; Bartolomé, C.; De Smet, L.; Higes, M.; Wenseleers, T.; et al. Differential diagnosis of the honey bee trypanosomatids Crithidia mellificae and Lotmaria passim. *J. Invertebr. Pathol.* **2015**, *130*, 21–27, doi:10.1016/j.jip.2015.06.007.

28. Cornman, R.S.; Chen, Y.P.; Schatz, M.C.; Street, C.; Zhao, Y.; Desany, B.; Egholm, M.; Hutchison, S.; Pettis, J.S.; Lipkin, W.I.; et al. Genomic Analyses of the Microsporidian Nosema ceranae, an Emergent Pathogen of Honey Bees. *PLoS Pathog.* **2009**, *5*, e1000466, doi:10.1371/journal.ppat.1000466.

29. Genersch, E. American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. *J. Invertebr. Pathol.* **2010**, *103*, S10–S19, doi:10.1016/j.jip.2009.06.015.

30. Forsgren, E. European foulbrood in honey bees. *J. Invertebr. Pathol.* **2010**, *103*, S5–S9, doi:10.1016/j.jip.2009.06.016.

31. Nazzi, F.; Le Conte, Y. Ecology of Varroa destructor, the Major Ectoparasite of the Western Honey Bee, Apis mellifera. *Annu. Rev. Entomol.* **2016**, *61*, 417–432, doi:10.1146/annurev-ento-010715-023731.

32. Beaurepaire, A.; Piot, N.; Doublet, V.; Antunez, K.; Campbell, E.; Chantawannakul, P.; Chejanovsky, N.; Gajda, A.; Heerman, M.; Panziera, D.; et al. Diversity and Global Distribution of Viruses of the Western Honey Bee, Apis mellifera. *Insects* **2020**, *11*, 239, doi:10.3390/insects11040239.

33. Daughenbaugh, K.F.; Kahnonitch, I.; Carey, C.C.; McMenamin, A.J.; Wiegand, T.; Erez, T.; Arkin, N.; Ross, B.; Wiedenheft, B.; Sadeh, A.; et al. Metatranscriptome Analysis of Sympatric Bee Species Identifies Bee Virus Variants and a New Virus, Andrena-Associated Bee Virus-1. *Viruses* **2021**, *13*, 291, doi:10.3390/v13020291.

34. Schoonvaere, K.; Smagghe, G.; Francis, F.; de Graaf, D.C. Study of the Metatranscriptome of Eight Social and Solitary Wild Bee Species Reveals Novel Viruses and Bee Parasites. *Front. Microbiol.* **2018**, *9*, 177, doi:10.3389/fmicb.2018.00177.

35. de Miranda, J.R.; Cornman, R.S.; Evans, J.D.; Semberg, E.; Haddad, N.; Neumann, P.; Gauthier, L. Genome Characterization, Prevalence and Distribution of a Macula-Like Virus from Apis mellifera and Varroa destructor. *Viruses* **2015**, *7*, 3586–3602, doi:10.3390/v7072789.

36. Remnant, E.J.; Shi, M.; Buchmann, G.; Blacquiere, T.; Holmes, E.C.; Beekman, M.; Ashe, A. A Diverse Range of Novel RNA Viruses in Geographically Distinct Honey Bee Populations. *J. Virol.* **2017**, *91*, e00158-17, doi:10.1128/JVI.00158-17.

37. Levin, S.; Sela, N.; Chejanovsky, N. Two novel viruses associated with the Apis mellifera pathogenic mite Varroa destructor. *Sci. Rep.* **2016**, *6*, 37710, doi:10.1038/srep37710.

38. Levin, S.; Galbraith, D.; Sela, N.; Erez, T.; Grozinger, C.M.; Chejanovsky, N. Presence of Apis Rhabdovirus-1 in Populations of Pollinators and Their Parasites from Two Continents. *Front. Microbiol.* **2017**, *8*, 2482, doi:10.3389/fmicb.2017.02482.

39. Galbraith, D.A.; Fuller, Z.L.; Ray, A.M.; Brockmann, A.; Frazier, M.; Gikungu, M.W.; Martinez, J.F.I.; Kapheim, K.M.; Kerby, J.T.; Kocher, S.D.; et al. Investigating the viral ecology of global bee communities with high-throughput metagenomics. *Sci. Rep.* **2018**, *8*, 8879, doi:10.1038/s41598-018-27164-z.

40. Runckel, C.; Flenniken, M.L.; Engel, J.C.; Ruby, J.G.; Ganem, D.; Andino, R.; DeRisi, J.L. Temporal Analysis of the Honey Bee Microbiome Reveals Four Novel Viruses and Seasonal Prevalence of Known Viruses, Nosema, and Crithidia. *PLoS ONE* **2011**, *6*, e20656, doi:10.1371/journal.pone.0020656.

41. Deboutte, W.; Beller, L.; Yinda, C.K.; Shi, C.; Smets, L.; Vanmechelen, B.; Conceição-Neto, N.; Dallmeier, K.; Maes, P.; de Graaf, D.C.; et al. Hymenoptera associated eukaryotic virome lacks host specificity. *BioRxiv* **2020**, doi:10.1101/2020.09.15.298042.

42. Govan, V.A.; Leat, N.; Allsopp, M.; Davison, S. Analysis of the Complete Genome Sequence of Acute Bee Paralysis Virus Shows That It Belongs to the Novel Group of Insect-Infecting RNA Viruses. *Virology* **2000**, *277*, 457–463, doi:10.1006/viro.2000.0616.

43. Leat, N.; Ball, B.; Govan, V.; Davison, S. Analysis of the complete genome sequence of black queen-cell virus, a picorna-like virus of honey bees. *J. Gen. Virol.* **2000**, *81*, 2111–2119, doi:10.1099/0022-1317-81-8-2111.

44. Olivier, V.; Blanchard, P.; Chaouch, S.; Lallemand, P.; Schurr, F.; Celle, O.; Dubois, E.; Tordo, N.; Thiéry, R.; Houlgatte, R.; et al. Molecular characterisation and phylogenetic analysis of Chronic bee paralysis virus, a honey bee virus. *Virus Res.* **2008**, *132*, 59–68, doi:10.1016/j.virusres.2007.10.014.

45. Ribière, M.; Olivier, V.; Blanchard, P. Chronic bee paralysis: A disease and a virus like no other? *J. Invertebr. Pathol.* **2010**, *103*, S120–S131, doi:10.1016/j.jip.2009.06.013.

46. Lanzi, G.; de Miranda, J.R.; Boniotti, M.B.; Cameron, C.E.; Lavazza, A.; Capucci, L.; Camazine, S.M.; Rossi, C. Molecular and biological characterization of deformed wing virus of honeybees (Apis mellifera L.). *J. Virol.* **2006**, *80*, 4998–5009, doi:10.1128/JVI.80.10.4998-5009.2006.

47. de Miranda, J.R.; Genersch, E. Deformed wing virus. *J. Invertebr. Pathol.* **2010**, *103*, S48–S61, doi:10.1016/j.jip.2009.06.012.

48. Maori, E.; Lavi, S.; Mozes-Koch, R.; Gantman, Y.; Peretz, Y.; Edelbaum, O.; Tanne, E.; Sela, I. Isolation and characterization of Israeli acute paralysis virus, a dicistrovirus affecting honeybees in Israel: Evidence for diversity due to intra- and inter-species recombination. *J. Gen. Virol.* **2007**, *88*, 3428–3438, doi:10.1099/vir.0.83284-0.

49. de Miranda, J.R.; Drebot, M.; Tyler, S.; Shen, M.; Cameron, C.E.; Stoltz, D.B.; Camazine, S.M. Complete nucleotide sequence of Kashmir bee virus and comparison with acute bee paralysis virus. *J. Gen. Virol.* **2004**, *85*, 2263–2270, doi:10.1099/vir.0.79990-0.

50. Ghosh, R.C.; Ball, B.V.; Willcocks, M.M.; Carter, M.J. The nucleotide sequence of sacbrood virus of the honey bee: An insect picorna-like virus. *J. Gen. Virol.* **1999**, *80*, 1541–1549, doi:10.1099/0022-1317-80-6-1541.

51. Bigot, D.; Dalmon, A.; Roy, B.; Hou, C.; Germain, M.; Romary, M.; Deng, S.; Diao, Q.; Weinert, L.A.; Cook, J.M.; et al. The discovery Halictivirus resolves the Sinaivirus phylogeny. *J. Gen. Virol.* **2017**, *98*, 2864–2875, doi:10.1099/jgv.0.000957.

52. Daughenbaugh, K.F.; Martin, M.; Brutscher, L.M.; Cavigli, I.; Garcia, E.; Lavin, M.; Flenniken, M.L. Honey bee infecting Lake Sinai viruses. *Viruses* **2015**, *7*, 3285–3309, doi:10.3390/v7062772.

53. Francis, R.M.; Nielsen, S.L.; Kryger, P. Patterns of viral infection in honey bee queens. *J. Gen. Virol.* **2013**, *94*, 668–676, doi:10.1099/vir.0.047019-0.

54. D'Alvise, P.; Seeburger, V.; Gihring, K.; Kieboom, M.; Hasselmann, M. Seasonal dynamics and co-occurrence patterns of honey bee pathogens revealed by high-throughput RT-qPCR analysis. *Ecol. Evol.* **2019**, *9*, 10241–10252, doi:10.1002/ece3.5544.

55. Allen, M.; Ball, B. The incidence and world distribution of honey bee viruses. *Bee World* **1996**, *77*, 141–162, doi:10.1080/0005772x.1996.11099306.

56. Bradbear, N. World Distribution of Major Honeybee Diseases and Pests. *Bee World* **1988**, *69*, 15–39, doi:10.1080/0005772X.1988.11098943.

57. Ellis, J.D.; Munn, P.A. The worldwide health status of honey bees. *Bee World* **2005**, *86*, 88–101, doi:10.1080/0005772X.2005.11417323.

58. Chen, Y.P.; Siede, R. Honey bee viruses. *Adv. Virus Res.* **2007**, *70*, 33–80, doi:10.1016/S0065-3527(07)70002-7.

59. Bailey, L. The multiplication and spread of sacbrood virus of bees. *Ann. Appl. Biol.* **1969**, *63*, 483–491, doi:10.1111/j.1744-7348.1969.tb02844.x.

60. Shen, M.; Cui, L.; Ostiguy, N.; Cox-Foster, D. Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite. *J. Gen. Virol.* **2005**, *86*, 2281–2289, doi:10.1099/vir.0.80824-0.

61. Bailey, L.; Ball, B.V.; Perry, J.N. The prevalence of viruses of honey bees in Britain. *Ann. Appl. Biol.* **1981**, *97*, 109–118, doi:10.1111/j.1744-7348.1981.tb02999.x.

62. Tentcheva, D.; Gauthier, L.; Zappulla, N.; Dainat, B.; Cousserans, F.; Colin, M.E.; Bergoin, M. Prevalence and seasonal variations of six bee viruses in Apis mellifera L. and Varroa destructor mite populations in France. *Appl. Environ. Microbiol.* **2004**, *70*, 7185–7191, doi:10.1128/AEM.70.12.7185-7191.2004.

63. Faurot-Daniels, C.; Glenny, W.; Daughenbaugh, K.F.; McMenamin, A.J.; Burkle, L.A.; Flenniken, M.L. Longitudinal monitoring of honey bee colonies reveals dynamic nature of virus abundance and indicates a negative impact of Lake Sinai virus 2 on colony health. *PLoS ONE* **2020**, *15*, e0237544, doi:10.1371/journal.pone.0237544.

64. Cavigli, I.; Daughenbaugh, K.F.; Martin, M.; Lerch, M.; Banner, K.; Garcia, E.; Brutscher, L.M.; Flenniken, M.L. Pathogen prevalence and abundance in honey bee colonies involved in almond pollination. *Apidologie* **2016**, *47*, 251–266, doi:10.1007/s13592-015-0395-5.

65. Gusachenko, O.N.; Woodford, L.; Balbirnie-Cumming, K.; Campbell, E.M.; Christie, C.R.; Bowman, A.S.; Evans, D.J. Green Bees: Reverse Genetic Analysis of Deformed Wing Virus Transmission, Replication, and Tropism. *Viruses* **2020**, *12*, 532, doi:10.3390/v12050532.

66. Gisder, S.; Aumeier, P.; Genersch, E. Deformed wing virus: Replication and viral load in mites (Varroa destructor). *J. Gen. Virol.* **2009**, *90*, 463–467, doi:10.1099/vir.0.005579-0.

67. Genersch, E.; Yue, C.; Fries, I.; de Miranda, J.R. Detection of Deformed wing virus, a honey bee viral pathogen, in bumble bees (Bombus terrestris and Bombus pascuorum) with wing deformities. *J. Invertebr. Pathol*. **2006**, *91*, 61–63, doi:10.1016/j.jip.2005.10.002.

68. Moeckel, N.; Gisder, S.; Genersch, E. Horizontal transmission of deformed wing virus: Pathological consequences in adult bees (Apis mellifera) depend on the transmission route. *J. Gen. Virol.* **2011**, *92*, 370–377, doi:10.1099/vir.0.025940-0.

69. Benaets, K.; Van Geystelen, A.; Cardoen, D.; De Smet, L.; de Graaf, D.C.; Schoofs, L.; Larmuseau, M.H.D.; Brettell, L.E.; Martin, S.J.; Wenseleers, T. Covert deformed wing virus infections have long-term deleterious effects on honeybee foraging and survival. *Proc. R. Soc. B Biol. Sci.* **2017**, *284*, 20162149, doi:10.1098/rspb.2016.2149.

70. Dainat, B.; Evans, J.D.; Chen, Y.P.; Gauthier, L.; Neumann, P. Dead or alive: Deformed wing virus and Varroa destructor reduce the life span of winter honeybees. *Appl. Environ. Microbiol*. **2012**, *78*, 981–987, doi:10.1128/AEM.06537-11.

71. Traniello, I.M.; Bukhari, S.A.; Kevill, J.; Ahmed, A.C.; Hamilton, A.R.; Naeger, N.L.; Schroeder, D.C.; Robinson, G.E. Meta-analysis of honey bee neurogenomic response links Deformed wing virus type A to precocious behavioral maturation. *Sci. Rep.* **2020**, *10*, 3101, doi:10.1038/s41598-020-59808-4.

72. Wells, T.; Wolf, S.; Nicholls, E.; Groll, H.; Lim, K.S.; Clark, S.J.; Swain, J.; Osborne, J.L.; Haughton, A.J. Flight performance of actively foraging honey bees is reduced by a common pathogen. *Environ. Microbiol. Rep.* **2016**, *8*, 728–737, doi:10.1111/1758-2229.12434.

73. Fujiyuki, T.; Takeuchi, H.; Ono, M.; Ohka, S.; Sasaki, T.; Nomoto, A.; Kubo, T. Novel Insect Picorna-Like Virus Identified in the Brains of Aggressive Worker Honeybees. *J. Virol.* **2004**, *78*, 1093–1100, doi:10.1128/JVI.78.3.1093-1100.2004.

74. Iqbal, J.; Mueller, U. Virus infection causes specific learning deficits in honeybee foragers. *Proc. Biol. Sci.* **2007**, *274*, 1517–1521, doi:10.1098/rspb.2007.0022.

75. Pizzorno, M.C.; Field, K.; Kobokovich, A.L.; Martin, P.L.; Gupta, R.A.; Mammone, R.; Rovnyak, D.; Capaldi, E.A. Transcriptomic Responses of the Honey Bee Brain to Infection with Deformed Wing Virus. *Viruses* **2021**, *13*, 287, doi:10.3390/v13020287.

76. Amiri, E.; Kryger, P.; Meixner, M.D.; Strand, M.K.; Tarpy, D.R.; Rueppell, O. Quantitative patterns of vertical transmission of deformed wing virus in honey bees. *PLoS ONE* **2018**, *13*, e0195283, doi:10.1371/journal.pone.0195283.

77. Yue, C.; Schröder, M.; Gisder, S.; Genersch, E. Vertical-transmission routes for deformed wing virus of honeybees (Apis mellifera). *J. Gen. Virol.* **2007**, *88*, 2329–2336, doi:10.1099/vir.0.83101-0.

78. Posada-Florez, F.; Lamas, Z.S.; Hawthorne, D.J.; Chen, Y.; Evans, J.D.; Ryabov, E.V. Pupal cannibalism by worker honey bees contributes to the spread of deformed wing virus. *Sci. Rep.* **2021**, *11*, 8989, doi:10.1038/s41598-021-88649-y.

79. Yañez, O.; Jaffé, R.; Jarosch, A.; Fries, I.; Moritz, R.F.A.; Paxton, R.J.; de Miranda, J.R. Deformed wing virus and drone mating flights in the honey bee (Apis mellifera): Implications for sexual transmission of a major honey bee virus. *Apidologie* **2012**, *43*, 17–30, doi:10.1007/s13592-011-0088-7.

80. Amiri, E.; Meixner, M.D.; Kryger, P. Deformed wing virus can be transmitted during natural mating in honey bees and infect the queens. *Sci. Rep.* **2016**, *6*, 33065, doi:10.1038/srep33065.

81. de Miranda, J.R.; Fries, I. Venereal and vertical transmission of deformed wing virus in honeybees (Apis mellifera L.). *J. Invertebr. Pathol.* **2008**, *98*, 184–189, doi:10.1016/j.jip.2008.02.004.

82. Yañez, O.; Piot, N.; Dalmon, A.; de Miranda, J.R.; Chantawannakul, P.; Panziera, D.; Amiri, E.; Smagghe, G.; Schroeder, D.; Chejanovsky, N. Bee Viruses: Routes of Infection in Hymenoptera. *Front. Microbiol.* **2020**, *11*, 943–943, doi:10.3389/fmicb.2020.00943.

83. Ramsey, S.D.; Ochoa, R.; Bauchan, G.; Gulbronson, C.; Mowery, J.D.; Cohen, A.; Lim, D.; Joklik, J.; Cicero, J.M.; Ellis, J.D.; et al. Varroa destructor feeds primarily on honey bee fat body tissue and not hemolymph. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 1792–1801, doi:10.1073/pnas.1818371116.

84. Posada-Florez, F.; Ryabov, E.V.; Heerman, M.C.; Chen, Y.; Evans, J.D.; Sonenshine, D.E.; Cook, S.C. Varroa destructor mites vector and transmit pathogenic honey bee viruses acquired from an artificial diet. *PLoS ONE* **2020**, *15*, e0242688, doi:10.1371/journal.pone.0242688.

85. Gisder, S.; Genersch, E.; Pfeiffer Julie, K. Direct Evidence for Infection of Varroa destructor Mites with the Bee-Pathogenic Deformed Wing Virus Variant B, but Not Variant A, via Fluorescence In Situ Hybridization Analysis. *J. Virol.* **2021**, *95*, e01786–01720, doi:10.1128/JVI.01786-20.

86. Ryabov, E.V.; Wood, G.R.; Fannon, J.M.; Moore, J.D.; Bull, J.C.; Chandler, D.; Mead, A.; Burroughs, N.; Evans, D.J. A Virulent Strain of Deformed Wing Virus (DWV) of Honeybees (Apis mellifera) Prevails after Varroa destructor-Mediated, or In Vitro, Transmission. *PLoS Pathog.* **2014**, *10*, e1004230, doi:10.1371/journal.ppat.1004230.

87. Posada-Florez, F.; Childers, A.K.; Heerman, M.C.; Egekwu, N.I.; Cook, S.C.; Chen, Y.; Evans, J.D.; Ryabov, E.V. Deformed wing virus type A, a major honey bee pathogen, is vectored by the mite Varroa destructor in a non-propagative manner. *Sci. Rep.* **2019**, *9*, 12445, doi:10.1038/s41598-019-47447-3.

88. Ongus, J.R.; Peters, D.; Bonmatin, J.M.; Bengsch, E.; Vlak, J.M.; van Oers, M.M. Complete sequence of a picorna-like virus of the genus Iflavirus replicating in the mite Varroa destructor. *J. Gen. Virol.* **2004**, *85*, 3747–3755, doi:10.1099/vir.0.80470-0.

89. Moore, J.; Jironkin, A.; Chandler, D.; Burroughs, N.; Evans, D.J.; Ryabov, E.V. Recombinants between Deformed wing virus and Varroa destructor virus-1 may prevail

in Varroa destructor-infested honeybee colonies. *J. Gen. Virol.* **2011**, *92*, 156–161, doi:10.1099/vir.0.025965-0.

90. Zioni, N.; Soroker, V.; Chejanovsky, N. Replication of Varroa destructor virus 1 (VDV-1) and a Varroa destructor virus 1–deformed wing virus recombinant (VDV-1–DWV) in the head of the honey bee. *Virology* **2011**, *417*, 106–112, doi:10.1016/j.virol.2011.05.009.

91. Mordecai, G.J.; Wilfert, L.; Martin, S.J.; Jones, I.M.; Schroeder, D.C. Diversity in a honey bee pathogen: First report of a third master variant of the Deformed Wing Virus quasispecies. *ISME J.* **2016**, *10*, 1264–1273, doi:10.1038/ismej.2015.178.

92. Kevill, J.L.; Highfield, A.; Mordecai, G.J.; Martin, S.J.; Schroeder, D.C. ABC Assay: Method Development and Application to Quantify the Role of Three DWV Master Variants in Overwinter Colony Losses of European Honey Bees. *Viruses* **2017**, *9*, 314.

93. Dalmon, A.; Desbiez, C.; Coulon, M.; Thomasson, M.; Conte, Y.L.; Alaux, C.; Vallon, J. Evidence for positive selection and recombination hotspots in Deformed wing virus (DWV). *Sci. Rep.* **2017**, 41045, doi:10.1038/srep41045.

94. Genersch, E.; Von Der Ohe, W.; Kaatz, H.; Schroeder, A.; Otten, C.; Büchler, R.; Berg, S.; Ritter, W.; Mühlen, W.; Gisder, S.; et al. The German bee monitoring project: A long term study to understand periodically high winter losses of honey bee colonies. *Apidologie* **2010**, *41*, 332–352, doi:10.1051/apido/2010014.

95. Nazzi, F.; Brown, S.P.; Annoscia, D.; Del Piccolo, F.; Di Prisco, G.; Varricchio, P.; Vedova, G.D.; Cattonaro, F.; Caprio, E.; Pennacchio, F. Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. *PLoS Pathog.* **2012**, *8*, e1002735, doi:10.1371/journal.ppat.1002735.

96. Di Prisco, G.; Annoscia, D.; Margiotta, M.; Ferrara, R.; Varricchio, P.; Zanni, V.; Caprio, E.; Nazzi, F.; Pennacchio, F. A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 201523515, doi:10.1073/pnas.1523515113.

97. Glenny, W.; Cavigli, I.; Daughenbaugh, K.F.; Radford, R.; Kegley, S.E.; Flenniken, M.L. Honey bee (Apis mellifera) colony health and pathogen composition in migratory beekeeping operations involved in California almond pollination. *PLoS ONE* **2017**, *12*, e0182814, doi:10.1371/journal.pone.0182814.

98. Nazzi, F.; Pennacchio, F. Disentangling multiple interactions in the hive ecosystem. *Trends Parasitol.* **2014**, *30*, 556–561, doi:10.1016/j.pt.2014.09.006.

99. Lamp, B.; Url, A.; Seitz, K.; Eichhorn, J.; Riedel, C.; Sinn, L.J.; Indik, S.; Köglberger, H.; Rümenapf, T. Construction and Rescue of a Molecular Clone of Deformed Wing Virus (DWV). *PLoS ONE* **2016**, *11*, e0164639, doi:10.1371/journal.pone.0164639.

100. Ryabov, E.V.; Fannon, J.M.; Moore, J.D.; Wood, G.R.; Evans, D.J. The Iflaviruses Sacbrood virus and Deformed wing virus evoke different transcriptional responses in the honeybee which may facilitate their horizontal or vertical transmission. *PeerJ* **2015**, *3*, e1405v1, doi:10.7717/peerj.1591.

101. Galbraith, D.A.; Yang, X.; Nino, E.L.; Yi, S.; Grozinger, C. Parallel epigenomic and transcriptomic responses to viral infection in honey bees (Apis mellifera). *PLoS Pathog.* **2015**, *11*, e1004713, doi:10.1371/journal.ppat.1004713.

102. Boncristiani, H.F.; Evans, J.D.; Chen, Y.; Pettis, J.; Murphy, C.; Lopez, D.L.; Simone-Finstrom, M.; Strand, M.; Tarpy, D.R.; Rueppell, O. In vitro infection of pupae with Israeli acute paralysis virus suggests disturbance of transcriptional homeostasis in honey bees (Apis mellifera). *PLoS ONE* **2013**, *8*, e73429, doi:10.1371/journal.pone.0073429.

103. Dolezal, A.G.; Hendrix, S.D.; Scavo, N.A.; Carrillo-tripp, J.; Harris, A.; Wheelock, M.J.; Neal, M.E.O.; Toth, A.L. Honey Bee Viruses in Wild Bees : Viral Prevalence , Loads , and Experimental Inoculation. *PLoS ONE* **2016**, *11*, e0166190, doi:10.1371/journal.pone.0166190.

104. Hsieh, E.M.; Carrillo-Tripp, J.; Dolezal, A.G. Preparation of Virus-Enriched Inoculum for Oral Infection of Honey Bees (Apis Mellifera). *J. Vis. Exp.* **2020**, *162*, e61725, doi:10.3791/61725.

105. O'Neal, S.T.; Brewster, C.C.; Bloomquist, J.R.; Anderson, T.D. Amitraz and its metabolite modulate honey bee cardiac function and tolerance to viral infection. *J. Invertebr. Pathol.* **2017**, *149*, 119–126, doi:10.1016/j.jip.2017.08.005.

106. O'Neal, S.T.; Swale, D.R.; Anderson, T.D. ATP-sensitive inwardly rectifying potassium channel regulation of viral infections in honey bees. *Sci. Rep.* **2017**, *7*, 8668–8668, doi:10.1038/s41598-017-09448-y.

107. Brutscher, L.M.; Daughenbaugh, K.F.; Flenniken, M.L. Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. *Sci. Rep.* **2017**, *7*, 6448, doi:10.1038/s41598-017-06623-z.

108. Flenniken, M.L.; Andino, R. Non-Specific dsRNA-Mediated Antiviral Response in the Honey Bee. *PLoS ONE* **2013**, *8*, e77263, doi:10.1371/journal.pone.0077263.

109. McMenamin, A.J.; Daughenbaugh, K.F.; Flenniken, M.L. The Heat Shock Response in the Western Honey Bee (Apis mellifera) is Antiviral. *Viruses* **2020**, *12*, 245, doi:10.3390/v12020245.

110. Dasgupta, R.; Cheng, L.L.; Bartholomay, L.C.; Christensen, B.M. Flock house virus replicates and expresses green fluorescent protein in mosquitoes. *J. Gen. Virol.* **2003**, *84*, 1789–1797, doi:10.1099/vir.0.18938-0.

111. Dasgupta, R.; Free, H.M.; Zietlow, S.L.; Paskewitz, S.M.; Aksoy, S.; Shi, L.; Fuchs, J.; Hu, C.; Christensen, B.M. Replication of flock house virus in three genera of medically important insects. *J. Med. Entomol.* **2007**, *44*, 102–110, doi:10.1603/0022-2585(2007)44[102:rofhvi]2.0.co;2.

112. Wang, X.-H.; Aliyari, R.; Li, W.-X.; Li, H.-W.; Kim, K.; Carthew, R.; Atkinson, P.; Ding, S.-W. RNA Interference Directs Innate Immunity Against Viruses in Adult Drosophila. *Science* **2006**, *312*, 452–454, doi:10.1126/science.1125694.

113. Lingel, A.; Simon, B.; Izaurralde, E.; Sattler, M. The structure of the flock house virus B2 protein, a viral suppressor of RNA interference, shows a novel mode of double-stranded RNA recognition. *EMBO Rep*. **2005**, *6*, 1149–1155, doi:10.1038/sj.embor.7400583.

114. Venter, P.A.; Schneemann, A. Recent insights into the biology and biomedical applications of Flock House virus. *Cell. Mol. Life Sci.* **2008**, *65*, 2675–2687, doi:10.1007/s00018-008-8037-y.

115. Gallagher, T.M.; Rueckert, R.R. Assembly-dependent maturation cleavage in provirions of a small icosahedral insect ribovirus. *J. Virol.* **1988**, *62*, 3399–3406, doi:10.1128/jvi.62.9.3399-3406.1988.

116. Wu, S.X.; Kaesberg, P. Synthesis of template-sense, single-strand Flockhouse virus RNA in a cell-free replication system. *Virology* **1991**, *183*, 392–396, doi:10.1016/0042-6822(91)90153-3.

117. Miller, D.J.; Ahlquist, P. Flock house virus RNA polymerase is a transmembrane protein with amino-terminal sequences sufficient for mitochondrial localization and membrane insertion. *J. Virol.* **2002**, *76*, 9856–9867, doi:10.1128/jvi.76.19.9856-9867.2002.

118. Miller, D.J.; Schwartz, M.D.; Ahlquist, P. Flock house virus RNA replicates on outer mitochondrial membranes in Drosophila cells. *J. Virol.* **2001**, *75*, 11664–11676, doi:10.1128/JVI.75.23.11664-11676.2001.

119. Albariño, C.G.; Price, B.D.; Eckerle, L.D.; Ball, L.A. Characterization and template properties of RNA dimers generated during flock house virus RNA replication. *Virology* **2001**, *289*, 269–282, doi:10.1006/viro.2001.1125.

120. Eckerle, L.D.; Albariño, C.G.; Ball, L.A. Flock House virus subgenomic RNA3 is replicated and its replication correlates with transactivation of RNA2. *Virology* **2003**, *317*, 95–108, doi:10.1016/j.virol.2003.08.029.

121. Dearing, S.C.; Scotti, P.D.; Wigley, P.J.; Dhana, S.D. A small RNA virus isolated from the grass grub, Costelytra zealandica (Coleoptera: Scarabaeidae). *N. Z. J. Zool.* **1980**, *7*, 267–269, doi:10.1080/03014223.1980.10423785.

122. Scotti, P.D.; Dearing, S.; Mossop, D.W. Flock house virus: A Nodavirus isolated fromCostelytra zealandica (White) (Coleoptera: Scarabaeida). *Arch. Virol.* **1983**, *75*, 181–189, doi:10.1007/BF01315272.

123. Bonning, B.C.; Saleh, M.-C. The Interplay Between Viruses and RNAi Pathways in Insects. *Annu. Rev. Entomol.* **2021**, *66*, 61–79, doi:10.1146/annurev-ento-033020-090410.

124. Bronkhorst, A.W.; van Rij, R.P. The long and short of antiviral defense: Small RNA-based immunity in insects. *Curr. Opin. Virol.* **2014**, *7*, 19–28, doi:10.1016/j.coviro.2014.03.010.

125. Gammon, D.B.; Mello, C.C. RNA interference-mediated antiviral defense in insects. *Curr. Opin. Insect Sci.* **2015**, *8*, 111–120, doi:10.1016/j.cois.2015.01.006.

126. Wu, Q.; Wang, X.; Ding, S.-W. Viral suppressors of RNA-based viral immunity: Host targets. *Cell Host Microbe* **2010**, *8*, 12–15, doi:10.1016/j.chom.2010.06.009.

127. Chao, J.A.; Lee, J.H.; Chapados, B.R.; Debler, E.W.; Schneemann, A.; Williamson, J.R. Dual modes of RNA-silencing suppression by Flock House virus protein B2. *Nat. Struct. Mol. Biol.* **2005**, *12*, 952–957, doi:10.1038/nsmb1005.

128. Lu, R.; Maduro, M.; Li, F.; Li, H.W.; Broitman-Maduro, G.; Li, W.X.; Ding, S.W. Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. *Nature* **2005**, *436*, 1040–1043, doi:10.1038/nature03870.

129. McMenamin, A.J.; Daughenbaugh, K.F.; Parekh, F.; Pizzorno, M.C.; Flenniken, M.L. Honey Bee and Bumble Bee Antiviral Defense. *Viruses* **2018**, *10*, 395, doi:10.3390/v10080395.

130. Kingsolver, M.B.; Hardy, R.W. Making connections in insect innate immunity. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 18639–18640, doi:10.1073/pnas.1216736109.

131. Kingsolver, M.B.; Huang, Z.; Hardy, R.W. Insect antiviral innate immunity: Pathways, effectors, and connections. *J. Mol. Biol.* **2013**, *425*, 4921–4936, doi:10.1016/j.jmb.2013.10.006.

132. Iwasaki, S.; Kobayashi, M.; Yoda, M.; Sakaguchi, Y.; Katsuma, S.; Suzuki, T.; Tomari, Y. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol. Cell.* **2010**, *39*, 292–299, doi:10.1016/j.molcel.2010.05.015.

133. Dorner, S.; Lum, L.; Kim, M.; Paro, R.; Beachy, P.A.; Green, R. A genomewide screen for components of the RNAi pathway in Drosophila cultured cells. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11880–11885.

134. Galiana-Arnoux, D.; Dostert, C.; Schneemann, A.; Hoffmann, J.A.; Imler, J.-L. Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. *Nat. Immunol.* **2006**, *7*, 590–597, doi:10.1038/ni1335.

135. van Rij, R.P.; Saleh, M.-C.; Berry, B.; Foo, C.; Houk, A.; Antoniewski, C.; Andino, R. The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. *Genes Dev.* **2006**, *20*, 2985–2995, doi:10.1101/gad.1482006.

136. Zambon, R.A.; Vakharia, V.N.; Wu, L.P. RNAi is an antiviral immune response against a dsRNA virus in Drosophila melanogaster. *Cell. Microbiol.* **2006**, *8*, 880–889, doi:10.1111/j.1462-5822.2006.00688.x.

137. Myles, K.M.; Wiley, M.R.; Morazzani, E.M.; Adelman, Z.N. Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 19938, doi:10.1073/pnas.0803408105.

138. Maori, E.; Paldi, N.; Shafir, S.; Kalev, H.; Tsur, E.; Glick, E.; Sela, I. IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. *Insect Mol. Biol.* **2009**, *18*, 55–60.

139. Chejanovsky, N.; Ophir, R.; Schwager, M.S.; Slabezki, Y.; Grossman, S.; Cox-Foster, D. Characterization of viral siRNA populations in honey bee colony collapse disorder. *Virology* **2014**, *454–455*, 176–183, doi:10.1016/j.virol.2014.02.012.

140. Niu, J.; Meeus, I.; Cappelle, K.; Piot, N.; Smagghe, G. The immune response of the small interfering RNA pathway in the defense against bee viruses. *Curr. Opin. Insect Sci.* **2014**, *6*, 22–27, doi:10.1016/j.cois.2014.09.014.

141. Piot, N.; Snoeck, S.; Vanlede, M.; Smagghe, G.; Meeus, I. The effect of oral administration of dsRNA on viral replication and mortality in Bombus terrestris. *Viruses* **2015**, *7*, 3172–3185, doi:10.3390/v7062765.

142. Niu, J.; Smagghe, G.; De Coninck, D.I.; Van Nieuwerburgh, F.; Deforce, D.; Meeus, I. In vivo study of Dicer-2-mediated immune response of the small interfering RNA pathway upon systemic infections of virulent and avirulent viruses in Bombus terrestris. *Insect. Biochem. Mol. Biol.* **2016**, *70*, 127–137, doi:10.1016/j.ibmb.2015.12.006.

143. Jarosch, A.; Moritz, R.F.A. RNA interference in honeybees: Off-target effects caused by dsRNA. *Apidologie* **2012**, *43*, 128–138, doi:10.1007/s13592-011-0092-y.

144. Nunes, F.M.F.; Aleixo, A.C.; Barchuk, A.R.; Bomtorin, A.D.; Grozinger, C.M.; Simões, Z.L.P. Non-Target Effects of Green Fluorescent Protein (GFP)-Derived Double-Stranded RNA (dsRNA-GFP) Used in Honey Bee RNA Interference (RNAi) Assays. *Insects* **2013**, *4*, 90–103.

145. Bhatia, S.; Baral, S.S.; Vega Melendez, C.; Amiri, E.; Rueppell, O. Comparing Survival of Israeli Acute Paralysis Virus Infection among Stocks of U.S. Honey Bees. *Insects* **2021**, *12*, 60.

146. Doublet, V.; Poeschl, Y.; Gogol-Döring, A.; Alaux, C.; Annoscia, D.; Aurori, C.; Barribeau, S.M.; Bedoya-Reina, O.C.; Brown, M.J.F.; Bull, J.C.; et al. Unity in defence: Honeybee workers exhibit conserved molecular responses to diverse pathogens. *BMC Genom.* **2017**, *18*, 207, doi:10.1186/s12864-017-3597-6.

147. Niu, J.; Meeus, I.; Smagghe, G. Differential expression pattern of Vago in bumblebee (Bombus terrestris), induced by virulent and avirulent virus infections. *Sci. Rep.* **2016**, *6*, 34200, doi:10.1038/srep34200.

148. Di Prisco, G.; Cavaliere, V.; Annoscia, D.; Varricchio, P.; Caprio, E.; Nazzi, F.; Gargiulo, G.; Pennacchio, F. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 18466–18471, doi:10.1073/pnas.1314923110.

149. Desai, S.D.; Eu, Y.J.; Whyard, S.; Currie, R.W. Reduction in deformed wing virus infection in larval and adult honey bees (Apis mellifera L.) by double-stranded RNA ingestion. *Insect Mol. Biol.* **2012**, *21*, 446–455, doi:10.1111/j.1365-2583.2012.01150.x.

150. Cappelle, K.; Smagghe, G.; Dhaenens, M.; Meeus, I. Israeli Acute Paralysis Virus Infection Leads to an Enhanced RNA Interference Response and Not Its Suppression in the Bumblebee Bombus terrestris. *Viruses* **2016**, *8*, 334, doi:10.3390/v8120334.

151. Erez, T.; Chejanovsky, N. Infection of a Lepidopteran Cell Line with Deformed Wing Virus. *Viruses* **2020**, *12*, 739, doi:10.3390/v12070739.

152. Genersch, E.; Gisder, S.; Hedtke, K.; Hunter, W.B.; Möckel, N.; Müller, U. Standard methods for cell cultures in Apis mellifera research. *J. Apic. Res.* **2013**, *52*, 1–8, doi:10.3896/IBRA.1.52.1.02.

153. Carrillo-Tripp, J.; Bonning, B.C.; Miller, W.A. Challenges associated with research on RNA viruses of insects. *Curr. Opin. Insect Sci.* **2015**, *8*, 62–68, doi:10.1016/j.cois.2014.11.002.

154. Arbour, N.; Ekandé, S.; Côté, G.; Lachance, C.; Chagnon, F.; Tardieu, M.; Cashman, N.R.; Talbot, P.J. Persistent infection of human oligodendrocytic and neuroglial cell lines by human coronavirus 229E. *J. Virol.* **1999**, *73*, 3326–3337, doi:10.1128/JVI.73.4.3326-3337.1999.

155. Stollar, V.; Thomas, V.L. An agent in the Aedes aegypti cell line (Peleg) which causes fusion of Aedes albopictus cells. *Virology* **1975**, *64*, 367–377, doi:10.1016/0042-6822(75)90113-0.

156. Maringer, K.; Yousuf, A.; Heesom, K.J.; Fan, J.; Lee, D.; Fernandez-Sesma, A.; Bessant, C.; Matthews, D.A.; Davidson, A.D. Proteomics informed by transcriptomics for characterising active transposable elements and genome annotation in Aedes aegypti. *BMC Genom.* **2017**, *18*, 101, doi:10.1186/s12864-016-3432-5.

157. Wu, Q.; Luo, Y.; Lu, R.; Lau, N.; Lai, E.C.; Li, W.-X.; Ding, S.-W. Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1606, doi:10.1073/pnas.0911353107.

158. Katsuma, S.; Tanaka, S.; Omuro, N.; Takabuchi, L.; Daimon, T.; Imanishi, S.; Yamashita, S.; Iwanaga, M.; Mita, K.; Maeda, S.; et al. Novel Macula-Like Virus Identified in Bombyx mori Cultured Cells. *J. Virol.* **2005**, *79*, 5577–5584, doi:10.1128/JVI.79.9.5577-5584.2005.

159. Li, T.-C.; Scotti Paul, D.; Miyamura, T.; Takeda, N. Latent Infection of a New Alphanodavirus in an Insect Cell Line. *J. Virol.* **2007**, *81*, 10890–10896, doi:10.1128/JVI.00807-07.

160. Ma, H.; Galvin, T.A.; Glasner, D.R.; Shaheduzzaman, S.; Khan, A.S. Identification of a novel rhabdovirus in Spodoptera frugiperda cell lines. *J. Virol.* **2014**, *88*, 6576–6585, doi:10.1128/jvi.00780-14.

161. Travanty, E.; Zhou, B.; Zhang, H.; Di, Y.P.; Alcorn, J.F.; Wentworth, D.E.; Mason, R.; Wang, J. Differential Susceptibilities of Human Lung Primary Cells to H1N1 Influenza Viruses. *J. Virol.* **2015**, *89*, 11935–11944, doi:10.1128/JVI.01792-15.

162. Chen, Y.-C.; Wang, S.-Y.; King, C.-C. Bacterial Lipopolysaccharide Inhibits Dengue Virus Infection of Primary Human Monocytes/Macrophages by Blockade of Virus Entry via a CD14-Dependent Mechanism. *J. Virol.* **1999**, *73*, 2650–2657, doi:10.1128/JVI.73.4.2650-2657.1999.

163. Lavine, M.D.; Strand, M.R. Insect hemocytes and their role in immunity. *Insect Biochem. Mol. Biol.* **2002**, *32*, 1295–1309, doi:10.1016/S0965-1748(02)00092-9.

164. Richardson, R.T.; Ballinger, M.N.; Qian, F.; Christman, J.W.; Johnson, R.M. Morphological and functional characterization of honey bee, Apis mellifera, hemocyte cell communities. *Apidologie* **2018**, *49*, 397–410, doi:10.1007/s13592-018-0566-2.

165. Marringa, W.J.; Krueger, M.J.; Burritt, N.L.; Burritt, J.B. Honey Bee Hemocyte Profiling by Flow Cytometry. *PLoS ONE* **2014**, *9*, e108486, doi:10.1371/journal.pone.0108486.

166. Lanot, R.; Zachary, D.; Holder, F.; Meister, M. Postembryonic Hematopoiesis in Drosophila. *Dev. Biol.* **2001**, *230*, 243–257, doi:10.1006/dbio.2000.0123.

167. Gábor, E.; Cinege, G.; Csordás, G.; Török, T.; Folkl-Medzihradszky, K.; Darula, Z.; Andó, I.; Kurucz, É. Hemolectin expression reveals functional heterogeneity in honey bee (Apis mellifera) hemocytes. *Dev. Comp. Immunol.* **2017**, *76*, 403–411, doi:10.1016/j.dci.2017.07.013.

168. Mussen, E.C.; Furgala, B. Replication of sacbrood virus in larval and adult honeybees, Apis mellifera. *J. Invertebr. Pathol.* **1977**, 30, 20–34, doi:10.1016/0022-2011(77)90033-7.

169. Annoscia, D.; Brown, S.P.; Di Prisco, G.; De Paoli, E.; Del Fabbro, S.; Frizzera, D.; Zanni, V.; Galbraith, D.A.; Caprio, E.; Grozinger, C.M.; et al. Haemolymph removal by Varroa mite destabilizes the dynamical interaction between immune effectors and virus in bees, as predicted by Volterra's model. *Proc. R. Soc. B Biol. Sci.* **2019**, *286*, 20190331, doi:10.1098/rspb.2019.0331.

170. Tassetto, M.; Kunitomi, M.; Andino, R. Circulating Immune Cells Mediate a Systemic RNAi-Based Adaptive Antiviral Response in Drosophila. *Cell* **2017**, *169*, 314–325.e313, doi:10.1016/j.cell.2017.03.033.

171. Saleh, M.-C.; Tassetto, M.; van Rij, R.P.; Goic, B.; Gausson, V.; Berry, B.; Jacquier, C.; Antoniewski, C.; Andino, R. Antiviral immunity in Drosophila requires

systemic RNA interference spread. *Nature* **2009**, *458*, 346–350, doi:10.1038/nature07712.

172. McMenamin, A.J.; Brutscher, L.M.; Daughenbaugh, K.F.; Flenniken, M.L. The honey bee gene, bee antiviral protein-1, formerly MF116383, is a taxonomically restricted antiviral immune gene. *Front. Insect Sci.* **2021**, in prep.

173. Milbrath, M. Varroa Mite Monitoring: Using a Sugar Roll to Identify Populations of Varroa Destructor in Honey Bee Colonies. Available online: https://pollinators.msu.edu/resources/beekeepers/varroa-mite-monitoring1/ (accessed on 27 June 2020).

174. Tools for Varroa Management: A Guide to Effective Varroa Sampling and Control. 2015, 8. Available online: https://honeybeehealthcoalition.org/wp-content/uploads/2015/08/HBHC-Guide_Varroa-Interactive-PDF.pdf (accessed 27 June 2021).

175. Hunter, W.B. Medium for development of bee cell cultures (Apis mellifera: Hymenoptera: Apidae). *Vitr. Cell. Dev. Biol. Anim.* **2010**, *46*, 83–86, doi:10.1007/s11626-009-9246-x.

176. de Miranda, J.R.; Bailey, L.; Ball, B.V.; Blanchard, P.; Budge, G.E.; Chejanovsky, N.; Chen, Y.-P.; Gauthier, L.; Genersch, E.; de Graaf, D.C.; et al. Standard methods for virus research in Apis mellifera. *J. Apic. Res.* **2013**, *52*, 1–56, doi:10.3896/IBRA.1.52.4.22.

177. Selling, B.H.; Rueckert, R.R. Plaque assay for black beetle virus. *J. Virol.* **1984**, *51*, 251–253, doi:10.1128/JVI.51.1.251-253.1984.

178. Marshall, D.; Schneemann, A. Specific packaging of nodaviral RNA2 requires the N-terminus of the capsid protein. *Virology* **2001**, *285*, 165–175, doi:10.1006/viro.2001.0951.

179. Yue, C.; Genersch, E. RT-PCR analysis of Deformed wing virus in honeybees (Apis mellifera) and mites (Varroa destructor). *J. Gen. Virol.* **2005**, *86*, 3419–3424, doi:10.1099/vir.0.81401-0.

180. Boncristiani, H.F.; Di Prisco, G.; Pettis, J.S.; Hamilton, M.; Chen, Y.P. Molecular approaches to the analysis of deformed wing virus replication and pathogenesis in the honey bee, Apis mellifera. *Virol. J.* **2009**, *6*, 221, doi:10.1186/1743-422X-6-221.

181. Ginzinger, D.G. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Exp. Hematol.* **2002**, *30*, 503–512, doi:10.1016/s0301-472x(02)00806-8.

182. Dunnett, C.W. A Multiple Comparison Procedure for Comparing Several Treatments with a Control. *J. Am. Stat. Assoc.* **1955**, *50*, 1096–1121, doi:10.2307/2281208.

183. Bauer, D.F. Constructing Confidence Sets Using Rank Statistics. *J. Am. Stat. Assoc.* **1972**, *67*, 687–690, doi:10.2307/2284469.

184. Buck, K.W. Comparison of The Replication of Positive-Stranded Rna Viruses of Plants and Animals. In *Advances in Virus Research*; Maramorosch, K., Murphy, F.A., Shatkin, A.J., Eds.; Academic Press: Cambridge, MA, 1996; Volume 47, pp. 159–251.

185. Provost, K.; Dancho, B.A.; Ozbay, G.; Anderson, R.S.; Richards, G.P.; Kingsley, D.H. Hemocytes are sites of enteric virus persistence within oysters. *Appl. Environ. Microbiol*. **2011**, *77*, 8360–8369, doi:10.1128/aem.06887-11.

186. Wang, Y.T.; Liu, W.; Seah, J.N.; Lam, C.S.; Xiang, J.H.; Korzh, V.; Kwang, J. White spot syndrome virus (WSSV) infects specific hemocytes of the shrimp Penaeus merguiensis. *Dis. Aquat. Organ.* **2002**, *52*, 249–259, doi:10.3354/dao052249.

187. Ravindran, M.S.; Bagchi, P.; Cunningham, C.N.; Tsai, B. Opportunistic intruders: How viruses orchestrate ER functions to infect cells. *Nat. Rev. Microbiol.* **2016**, *14*, 407–420, doi:10.1038/nrmicro.2016.60.

188. Fan, Y.; Sanyal, S.; Bruzzone, R. Breaking Bad: How Viruses Subvert the Cell Cycle. *Front. Cell. Infect. Microbiol.* **2018**, *8*, 396–396, doi:10.3389/fcimb.2018.00396.

189. Lamiable, O.; Arnold, J.; de Faria, I.; Olmo, R.P.; Bergami, F.; Meignin, C.; Hoffmann, J.A.; Marques, J.T.; Imler, J.L. Analysis of the Contribution of Hemocytes and Autophagy to Drosophila Antiviral Immunity. *J. Virol.* **2016**, *90*, 5415–5426, doi:10.1128/jvi.00238-16.

190. Chen, Y.P.; Pettis, J.S.; Corona, M.; Chen, W.P.; Li, C.J.; Spivak, M.; Visscher, P.K.; DeGrandi-Hoffman, G.; Boncristiani, H.; Zhao, Y.; et al. Israeli Acute Paralysis Virus: Epidemiology, Pathogenesis and Implications for Honey Bee Health. *PLoS Pathog*. **2014**, *10*, e1004261, doi:10.1371/journal.ppat.1004261.

191. Li, H.; Li, W.X.; Ding, S.W. Induction and Suppression of RNA Silencing by an Animal Virus. *Science* **2002**, *296*, 1319, doi:10.1126/science.1070948.

192. Nayak, A.; Berry, B.; Tassetto, M.; Kunitomi, M.; Acevedo, A.; Deng, C.; Krutchinsky, A.; Gross, J.; Antoniewski, C.; Andino, R. Cricket paralysis virus antagonizes Argonaute 2 to modulate antiviral defense in Drosophila. *Nat. Struct. Mol. Biol.* **2010**, *17*, 547–554, doi:10.1038/nsmb.1810.

193. Nayak, A.; Kim, D.Y.; Trnka, M.J.; Kerr, C.H.; Lidsky, P.V.; Stanley, D.J.; Rivera, B.M.; Li, K.H.; Burlingame, A.L.; Jan, E.; et al. A Viral Protein Restricts Drosophila RNAi Immunity by Regulating Argonaute Activity and Stability. *Cell Host Microbe* **2018**, *24*, 542–557.e549, doi:10.1016/j.chom.2018.09.006.

CHAPTER SEVEN

GENERAL CONCLUSIONS AND DISCUSSION

Honey Bee Host-virus Interactions At The Individual And Cellular Level

Studies at the individual and cellular levels were carried out to further elucidate honey bee (*Apis mellifera*) host-virus interactions. First, the heat shock response (HSR) is described as a novel antiviral immune pathway in the Western honey bee, though precisely how it contributes to antiviral immunity is an exciting topic for future investigation. Then, *bee antiviral protein-1* (*bap1*), a recently discovered antiviral protein, was further characterized *in silico* and it was determined to be a taxonomically restricted, rapidly evolving, disordered antiviral protein that is highly coexpressed with *argonaute-2* (*Ago2*), the catalytic component of the RNA induced silencing complex (RISC). Lastly, it was demonstrated that primary cell cultures derived from larval immune cells and explanted pupal tissues support viral infection, which mount virus-specific transcriptional responses, as assessed by the evaluation of the expression of three immune genes (i.e., *bap1*, *ago2*, and *dicer-like*).

Honey bees have evolved a wide range of strategies to control pathogens [1-7]. The outcomes of viral infections in individual honey bees are primarily governed by cellular immune responses, which include double stranded RNA (dsRNA)-triggered immune responses (i.e., RNAi and non-sequence specific dsRNA mediated antiviral mechanisms) [1,8-15] and canonical immune signaling pathways, including the JAK/STAT, JNK, and Imd pathways, and the NF-κB/Dorsal mediated Toll pathway, which has been further characterized in vivo [16,17]. The mechanisms and genes involved in honey bee antiviral responses require further investigation to

rationally develop therapeutics and breeding targets to mitigate colony losses due to viral infection [4,8,10,11,17-25]. Herein, we presented evidence that the heat shock response is involved in honey bee antiviral defense. First, we demonstrated that heat shock (i.e., exposure to 42 °C for 4 h) reduced the abundance of the model virus, Sindbis-GFP (SINV), compared to bees maintained at a constant temperature. One hypothesis that might explain the 74–90% reduction in virus abundance in heat-shocked honey bees is that there is a general disruption of protein synthesis in heat-stressed bees. However, drosophila cells completely recover normal protein synthesis upon return to physiologically normal temperatures after heat shock (four treatments at 37 °C for 25 min each) [26]. Furthermore, honey bee thoraces can reach in excess of 45 °C during foraging and aggression, suggesting they are adapted to cope with these high temperatures for short durations of time [27-31]. Therefore, disruption of protein synthesis is unlikely to be sufficient to explain the near log-reduction in viral abundance in heat-shocked bees at 72 h post-infection. Instead, it is likely that the transcriptional regulation of heat shock proteins is at least partially responsible for the antiviral effect of heat shock. Whether heat shock proteins (HSPs) contribute to antiviral immunity directly or indirectly by chaperoning immune proteins requires further investigation.

Similar to other insects, RNA interference is antiviral in honey bees, as co-administration of a virus with virus-sequence specific dsRNA results in a reduction of virus abundance [1,9,12-15,32,33]. Additionally, *bee antiviral protein* 1 (*bap1*), a novel immune gene, which had increased expression in SINV-infected bees, was demonstrated to limit virus infection *in vivo* [1]. To determine whether the increased expression of select immune genes explains the antiviral

effect of heat shock, the expression of three immune genes (i.e., *bap1*, *ago-2*, *dcr-like*) was also measured in heat shocked bees, SINV-infected bees and bees that were infected and exposed to heat shock (42ºC for 4 hours). In contrast to the impact of heat shock on *bap1* expression, heat shock had a variable effect on the expression of RNAi machinery across replicates. In some cases, the expression of *dcr-like* and *ago2* was reduced in heat shocked honey bees when compared to honey bees that were maintained at 32 °C. This implies that the mitigating effect of heat-treatment on virus infection is not simply explained by greater expression of the RNAi machinery. Instead, the protective effect of HSPs may be in part due to more efficient chaperone-mediated loading of the RISC [34,35]. Increased availability of chaperone proteins following heat shock may also ensure there are chaperones available for host-proteins as opposed to being occupied by viral proteins [36,37]. In addition to the expression of heat shock protein-encoding genes correlating with each other, as expected, the expression of some HSP-encoding genes (e.g., *hsc70-3*, *hsc70-4*, and *hsp90*) was positively correlated with *dcr-like* and *ago2.* This suggests co-regulation of these genes. Though further studies are needed to determine the mechanisms leading to co-regulation of immune genes and HSP-encoding genes, it may be advantageous to co-regulate HSPs and HSP client proteins [34,35]. In addition to HSPs serving as chaperones for RNAi proteins, they may also play a direct antiviral role by interacting with viral proteins or may participate in, or mediate, broader stress-response pathways involved in antiviral defense. Interestingly, *bap1* was the only immune gene assessed that showed consistent upregulation by heat shock, suggesting *bap1* may serve as a point of point of crosstalk between the HSR and canonical antiviral pathways, or that it is a client of HSPs.

As an antiviral immune protein, *bap1* exists at the interface between host and parasite. Therefore, one would expect *bap1* to be under positive selection as the conflict between host and parasite drives rapid diversification between lineages [38,39]. For example, RNAi genes across multiple insect species are under higher rates of adaptive evolution and experience more selective sweeps compared to paralogous housekeeping genes [40,41]. Bayesian inference phylogenetic analysis reveals that *bap1* is taxonomically restricted to Hymenopterans (bees, ants, wasps and sawflies) and *Blatella germanica* (the German cockroach). Additionally, while there are sites in Bap1 under positive selection (dN/dS ~ 1.94), the majority of the protein is under neutral selection (797 sites out of 1511 with a confidence of 0.8). As compared to RPB1, the largest subunit of DNA-directed RNA polymerase II, which has 191 sites under neutral selection and 1,314 sites under strong negative selection (dN/dS ~ 0.0006). This relaxed selection on *bap1* is likely leading to the rapid diversification of *bap1* that we observed and is probably due to relaxed structural constraints [42-45]. The large percentage of disorder predicted in Bap1 (67.2%) is interesting because disordered proteins often serve as interaction hubs in protein networks due to their highly modular structure which enables binding of multiple partners which may be protein or nucleic acid [46-49]. It is tempting to speculate that bap1 mediates detection of virus infection by either binding viral nucleic acids, or another protein that binds viral nucleic acids and then transducing the signal to additional proteins that drive a transcriptional response. As a participator in an as yet unidentified antiviral pathway, one would expect *bap1* to be coexpressed with other antiviral genes. Indeed, in a *post hoc* analysis of a data set consisting of the sequenced transcriptomes of bees receiving various treatments (e.g., SINV and/or ds-RNA)

bap1 is highly coexpressed with *TEP7*, *tudor-sn*, and *ago-2*. In fruit flies and mosquitoes, TEP expression is likely regulated by JAK in response to bacterial infection and they function in bacterial recognition, opsonization and phagocytosis (reviewed in [50,51]). However, in *Aedes aegypti* TEP1 and TEP2 limit West Nile virus infection [54] and TEP is indirectly involved in combatting Dengue virus [55]. Furthermore, while Tudor-SN is a nuclease and a core component of RISC of Drosophila [52] it is also involved in cleavage of hyper-ADAR-edited dsRNAs [53]. Therefore, *bap1* is a rapidly evolving, taxonomically restricted antiviral immune genes that is coexpressed with several genes implicated in antiviral immunity. However, the specific molecular function of bap1 is not yet elucidated. The development of additional tools and techniques will be crucial to enabling the discovery and description of novel antiviral immune pathways in honey bees. For example, the development of cell culture techniques will facilitate these efforts because cell cultures are highly manipulable. Additionally, experiments performed on multiple cultures from a single individual should have less variability than individual bee-level studies because between-individual variation is exacerbated by the fact that honey bees are an outbred livestock species [56].

In spite of their utility and importance, robust cell culture systems are not available for many non-model organisms, including honey bees (*Apis mellifera*). While approximately 1,000 cell lines have been derived from insect tissues, including 19 hymenopteran cell lines, only one immortalized honey bee cell line, AmE-711, has been established [57-59]. While an important step towards the development of immortalized honey bee cell lines, AmE-711 has a long doubling time, is difficult to maintain in other labs, and it is persistently infected with DWV

[57]. Additionally, while immortalized cell cultures provide a convenient and consistent tool, they often do not fully recapitulate in situ biology. For example, the immune response that AmE-711, a honey bee fibroblast-type cell line, mounts against viruses may not represent the immune response in other honey bee tissues or cell types (i.e., fat body, endothelium, or hemocytes). Therefore, the development of primary cell cultures alongside immortalized cell cultures is an important endeavor. Herein, we demonstrate that primary hemocyte cultures and explanted pupal tissue cultures susceptible to infection and pupal cultures showed virus-specific immune responses to different viruses.

Hemocytes are the primary insect immune cells that phagocytose or encapsulate pathogens [60]. However, in *Drosophila*, hemocytes mediate a systemic adaptive antiviral response [61-63]. In honey bees, the role of hemocytes in antiviral immunity is less defined. Specifically, genes involved in migration, phagocytosis, and expression immunoglobulin domain encoding, were expressed at greater levels in honey bees infected with a model virus, SINV, relative to mock-infected bees [1]. We demonstrated that hemocytes were productively infected by sacbrood virus (SBV) and Flock House virus, post inoculation. In addition, we demonstrated that hemocytes extracted from larvae were naturally infected by SBV. This observation that hemocytes are infected by viruses in honey bees has important implications for virus pathogenesis, since viruses subvert cellular processes and disrupt cellular homeostasis [64,65]. The infection of hemocytes by a model virus, FHV, provides a useful tool for studying honey bee hemocyte-virus interaction because FHV is relatively easy to propagate and purify. Additionally, SBV, FHV, and deformed wing virus (DWV) productively replicate in mixed cell type cultures

derived from explanted pupal tissues. Infected honey bee pupal cell cultures exhibited unique differential expression profiles of *bap1*, *ago2,* and *dcr-like* in response to SBV, FHV, and DWV, suggesting these cultures are useful tools for studying honey bee transcriptional responses to viral infection.

Herein, a previously unappreciated honey antiviral immune pathway is identified, a novel immune protein is further characterized and cell culture tools are developed to study honey bee host-virus interactions. This work sets the stage and further develops the tools to identify and elucidate new mechanisms by which honey bees combat virus infection. These efforts may lead to the development of strategies to mitigate honey bee colony losses by virus infection. This might occur through the development of therapeutics, such as the identification of immune-stimulating small molecules, or the identification of heritable variability in antiviral immune genes that can be bred for resistance to virus infection.

References Cited

1. Brutscher, L.M.; Daughenbaugh, K.F.; Flenniken, M.L. Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. Scientific Reports **2017**, 7, 6448, doi:10.1038/s41598-017-06623-z.

2. McMenamin, A.J.; Daughenbaugh, K.F.; Parekh, F.; Pizzorno, M.C.; Flenniken, M.L. Honey Bee and Bumble Bee Antiviral Defense. Viruses **2018**, 10, 395, doi:10.3390/v10080395.

3. Simone, M.; Evans, J.D.; Spivak, M. Resin collection and social immunity in honey bees. Evolution; international journal of organic evolution **2009**, 63, 3016-3022, doi:10.1111/j.1558-5646.2009.00772.x.

4. Ryabov, E.V.; Fannon, J.M.; Moore, J.D.; Wood, G.R.; Evans, D.J. The Iflaviruses Sacbrood virus and Deformed wing virus evoke different transcriptional responses in the

honeybee which may facilitate their horizontal or vertical transmission. PeerJ **2016**, 4, e1591, doi:10.7717/peerj.1591.

5. Brutscher, L.M.; Daughenbaugh, K.F.; Flenniken, M.L. Antiviral defense mechanisms in honey bees. Current Opinion in Insect Science **2015**, 2, 1-12, doi:10.1016/j.cois.2015.04.016.

6. Simone-Finstrom, M.D.; Spivak, M. Increased resin collection after parasite challenge: a case of self-medication in honey bees? PloS one **2012**, 7, e34601, doi:10.1371/journal.pone.0034601.

7. Spivak, M.; Goblirsch, M.; Simone-Finstrom, M. Social-medication in bees: the line between individual and social regulation. Current Opinion in Insect Science **2019**, 33, 49-55, doi:https://doi.org/10.1016/j.cois.2019.02.009.

8. Chen, Y.P.; Pettis, J.S.; Corona, M.; Chen, W.P.; Li, C.J.; Spivak, M.; Visscher, P.K.; DeGrandi-Hoffman, G.; Boncristiani, H.; Zhao, Y.; et al. Israeli Acute Paralysis Virus: Epidemiology, Pathogenesis and Implications for Honey Bee Health. PLOS Pathogens **2014**, 10, e1004261, doi:10.1371/journal.ppat.1004261.

9. Flenniken, M.L.; Andino, R. Non-Specific dsRNA-Mediated Antiviral Response in the Honey Bee. PLoS ONE **2013**, 8, 1-16, doi:10.1371/journal.pone.0077263.

10. Galbraith, D.A.; Yang, X.; Nino, E.L.; Yi, S.; Grozinger, C. Parallel epigenomic and transcriptomic responses to viral infection in honey bees (Apis mellifera). PLoS Pathog **2015**, 11, e1004713, doi:10.1371/journal.ppat.1004713.

11. Ryabov, E.V.; Wood, G.R.; Fannon, J.M.; Moore, J.D.; Bull, J.C.; Chandler, D.; Mead, A.; Burroughs, N.; Evans, D.J. A Virulent Strain of Deformed Wing Virus (DWV) of Honeybees (Apis mellifera) Prevails after Varroa destructor-Mediated, or In Vitro, Transmission. PLoS Pathogens **2014**, 10, doi:10.1371/journal.ppat.1004230.

12. Maori, E.; Paldi, N.; Shafir, S.; Kalev, H.; Tsur, E.; Glick, E.; Sela, I. IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. Insect Molecular Biology **2009**, 18, 55-60.

13. Chejanovsky, N.; Ophir, R.; Schwager, M.S.; Slabezki, Y.; Grossman, S.; Cox-Foster, D. Characterization of viral siRNA populations in honey bee colony collapse disorder. Virology **2014**, 454-455, 176-183, doi:10.1016/j.virol.2014.02.012.

14. Desai, S.D.; Eu, Y.J.; Whyard, S.; Currie, R.W. Reduction in deformed wing virus infection in larval and adult honey bees (Apis mellifera L.) by double-stranded RNA ingestion. Insect Molecular Biology **2012**, 21, 446-455, doi:10.1111/j.1365-2583.2012.01150.x.

15. Liu, X.; Zhang, Y.; Yan, X.; Han, R. Prevention of chinese sacbrood virus infection in apis cerana using rna interference. Current Microbiology **2010**, 61, 422-428, doi:10.1007/s00284-010-9633-2.

16. Di Prisco, G.; Annoscia, D.; Margiotta, M.; Ferrara, R.; Varricchio, P.; Zanni, V.; Caprio, E.; Nazzi, F.; Pennacchio, F. A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. Proceedings of the National Academy of Sciences **2016**, 113, 201523515, doi:10.1073/pnas.1523515113.

17. Nazzi, F.; Brown, S.P.; Annoscia, D.; Del Piccolo, F.; Di Prisco, G.; Varricchio, P.; Della Vedova, G.; Cattonaro, F.; Caprio, E.; Pennacchio, F. Synergistic Parasite-Pathogen Interactions Mediated by Host Immunity Can Drive the Collapse of Honeybee Colonies. PLOS Pathogens **2012**, 8, e1002735, doi:10.1371/journal.ppat.1002735.

18. McMenamin, A.J.; Genersch, E. Honey bee colony losses and associated viruses. Current Opinion in Insect Science **2015**, 8, 121-129, doi:10.1016/j.cois.2015.01.015.

19. Dainat, B.; Evans, J.D.; Chen, Y.P.; Gauthier, L.; Neumann, P. Dead or alive: deformed wing virus and Varroa destructor reduce the life span of winter honeybees. Appl Environ Microbiol **2012**, 78, 981-987, doi:10.1128/AEM.06537-11.

20. Dainat, B.; Evans, J.D.; Chen, Y.P.; Gauthier, L.; Neumann, P. Predictive markers of honey bee colony collapse. PloS one **2012**, 7, e32151, doi:10.1371/journal.pone.0032151.

21. Dainat, B.; Neumann, P. Clinical signs of deformed wing virus infection are predictive markers for honey bee colony losses. Journal of invertebrate pathology **2013**, 112, 278-280, doi:10.1016/j.jip.2012.12.009.

22. Brutscher, L.M.; McMenamin, A.J.; Flenniken, M.L. The buzz about honey bee viruses. PLoS Pathogens **2016**, 12, e1005757, doi:10.1371/journal.ppat.1005757.

23. Bull, J.C.; Ryabov, E.V.; Prince, G.; Mead, A.; Zhang, C.; Baxter, L.A.; Pell, J.K.; Osborne, J.L.; Chandler, D. A Strong Immune Response in Young Adult Honeybees Masks Their Increased Susceptibility to Infection Compared to Older Bees. PLoS Pathogens **2012**, 8, 11-14, doi:10.1371/journal.ppat.1003083.

24. Doublet, V.; Poeschl, Y.; Gogol-Döring, A.; Alaux, C.; Annoscia, D.; Aurori, C.; Barribeau, S.M.; Bedoya-Reina, O.C.; Brown, M.J.F.; Bull, J.C.; et al. Unity in defence: honeybee workers exhibit conserved molecular responses to diverse pathogens. BMC Genomics **2017**, 18, 207, doi:10.1186/s12864-017-3597-6.

25. Genersch, E.; Von Der Ohe, W.; Kaatz, H.; Schroeder, A.; Otten, C.; Büchler, R.; Berg, S.; Ritter, W.; Mühlen, W.; Gisder, S.; et al. The German bee monitoring project: A long term

study to understand periodically high winter losses of honey bee colonies. Apidologie **2010**, 41, 332-352, doi:10.1051/apido/2010014.

26. Lindquist, S. Regulation of protein synthesis during heat shock. Nature **1981**, 293, 311-314, doi:10.1038/293311a0.

27. Cooper, P.D.; Schaffer, W.M. Temperature Regulation of Honey Bees (Apis mellifera) foraging in the Sonoran Desert. Journal of Experimental Biology **1985**, 1-15.

28. Stabentheiner, A.; Kovac, H.; Schmaranzer, S. Honeybee nestmate recognition: the thermal behaviour of guards and their examinees. Journal of Experimental Biology **2002**, 205, 2637.

29. Stabentheiner, A.; Kovac, H.; Schmaranzer, S. Thermal Behaviour of Honeybees During Aggressive Interactions. Ethology **2007**, 113, 995-1006, doi:10.1111/j.1439-0310.2007.01403.x.

30. Kovac, H.; Stabentheiner, A.; Schmaranzer, S. Thermoregulation of water foraging honeybees—Balancing of endothermic activity with radiative heat gain and functional requirements. Journal of Insect Physiology **2010**, 56, 1834-1845, doi:https://doi.org/10.1016/j.jinsphys.2010.08.002.

31. Kovac, H.; Käfer, H.; Stabentheiner, A.; Costa, C. Metabolism and upper thermal limits of Apis mellifera carnica and A. m. ligustica. Apidologie **2014**, 45, 664-677, doi:10.1007/s13592-014-0284-3.

32. Niu, J.; Meeus, I.; Cappelle, K.; Piot, N.; Smagghe, G. The immune response of the small interfering RNA pathway in the defense against bee viruses. Current Opinion in Insect Science **2014**, 6, 22-27, doi:10.1016/j.cois.2014.09.014.

33. Leonard, S.P.; Powell, J.E.; Perutka, J.; Geng, P.; Heckmann, L.C.; Horak, R.D.; Davies, B.W.; Ellington, A.D.; Barrick, J.E.; Moran, N.A. Engineered symbionts activate honey bee immunity and limit pathogens. Science **2020**, 367, 573, doi:10.1126/science.aax9039.

34. Iwasaki, S.; Kobayashi, M.; Yoda, M.; Sakaguchi, Y.; Katsuma, S.; Suzuki, T.; Tomari, Y. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. Mol Cell **2010**, 39, 292-299, doi:10.1016/j.molcel.2010.05.015.

35. Dorner, S.; Lum, L.; Kim, M.; Paro, R.; Beachy, P.A.; Green, R. A genomewide screen for components of the RNAi pathway in Drosophila cultured cells. Proceedings of the National Academy of Sciences **2006**, 103, 11880–11885.

36. Kampmueller, K.M.; Miller, D.J. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells. Journal of virology **2005**, 79, 6827-6837, doi:10.1128/JVI.79.11.6827-6837.2005.

37. Weeks, S.A.; Shield, W.P.; Sahi, C.; Craig, E.A.; Rospert, S.; Miller, D.J. A Targeted Analysis of Cellular Chaperones Reveals Contrasting Roles for Heat Shock Protein 70 in Flock House Virus RNA Replication. Journal of Virology **2010**, 84, 330, doi:10.1128/JVI.01808-09.

38. Paterson, S.; Vogwill, T.; Buckling, A.; Benmayor, R.; Spiers, A.J.; Thomson, N.R.; Quail, M.; Smith, F.; Walker, D.; Libberton, B.; et al. Antagonistic coevolution accelerates molecular evolution. Nature **2010**, 464, 275-278, doi:10.1038/nature08798.

39. Daugherty, M.D.; Malik, H.S. Rules of Engagement: Molecular Insights from Host-Virus Arms Races. Annual Review of Genetics **2012**, 46, 677-700, doi:10.1146/annurev-genet-110711-155522.

40. Obbard, D.J.; Jiggins, F.M.; Halligan, D.L.; Little, T.J. Natural Selection Drives Extremely Rapid Evolution in Antiviral RNAi Genes. Current Biology **2006**, 16, 580-585, doi:https://doi.org/10.1016/j.cub.2006.01.065.

41. Palmer, W.H.; Hadfield, J.D.; Obbard, D.J. RNA-Interference Pathways Display High Rates of Adaptive Protein Evolution in Multiple Invertebrates. Genetics **2018**, 208, 1585-1599, doi:10.1534/genetics.117.300567.

42. Lin, Y.S.; Hsu, W.L.; Hwang, J.K.; Li, W.H. Proportion of solvent-exposed amino acids in a protein and rate of protein evolution. Mol Biol Evol **2007**, 24, 1005-1011, doi:10.1093/molbev/msm019.

43. Xia, Y.; Franzosa, E.A.; Gerstein, M.B. Integrated Assessment of Genomic Correlates of Protein Evolutionary Rate. PLOS Computational Biology **2009**, 5, e1000413, doi:10.1371/journal.pcbi.1000413.

44. Brown, C.J.; Johnson, A.K.; Daughdrill, G.W. Comparing models of evolution for ordered and disordered proteins. Mol Biol Evol **2010**, 27, 609-621, doi:10.1093/molbev/msp277.

45. Brown, C.J.; Johnson, A.K.; Dunker, A.K.; Daughdrill, G.W. Evolution and disorder. Curr Opin Struct Biol **2011**, 21, 441-446, doi:10.1016/j.sbi.2011.02.005.

46. Wright, P.E.; Dyson, H.J. Intrinsically disordered proteins in cellular signalling and regulation. Nature Reviews Molecular Cell Biology **2014**, 16, 18, doi:10.1038/nrm3920.

47. Dunker, A.K.; Cortese, M.S.; Romero, P.; Iakoucheva, L.M.; Uversky, V.N. Flexible nets. The roles of intrinsic disorder in protein interaction networks. Febs j **2005**, 272, 5129-5148, doi:10.1111/j.1742-4658.2005.04948.x.

48. Higurashi, M.; Ishida, T.; Kinoshita, K. Identification of transient hub proteins and the possible structural basis for their multiple interactions. Protein Sci **2008**, 17, 72-78, doi:10.1110/ps.073196308.

49. Kim, P.M.; Sboner, A.; Xia, Y.; Gerstein, M. The role of disorder in interaction networks: a structural analysis. Mol Syst Biol **2008**, 4, 179-179, doi:10.1038/msb.2008.16.

50. Blandin, S.; Levashina, E.A. Thioester-containing proteins and insect immunity. Molecular Immunology **2004**, 40, 903-908, doi:https://doi.org/10.1016/j.molimm.2003.10.010.

51. Shokal, U.; Eleftherianos, I. Evolution and Function of Thioester-Containing Proteins and the Complement System in the Innate Immune Response. Frontiers in immunology **2017**, 8, 759-759, doi:10.3389/fimmu.2017.00759.

52. Caudy, A.A.; Ketting, R.F.; Hammond, S.M.; Denli, A.M.; Bathoorn, A.M.P.; Tops, B.B.J.; Silva, J.M.; Myers, M.M.; Hannon, G.J.; Plasterk, R.H.A. A micrococcal nuclease homologue in RNAi effector complexes. Nature **2003**, 425, 411-414, doi:10.1038/nature01956.

53. Scadden, A.D.J. The RISC subunit Tudor-SN binds to hyper-edited double-stranded RNA and promotes its cleavage. Nature Structural & Molecular Biology **2005**, 12, 489-496, doi:10.1038/nsmb936.

54. Cheng, G.; Liu, L.; Wang, P.; Zhang, Y.; Zhao, Y.O.; Colpitts, T.M.; Feitosa, F.; Anderson, J.F.; Fikrig, E. An In Vivo Transfection Approach Elucidates a Role for Aedes aegypti Thioester-Containing Proteins in Flaviviral Infection. PLOS ONE **2011**, 6, e22786, doi:10.1371/journal.pone.0022786.

55. Xiao, X.; Liu, Y.; Zhang, X.; Wang, J.; Li, Z.; Pang, X.; Wang, P.; Cheng, G. Complement-Related Proteins Control the Flavivirus Infection of Aedes aegypti by Inducing Antimicrobial Peptides. PLOS Pathogens **2014**, 10, e1004027, doi:10.1371/journal.ppat.1004027.

56. Cobey, S.; Sheppard, W.S.; Tarpy, D.R. Honey Bee Colony Health: Challenges and Sustainable Solutions; Sammataro, D., Yoder, J.A., Eds.; CRC Press: Boca Raton, United States, 2011.

57. Carrillo-Tripp, J.; Dolezal, A.G.; Goblirsch, M.J.; Miller, W.A.; Toth, A.L.; Bonning, B.C. In vivo and in vitro infection dynamics of honey bee viruses. Scientific Reports **2016**, 6, 22265, doi:10.1038/srep22265.

58. Goblirsch, M.J.; Spivak, M.S.; Kurtti, T.J. A Cell Line Resource Derived from Honey Bee (Apis mellifera) Embryonic Tissues. PLoS ONE **2013**, 8, 1-13, doi:10.1371/journal.pone.0069831.

59. Guo, Y.; Goodman, C.L.; Stanley, D.W.; Bonning, B.C. Cell Lines for Honey Bee Virus Research. Viruses **2020**, 12, 236, doi:10.3390/v12020236.

60. Lavine, M.D.; Strand, M.R. Insect hemocytes and their role in immunity. Insect Biochemistry and Molecular Biology **2002**, 32, 1295-1309, doi:https://doi.org/10.1016/S0965-1748(02)00092-9.

61. Tassetto, M.; Kunitomi, M.; Andino, R. Circulating Immune Cells Mediate a Systemic RNAi-Based Adaptive Antiviral Response in Drosophila. Cell **2017**, 169, 314-325.e313, doi:10.1016/j.cell.2017.03.033.

62. Saleh, M.-c.; Tassetto, M.; van Rij, R.P.; Goic, B.; Gausson, V.; Berry, B.; Jacquier, C.; Antoniewski, C.; Andino, R. Antiviral immunity in Drosophila requires systemic RNA interference spread. Nature **2009**, 458, 346-350, doi:10.1038/nature07712.

63. Poirier, E.Z.; Goic, B.; Tomé-Poderti, L.; Frangeul, L.; Boussier, J.; Gausson, V.; Blanc, H.; Vallet, T.; Loyd, H.; Levi, L.I.; et al. Dicer-2-Dependent Generation of Viral DNA from Defective Genomes of RNA Viruses Modulates Antiviral Immunity in Insects. Cell Host Microbe **2018**, 23, 353-365.e358, doi:10.1016/j.chom.2018.02.001.

64. Ravindran, M.S.; Bagchi, P.; Cunningham, C.N.; Tsai, B. Opportunistic intruders: how viruses orchestrate ER functions to infect cells. Nat Rev Microbiol **2016**, 14, 407-420, doi:10.1038/nrmicro.2016.60.

65. Fan, Y.; Sanyal, S.; Bruzzone, R. Breaking Bad: How Viruses Subvert the Cell Cycle. Front Cell Infect Microbiol **2018**, 8, 396-396, doi:10.3389/fcimb.2018.00396.

REFERENCES CITED

(GRPC), G. P. R. C. GRPC Canadian National Honey Bee Health Survey. GPRC National Bee Diagnostic Centre (May 23, 2018),

Aizen, M.A.; Garibaldi, L.A.; Cunningham, S.A.; Klein, A.M. Long-Term Global Trends in Crop Yield and Production Reveal No Current Pollination Shortage but Increasing Pollinator Dependency. Current Biology 2008, 18, 1572-1575, doi:https://doi.org/10.1016/j.cub.2008.08.066.

Aizen, M.a.; Harder, L.D. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Current biology : CB 2009, 19, 915-918, doi:10.1016/j.cub.2009.03.071.

Akopyants NS, Lye L, Dobson DE, Lukeš J, Beverley S: A novel Bunyavirus-like virus of trypanosomatid protist parasites. Genome Announc. 2016, 4:e00715-16.

Albariño, C.G.; Price, B.D.; Eckerle, L.D.; Ball, L.A. Characterization and template properties of RNA dimers generated during flock house virus RNA replication. Virology 2001, 289, 269–282, doi:10.1006/viro.2001.1125.

Allen, M.; Ball, B. The incidence and world distribution of honey bee viruses. Bee World 1996, 77, 141–162, doi:10.1080/0005772x.1996.11099306.

Almagro Armenteros, J.J.; Tsirigos, K.D.; Sønderby, C.K.; Petersen, T.N.; Winther, O.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol 2019, 37, 420-423, doi:10.1038/s41587-019-0036-z.

Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. J Mol Biol 1990, 215, 403-410, doi:10.1016/s0022-2836(05)80360-2.

Altschul, S.F.; Madden, T.L.; Schäffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997, 25, 3389-3402, doi:10.1093/nar/25.17.3389.

Amiri, E.; Kryger, P.; Meixner, M.D.; Strand, M.K.; Tarpy, D.R.; Rueppell, O. Quantitative patterns of vertical transmission of deformed wing virus in honey bees. PLoS ONE 2018, 13, e0195283, doi:10.1371/journal.pone.0195283.

Amiri, E.; Meixner, M.; Büchler, R.; Kryger, P. Chronic Bee Paralysis Virus in Honeybee Queens: Evaluating Susceptibility and Infection Routes. Viruses 2014, 6, (3), 1188-1201.

Amiri, E.; Meixner, M.; Nielsen, S. L.; Kryger, P. Four Categories of Viral Infection Describe the Health Status of Honey Bee Colonies. PLoS ONE 2015, 10, (10), e0140272.

Amiri, E.; Meixner, M.D.; Kryger, P. Deformed wing virus can be transmitted during natural mating in honey bees and infect the queens. Sci. Rep. 2016, 6, 33065, doi:10.1038/srep33065.

Amsalem, E.; Galbraith, D. A.; Cnaani, J.; Teal, P. E. A.; Grozinger, C. M. Conservation and modification of genetic and physiological toolkits underpinning diapause in bumble bee queens. Mol Ecol 2015, 24, (22), 5596-5615.

Amsalem, E.; Grozinger, C. M.; Padilla, M.; Hefetz, A., The Physiological and Genomic Bases of Bumble Bee Social Behaviour. In Genomics, Physiology and Behaviour of Social Insects, Elsevier: London, UK, 2015; Vol. 48, pp 37-93.

Andino R, Domingo E: Viral quasispecies. Virology 2015, 479–480:46–51.

Annoscia, D.; Brown, S.P.; Di Prisco, G.; De Paoli, E.; Del Fabbro, S.; Frizzera, D.; Zanni, V.; Galbraith, D.A.; Caprio, E.; Grozinger, C.M.; et al. Haemolymph removal by Varroa mite destabilizes the dynamical interaction between immune effectors and virus in bees, as predicted by Volterra's model. Proc. R. Soc. B Biol. Sci. 2019, 286, 20190331, doi:10.1098/rspb.2019.0331.

Antúnez, K.; Anido, M.; Branchiccela, B.; Harriet, J.; Campa, J.; Invernizzi, C.; Santos, E.; Higes, M.; Martín-Hernández, R.; Zunino, P. Seasonal Variation of Honeybee Pathogens and its Association with Pollen Diversity in Uruguay. Microbial Ecology 2015, 70, 522-533, doi:10.1007/s00248-015-0594-7.

Arbetman MP, Gleiser G, Morales CL, Williams P, Aizen MA: Global decline of bumblebees is phylogenetically structured and inversely related to species range size and pathogen incidence. Proc. R. Soc. B 2017, 284:20170204.

Arbour, N.; Ekandé, S.; Côté, G.; Lachance, C.; Chagnon, F.; Tardieu, M.; Cashman, N.R.; Talbot, P.J. Persistent infection of human oligodendrocytic and neuroglial cell lines by human coronavirus 229E. J. Virol. 1999, 73, 3326–3337, doi:10.1128/JVI.73.4.3326-3337.1999.

Armitage, S.A.; Peuss, R.; Kurtz, J. Dscam and pancrustacean immune memory - a review of the evidence. Dev Comp Immunol 2015, 48, 315-323, doi:10.1016/j.dci.2014.03.004.

Armitage, S.A.O.; Kurtz, J.; Brites, D.; Dong, Y.; Du Pasquier, L.; Wang, H.C. Dscam1 in Pancrustacean Immunity: Current Status and a Look to the Future. Front Immunol 2017, 8, 662, doi:10.3389/fimmu.2017.00662.

Aronstein, K.; Saldivar, E. Characterization of a honey bee Toll related receptor gene Am18w and its potential involvement in antimicrobial immune defense. Apidologie 2005, 36, (1), 3-14.

Asensio, I.; Vicente-Rubiano, M.; Munoz, M. J.; Fernandez-Carrion, E.; Sanchez-Vizcaino, J. M.; Carballo, M. Importance of Ecological Factors and Colony Handling for Optimizing Health Status of Apiaries in Mediterranean Ecosystems. PLoS ONE 2016, 11, (10).

Ashe, A.; Sarkies, P.; Le Pen, J.; Tanguy, M.; Miska, E. A. Antiviral RNA Interference against Orsay Virus Is neither Systemic nor Transgenerational in Caenorhabditis elegans. J Virol 2015, 89, (23), 12035-12046.

Avadhanula, V.; Weasner, B.P.; Hardy, G.G.; Kumar, J.P.; Hardy, R.W. A Novel System for the Launch of Alphavirus RNA Synthesis Reveals a Role for the Imd Pathway in Arthropod Antiviral Response. PLOS Pathogens 2009, 5, e1000582, doi:10.1371/journal.ppat.1000582.

Bailey L, Ball B V.: Honey Bee Pathology. Elsevier Ltd; 1991.

Bailey L, Gibbs AJ: Acute infection of bees with paralysis virus. J. Insect Pathol. 1964, 6:395–407.

Bailey L: Viruses attacking the honey bee. Adv. Virus Res. 1976, 20:271–304.

Bailey, L. Acute bee-paralysis virus in adult honey bees injected with sacbrood virus. Virology 33, (2), 368.

Bailey, L. Honey Bee Pathology. Annu Rev Entomol 1968, 13, 191-212.

Bailey, L. The multiplication and spread of sacbrood virus of bees. Ann. Appl. Biol. 1969, 63, 483–491, doi:10.1111/j.1744-7348.1969.tb02844.x.

Bailey, L.; Ball, B.V.; Perry, J.N. The prevalence of viruses of honey bees in Britain. Ann. Appl. Biol. 1981, 97, 109–118, doi:10.1111/j.1744-7348.1981.tb02999.x.

Bailey, L.; Fernando, E. F. Effects of Sacbrood Virus on Adult Honey-Bees. Annals of Applied Biology 1972, 72, (1), 27-&.

Barchuk, A. R.; Figueiredo, V. L. C.; Simões, Z. L. P. Downregulation of ultraspiracle gene expression delays pupal development in honeybees. J Insect Physiol 2008, 54, (6), 1035-1040.

Barribeau, S. M.; Sadd, B. M.; du Plessis, L.; Schmid-Hempel, P. Gene expression differences underlying genotype-by-genotype specificity in a host–parasite system. Proc Natl Acad Sci USA 2014, 111, (9), 3496-3501.

Barribeau, S. M.; Schmid-Hempel, P. Qualitatively different immune response of the bumblebee host, Bombus terrestris, to infection by different genotypes of the trypanosome gut parasite, Crithidia bombi. Infection, Genetics and Evolution 2013, 20, 249-256.

Barron, A. B. Death of the bee hive: understanding the failure of an insect society. Curr Opin Insect Sci 2015, 10, 45-50.

Batch Entrez. In Encyclopedic Reference of Genomics and Proteomics in Molecular Medicine; Springer Berlin Heidelberg: Berlin, Heidelberg, 2006; pp. 131-131.

Bauer, D.F. Constructing Confidence Sets Using Rank Statistics. J. Am. Stat. Assoc. 1972, 67, 687–690, doi:10.2307/2284469.

Baulcombe, D. RNA silencing in plants. Nature 2004, 431, (7006), 356-363.

Baum, B.; Cherbas, L. Drosophila Cell Lines as Model Systems and as an Experimental Tool. In Drosophila: Methods and Protocols, Dahmann, C., Ed.; Humana Press: Totowa, NJ, 2008; pp. 391-424.

Beaurepaire, A.; Piot, N.; Doublet, V.; Antunez, K.; Campbell, E.; Chantawannakul, P.; Chejanovsky, N.; Gajda, A.; Heerman, M.; Panziera, D.; et al. Diversity and Global Distribution of Viruses of the Western Honey Bee, Apis mellifera. Insects 2020, 11, 239, doi:10.3390/insects11040239.

Benaets, K.; Van Geystelen, A.; Cardoen, D.; De Smet, L.; de Graaf, D.C.; Schoofs, L.; Larmuseau, M.H.D.; Brettell, L.E.; Martin, S.J.; Wenseleers, T. Covert deformed wing virus infections have long-term deleterious effects on honeybee foraging and survival. Proc. R. Soc. B Biol. Sci. 2017, 284, 20162149, doi:10.1098/rspb.2016.2149.

Benjeddou, M.; Leat, N.; Allsopp, M.; Davison, S. Development of infectious transcripts and genome manipulation of Black queen-cell virus of honey bees. J Gen Virol 2002, 83, (Pt 12), 3139-46.

Beye, M.; Hartel, S.; Hagen, A.; Hasselmann, M.; Omholt, S. W. Specific developmental gene silencing in the honey bee using a homeobox motif. Insect Mol Biol 2002, 11, (6), 527-32.

Bhatia, S.; Baral, S.S.; Vega Melendez, C.; Amiri, E.; Rueppell, O. Comparing Survival of Israeli Acute Paralysis Virus Infection among Stocks of U.S. Honey Bees. Insects 2021, 12, 60.

Biesmeijer JC, Roberts SP., Reemer M, Ohlemüller R, Edwards M, Peeters T, Schaffers AP, Potts SG, Kleukers R, Thomas CD, et al.: Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 2006, 313:351–354.

Bigot, D.; Dalmon, A.; Roy, B.; Hou, C.; Germain, M.; Romary, M.; Deng, S.; Diao, Q.; Weinert, L. A.; Cook, J. M.; Herniou, E. A.; Gayral, P. The discovery of Halictivirus resolves the Sinaivirus phylogeny. Journal of General Virology 2017, 98, (11), 2864-2875.

Bigot, D.; Dalmon, A.; Roy, B.; Hou, C.; Germain, M.; Romary, M.; Deng, S.; Diao, Q.; Weinert, L.A.; Cook, J.M., et al. The discovery Halictivirus resolves the Sinaivirus phylogeny. Journal of General Virology 2017, 98, 2864-2875, doi:10.1099/jgv.0.000957.

Blair, C. D. Mosquito RNAi is the major innate immune pathway controlling arbovirus infection and transmission. Future Microbiol 2011, 6, (3), 265-277.

Blair, C.; Olson, K. The Role of RNA Interference (RNAi) in Arbovirus-Vector Interactions. Viruses 2015, 7, (2), 820-843.

Blandin, S.; Levashina, E.A. Thioester-containing proteins and insect immunity. Molecular Immunology 2004, 40, 903-908, doi:https://doi.org/10.1016/j.molimm.2003.10.010.

Boisson, B.; Jacques, J. C.; Choumet, V.; Martin, E.; Xu, J.; Vernick, K.; Bourgouin, C. Gene silencing in mosquito salivary glands by RNAi. Febs Lett 2006, 580, (8), 1988-92.

Boncristiani, H. F.; Di Prisco, G.; Pettis, J. S.; Hamilton, M.; Chen, Y. Molecular approaches to the analysis of deformed wing virus replication and pathogenesis in the honey bee, Apis mellifera. Virol J 2009, 6, (1), 221.

Boncristiani, H. F.; Evans, J. D.; Chen, Y.; Pettis, J.; Murphy, C.; Lopez, D. L.; Simone-Finstrom, M.; Strand, M.; Tarpy, D. R.; Rueppell, O. In Vitro Infection of Pupae with Israeli Acute Paralysis Virus Suggests Disturbance of Transcriptional Homeostasis in Honey Bees (Apis mellifera). PLoS ONE 2013, 8, (9), e73429.

Boncristiani, H.F.; Di Prisco, G.; Pettis, J.S.; Hamilton, M.; Chen, Y.P. Molecular approaches to the analysis of deformed wing virus replication and pathogenesis in the honey bee, Apis mellifera. Virol. J. 2009, 6, 221, doi:10.1186/1743-422X-6-221.

Boncristiani, H.F.; Evans, J.D.; Chen, Y.; Pettis, J.; Murphy, C.; Lopez, D.L.; Simone-Finstrom, M.; Strand, M.; Tarpy, D.R.; Rueppell, O. In vitro infection of pupae with Israeli acute paralysis virus suggests disturbance of transcriptional homeostasis in honey bees (Apis mellifera). PLoS ONE 2013, 8, e73429, doi:10.1371/journal.pone.0073429.

Bonifacino, J.S.; Traub, L.M. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 2003, 72, 395-447, doi:10.1146/annurev.biochem.72.121801.161800.

Bonning, B.C.; Saleh, M.-C. The Interplay Between Viruses and RNAi Pathways in Insects. Annu. Rev. Entomol. 2021, 66, 61–79, doi:10.1146/annurev-ento-033020-090410.

Bordier, C.; Dechatre, H.; Suchail, S.; Peruzzi, M.; Soubeyrand, S.; Pioz, M.; Pélissier, M.; Crauser, D.; Conte, Y.L.; Alaux, C. Colony adaptive response to simulated heat waves and consequences at the individual level in honeybees (Apis mellifera). Scientific Reports 2017, 7, 3760, doi:10.1038/s41598-017-03944-x.

Bowen-Walker, P. L.; Martin, S. J.; Gunn, A. The transmission of deformed wing virus between honeybees (Apis mellifera L.) by the ectoparasitic mite Varroa jacobsoni Oud. J Invertebr Pathol 1999, 73, (1), 101-106.

Brackney, D.E.; Beane, J.E.; Ebel, G.D. RNAi Targeting of West Nile Virus in Mosquito Midguts Promotes Virus Diversification. PLOS Pathogens 2009, 5, e1000502, doi:10.1371/journal.ppat.1000502.

Bradbear, N. World Distribution of Major Honeybee Diseases and Pests. Bee World 1988, 69, 15–39, doi:10.1080/0005772X.1988.11098943.

Brennan, C.A.; Anderson, K.V. Drosophila: The Genetics of Innate Immune Recognition and Response. Annual Review of Immunology 2004, 22, 457-483, doi:10.1146/annurev.immunol.22.012703.104626.

Brittain, C.; Williams, N.; Kremen, C.; Klein, A. M. Synergistic effects of non-Apis bees and honey bees for pollination services. P R Soc B 2013, 280, (1754), 20122767-20122767.

Bronkhorst, A.W.; van Rij, R.P. The long and short of antiviral defense: small RNA-based immunity in insects. Current Opinion in Virology 2014, 7, 19-28, doi:https://doi.org/10.1016/j.coviro.2014.03.010.

Brosi, B. J. Pollinator specialization: from the individual to the community. New Phytol. 2016, 210, (4), 1190-1194.

Brown, C.J.; Johnson, A.K.; Daughdrill, G.W. Comparing models of evolution for ordered and disordered proteins. Mol Biol Evol 2010, 27, 609-621, doi:10.1093/molbev/msp277.

Brown, C.J.; Johnson, A.K.; Dunker, A.K.; Daughdrill, G.W. Evolution and disorder. Curr Opin Struct Biol 2011, 21, 441-446, doi:10.1016/j.sbi.2011.02.005.

Bruckner, S.; Steinhauer, N.; Aurell, S.D.; Caron, D.M.; Ellis, J.D.; Fauvel, A.M.; Kulhanek, K.; McArt, S.H.; Mullen, E.; Rangel, J.; et al. 2018–2019 Honey Bee Colony Losses in the United States: Preliminary Results; Bee Informed Partnership, 2019. Available online: https://beeinformed.org/citizen-science/loss-and-management-survey/ (accessed on 27 June 2021).

Bruckner, S.; Steinhauer, N.; Rennich, K.; Aurell, D.D.; Caron, D.M.; Ellis, J.D.; Fauvel, A.M.; Kulhanek, K.; Nelson, K.C.; Rangel, J.; et al. United States Honey Bee Colony Losses 2017–2018: Preliminary Results; Bee Informed Partnership, 2018. Available online: https://beeinformed.org/2018/06/21/preliminary-results-2017-2018-total-and-average-honey-bee-colony-losses-by-state-and-the-district-of-columbia/ (accessed on 27 June 2021).

Bruscella, P.; Bottini, S.; Baudesson, C.; Pawlotsky, J.M.; Feray, C.; Trabucchi, M. Viruses and miRNAs: More Friends than Foes. Front Microbiol 2017, 8, 824, doi:10.3389/fmicb.2017.00824.

Brutscher, L.M.; Daughenbaugh, K.F.; Flenniken, M.L. Antiviral defense mechanisms in honey bees. Current Opinion in Insect Science 2015, 2, 1-12, doi:10.1016/j.cois.2015.04.016.

Brutscher, L.M.; Daughenbaugh, K.F.; Flenniken, M.L. Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. Sci. Rep. 2017, 7, 6448, doi:10.1038/s41598-017-06623-z.

Brutscher, L.M.; Flenniken, M.L. RNAi and antiviral defense in the honey bee. Journal of Immunology Research 2015, 2015, doi:10.1155/2015/941897.

Brutscher, L.M.; McMenamin, A.J.; Flenniken, M.L. The buzz about honey bee viruses. PLoS Pathogens 2016, 12, e1005757, doi:10.1371/journal.ppat.1005757.

Buchon, N.; Silverman, N.; Cherry, S. Immunity in Drosophila melanogaster-from microbial recognition to whole-organism physiology. Nature Reviews Immunology 2014, 14, 796-810, doi:10.1038/nri3763.

Buck, K.W. Comparison of The Replication of Positive-Stranded Rna Viruses of Plants and Animals. In Advances in Virus Research; Maramorosch, K., Murphy, F.A., Shatkin, A.J., Eds.; Academic Press: Cambridge, MA, 1996; Volume 47, pp. 159–251.

Budge, G.E.; Pietravalle, S.; Brown, M.; Laurenson, L.; Jones, B.; Tomkies, V.; Delaplane, K.S. Pathogens as Predictors of Honey Bee Colony Strength in England and Wales. PLOS ONE 2015, 10, e0133228, doi:10.1371/journal.pone.0133228.

Bull, J.C.; Ryabov, E.V.; Prince, G.; Mead, A.; Zhang, C.; Baxter, L.A.; Pell, J.K.; Osborne, J.L.; Chandler, D. A Strong Immune Response in Young Adult Honeybees Masks Their Increased Susceptibility to Infection Compared to Older Bees. PLoS Pathogens 2012, 8, 11-14, doi:10.1371/journal.ppat.1003083.

Burger, W.C. Why Are There So Many Kinds of Flowering Plants? BioScience 1981, 31, 572-581, doi:10.2307/1308218.

Burkle, L. A.; Marlin, J. C.; Knight, T. M. Plant-Pollinator Interactions over 120 Years: Loss of Species, Co-Occurrence, and Function. Science 2013, 339, (6127), 1611-1615.

Calderone, N.W. Insect pollinated crops, insect pollinators and US agriculture: Trend analysis of aggregate data for the period 1992-2009. PLoS ONE 2012, 7, 24-28, doi:10.1371/journal.pone.0037235.

California Almond Board Almond Almanac; 2014.

Cameron, S. A.; Lozier, J. D.; Strange, J. P.; Koch, J. B.; Cordes, N.; Solter, L. F.; Griswold, T. L. Patterns of widespread decline in North American bumble bees. Proc Natl Acad Sci USA 2011, 108, (2), 662-667.

Cappelle, K.; Smagghe, G.; Dhaenens, M.; Meeus, I. Israeli Acute Paralysis Virus Infection Leads to an Enhanced RNA Interference Response and Not Its Suppression in the Bumblebee Bombus terrestris. Viruses 2016, 8, 334, doi:10.3390/v8120334.

Cardinal, S.; Straka, J.; Danforth, B. N. Comprehensive phylogeny of apid bees reveals the evolutionary origins and antiquity of cleptoparasitism. Proc Natl Acad Sci USA 2010, 107, (37), 16207-16211.

Carrillo-Tripp, J.; Dolezal, A.G.; Goblirsch, M.J.; Miller, W.A.; Toth, A.L.; Bonning, B.C. In vivo and in vitro infection dynamics of honey bee viruses. Scientific Reports 2016, 6, 22265, doi:10.1038/srep22265.

Caudy, A.A.; Ketting, R.F.; Hammond, S.M.; Denli, A.M.; Bathoorn, A.M.P.; Tops, B.B.J.; Silva, J.M.; Myers, M.M.; Hannon, G.J.; Plasterk, R.H.A. A micrococcal nuclease homologue in RNAi effector complexes. Nature 2003, 425, 411-414, doi:10.1038/nature01956.

Cavigli, I.; Daughenbaugh, K.F.; Martin, M.; Lerch, M.; Banner, K.; Garcia, E.; Brutscher, L.M.; Flenniken, M.L. Pathogen prevalence and abundance in honey bee colonies involved in almond pollination. Apidologie 2016, 47, 251–266, doi:10.1007/s13592-015-0395-5.

Celle O, Blanchard P, Olivier V, Schurr F, Cougoule N, Faucon JP, Ribière M: Detection of Chronic bee paralysis virus (CBPV) genome and its replicative RNA form in various hosts and possible ways of spread. Virus Res. 2008, 133:280–284.

Cepero A, Ravoet J, Gómez-Moracho T, Bernal J, Del Nozal MJ, Bartolomé C, Maside X, Meana A, González-Porto A V, de Graaf DC, et al.: Holistic screening of collapsing honey bee colonies in Spain: A case study. BMC Res. Notes 2014, 7:649.

Chan, M.M.; Choi, S.Y.; Chan, Q.W.; Li, P.; Guarna, M.M.; Foster, L.J. Proteome profile and lentiviral transduction of cultured honey bee (Apis mellifera L.) cells. Insect Mol Biol 2010, 19, 653-658, doi:10.1111/j.1365-2583.2010.01022.x.

Chandra, S.; Chandra, R.K. Nutrition, immune response, and outcome. Prog Food Nutr Sci 1986, 10, 1-65.

Chantawannakul P, Ward L, Boonham N, Brown M: A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in Varroa mites collected from a Thai honeybee (Apis mellifera) apiary. J. Invertebr. Pathol. 2006, 91:69–73.

Chao, J.A.; Lee, J.H.; Chapados, B.R.; Debler, E.W.; Schneemann, A.; Williamson, J.R. Dual modes of RNA-silencing suppression by Flock House virus protein B2. Nat. Struct. Mol. Biol. 2005, 12, 952–957, doi:10.1038/nsmb1005.

Chauzat, M.-P.; Jacques, A.; Laurent, M.; Bougeard, S.; Hendrikx, P.; Ribière-Chabert, M.; De Graaf, D.; Méroc, E.; Nguyen, B.K.; Roelandt, S., et al. Risk indicators affecting honeybee colony survival in Europe: one year of surveillance. Apidologie 2016, 47, 348-378, doi:10.1007/s13592-016-0440-z.

Chejanovsky, N.; Ophir, R.; Schwager, M.S.; Slabezki, Y.; Grossman, S.; Cox-Foster, D. Characterization of viral siRNA populations in honey bee colony collapse disorder. Virology 2014, 454-455, 176-183, doi:10.1016/j.virol.2014.02.012.

Chen Y, Pettis JS, Evans JD, Kramer M, Feldlaufer M: Transmission of Kashmir bee virus by the ectoparasitic mite Varroa destructor. Apidologie 2004, 35:441–448.

Chen, J.; Xie, C.; Tian, L.; Hong, L.; Wu, X.; Han, J. Participation of the p38 pathway in Drosophila host defense against pathogenic bacteria and fungi. Proceedings of the National Academy of Sciences 2010, 107, 20774, doi:10.1073/pnas.1009223107.

Chen, Y. P.; Pettis, J. S.; Corona, M.; Chen, W. P.; Li, C. J.; Spivak, M.; Visscher, P. K.; DeGrandi-Hoffman, G.; Boncristiani, H.; Zhao, Y.; vanEngelsdorp, D.; Delaplane, K.; Solter, L.; Drummond, F.; Kramer, M.; Lipkin, W. I.; Palacios, G.; Hamilton, M. C.; Smith, B.; Huang, S. K.; Zheng, H. Q.; Li, J. L.; Zhang, X.; Zhou, A. F.; Wu, L. Y.; Zhou, J. Z.; Lee, M.-L.; Teixeira, E. W.; Li, Z. G.; Evans, J. D. Israeli Acute Paralysis Virus: Epidemiology, Pathogenesis and Implications for Honey Bee Health. Plos Pathog 2014, 10, (7), e1004261.

Chen, Y.-C.; Wang, S.-Y.; King, C.-C. Bacterial Lipopolysaccharide Inhibits Dengue Virus Infection of Primary Human Monocytes/Macrophages by Blockade of Virus Entry via a CD14-Dependent Mechanism. J. Virol. 1999, 73, 2650–2657, doi:10.1128/JVI.73.4.2650-2657.1999.

Chen, Y.P.; Evans, J.; Feldlaufer, M. Horizontal and vertical transmission of viruses in the honey bee, Apis mellifera. Journal of Invertebrate Pathology 2006, 92, 152-159, doi:10.1016/j.jip.2006.03.010.

Chen, Y.P.; Siede, R. Honey bee viruses. Adv. Virus Res. 2007, 70, 33–80, doi:10.1016/S0065-3527(07)70002-7.

Cheng, G.; Liu, L.; Wang, P.; Zhang, Y.; Zhao, Y.O.; Colpitts, T.M.; Feitosa, F.; Anderson, J.F.; Fikrig, E. An In Vivo Transfection Approach Elucidates a Role for Aedes aegypti Thioester-Containing Proteins in Flaviviral Infection. PLOS ONE 2011, 6, e22786, doi:10.1371/journal.pone.0022786.

Choi, Y. S.; Choo, Y. M.; Lee, K. S.; Yoon, H. J.; Kim, I.; Je, Y. H.; Sohn, H. D.; Jin, B. R. Cloning and expression profiling of four antibacterial peptide genes from the bumblebee Bombus ignitus. Comp Biochem Phys B 2008, 150, (2), 141-146.

Cobey, S.; Sheppard, W.S.; Tarpy, D.R. Honey Bee Colony Health: Challenges and Sustainable Solutions; Sammataro, D., Yoder, J.A., Eds.; CRC Press: Boca Raton, United States, 2011.

Coffman, S. R.; Lu, J.; Guo, X.; Zhong, J.; Jiang, H.; Broitman-Maduro, G.; Li, W.-X.; Lu, R.; Maduro, M.; Ding, S.-W. Caenorhabditis elegans RIG-I Homolog Mediates Antiviral

RNA Interference Downstream of Dicer-Dependent Biogenesis of Viral Small Interfering RNAs. MBio 2017, 8, (2), e00264-17.

Coleman, J.; Inukai, M.; Inouye, M. Dual functions of the signal peptide in protein transfer across the membrane. Cell 1985, 43, 351-360, doi:https://doi.org/10.1016/0092-8674(85)90040-6.

Cooper, M.D.; Alder, M.N. The evolution of adaptive immune systems. Cell 2006, 124, 815-822, doi:10.1016/j.cell.2006.02.001.

Cooper, P.D.; Schaffer, W.M. Temperature Regulation of Honey Bees (Apis mellifera) foraging in the Sonoran Desert. Journal of Experimental Biology 1985, 1-15.

Cordes, N.; Huang, W.-F.; Strange, J. P.; Cameron, S. A.; Griswold, T. L.; Lozier, J. D.; Solter, L. F. Interspecific geographic distribution and variation of the pathogens Nosema bombi and Crithidia species in United States bumble bee populations. J Invertebr Pathol 2012, 109, (2), 209-216.

Cordin, O.; Banroques, J.; Tanner, N.K.; Linder, P. The DEAD-box protein family of RNA helicases. Gene 2006, 367, 17-37, doi:https://doi.org/10.1016/j.gene.2005.10.019.

Cornman RS: Relative abundance of Deformed wing virus, Varroa destructor virus 1, and their recombinants in honey bees (Apis mellifera) assessed by kmer analysis of public RNA-Seq data. J. Invertebr. Pathol. 2017, 149:44–50.

Cornman, R.S. Relative abundance and molecular evolution of Lake Sinai Virus (Sinaivirus) clades. PeerJ 2019, 7, e6305, doi:https://doi.org/10.7717/peerj.6305.

Cornman, R.S.; Chen, Y.P.; Schatz, M.C.; Street, C.; Zhao, Y.; Desany, B.; Egholm, M.; Hutchison, S.; Pettis, J.S.; Lipkin, W.I.; et al. Genomic Analyses of the Microsporidian Nosema ceranae, an Emergent Pathogen of Honey Bees. PLOS Pathogens 2009, 5, e1000466, doi:10.1371/journal.ppat.1000466.

Cornman, R.S.; Tarpy, D.R.; Chen, Y.; Jeffreys, L.; Lopez, D.; Pettis, J.S.; VanEngelsdorp, D.; Evans, J.D. Pathogen webs in collapsing honey bee colonies. PLoS ONE 2012, 7, e43562, doi:10.1371/journal.pone.0043562.

Costa, A.; Jan, E.; Sarnow, P.; Schneider, D. The Imd pathway is involved in antiviral immune responses in Drosophila. PLoS ONE 2009, 4, doi:10.1371/journal.pone.0007436.

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

Craggs JK, Ball JK, Thomson BJ, Irving WL, Grabowska AM: Development of a strand-specic RT-PCR based assay to detect the replicative form of hepatitis C virus RNA. J. Virol. Methods 2001, 94:111–120.

Crane, P.R.; Friis, E.M.; Pedersen, K.R. The origin and early diversification of angiosperms. Nature 1995, 374, 27-33, doi:10.1038/374027a0.

Cridland, J. M.; Tsutsui, N. D.; Ramirez, S. R. The Complex Demographic History and Evolutionary Origin of the Western Honey Bee, Apis Mellifera. Genome Biology and Evolution 2017, 9, (2), 457-472.

D'Alvise, P.; Seeburger, V.; Gihring, K.; Kieboom, M.; Hasselmann, M. Seasonal dynamics and co-occurrence patterns of honey bee pathogens revealed by high-throughput RT-qPCR analysis. Ecology and Evolution 2019, 9, 10241-10252, doi:10.1002/ece3.5544.

Dainat B, Evans JD, Chen YP, Gauthier L, Neumann P: Predictive markers of honey bee colony collapse. PLoS One 2012, 7:e32151.

Dainat, B.; Evans, J.D.; Chen, Y.P.; Gauthier, L.; Neumann, P. Dead or alive: deformed wing virus and Varroa destructor reduce the life span of winter honeybees. Appl Environ Microbiol 2012, 78, 981-987, doi:10.1128/AEM.06537-11.

Dainat, B.; Evans, J.D.; Chen, Y.P.; Gauthier, L.; Neumann, P. Predictive markers of honey bee colony collapse. PloS one 2012, 7, e32151, doi:10.1371/journal.pone.0032151.

Dainat, B.; Neumann, P. Clinical signs of deformed wing virus infection are predictive markers for honey bee colony losses. Journal of invertebrate pathology 2013, 112, 278-280, doi:10.1016/j.jip.2012.12.009.

Dalmon, A.; Desbiez, C.; Coulon, M.; Thomasson, M.; Conte, Y.L.; Alaux, C.; Vallon, J. Evidence for positive selection and recombination hotspots in Deformed wing virus (DWV). Sci. Rep. 2017, 41045, doi:10.1038/srep41045.

Dalmon, A.; Peruzzi, M.; Le Conte, Y.; Alaux, C.; Pioz, M. Temperature-driven changes in viral loads in the honey bee Apis mellifera. Journal of Invertebrate Pathology 2018, doi:https://doi.org/10.1016/j.jip.2018.12.005.

Danforth, B.N.; Sipes, S.; Fang, J.; Brady, S.G. The history of early bee diversification based on five genes plus morphology. Proceedings of the National Academy of Sciences 2006, 103, 15118, doi:10.1073/pnas.0604033103.

Danihlík, J.; Aronstein, K.; Petřivalský, M. Antimicrobial peptides: a key component of honey bee innate immunity. J Apicult Res 2016, 54, (2), 123-136.

Dasgupta, R.; Cheng, L.L.; Bartholomay, L.C.; Christensen, B.M. Flock house virus replicates and expresses green fluorescent protein in mosquitoes. J. Gen. Virol. 2003, 84, 1789–1797, doi:10.1099/vir.0.18938-0.

Dasgupta, R.; Free, H.M.; Zietlow, S.L.; Paskewitz, S.M.; Aksoy, S.; Shi, L.; Fuchs, J.; Hu, C.; Christensen, B.M. Replication of flock house virus in three genera of medically important insects. J. Med. Entomol. 2007, 44, 102–110, doi:10.1603/0022-2585(2007)44[102:rofhvi]2.0.co;2.

Daughenbaugh, K.F.; Kahnonitch, I.; Carey, C.C.; McMenamin, A.J.; Wiegand, T.; Erez, T.; Arkin, N.; Ross, B.; Wiedenheft, B.; Sadeh, A.; et al. Metatranscriptome Analysis of Sympatric Bee Species Identifies Bee Virus Variants and a New Virus, Andrena-Associated Bee Virus-1. Viruses 2021, 13, 291, doi:10.3390/v13020291.

Daughenbaugh, K.F.; Martin, M.; Brutscher, L.M.; Cavigli, I.; Garcia, E.; Lavin, M.; Flenniken, M.L. Honey bee infecting Lake Sinai viruses. Viruses 2015, 7, 3285-3309, doi:10.3390/v7062772.

Daugherty, M.D.; Malik, H.S. Rules of Engagement: Molecular Insights from Host-Virus Arms Races. Annual Review of Genetics 2012, 46, 677-700, doi:10.1146/annurev-genet-110711-155522.

De Maio, F. A.; Risso, G.; Iglesias, N. G.; Shah, P.; Pozzi, B.; Gebhard, L. G.; Mammi, P.; Mancini, E.; Yanovsky, M. J.; Andino, R.; Krogan, N.; Srebrow, A.; Gamarnik, A. V. The Dengue Virus NS5 Protein Intrudes in the Cellular Spliceosome and Modulates Splicing. Plos Pathog 2016, 12, (8), e1005841.

de Miranda JR, Cordoni G, Budge G: The Acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex. J. Invertebr. Pathol. 2010, 103:S30–S47.

de Miranda JR, Cornman SR, Evans JD, Semberg E, Haddad NJ, Neumann P, Gauthier L: Genome characterization, prevalence and distribution of a Macula-like virus from Apis mellifera and Varroa destructor. Viruses 2015, 7:3586–3602.

de Miranda, J. R.; Bailey, L.; Ball, B. V.; Blanchard, P.; Budge, G. E.; Chejanovsky, N.; Chen, Y.-P.; Gauthier, L.; Genersch, E.; de Graaf, D. C.; Ribière, M.; Ryabov, E.; De Smet, L.; van der Steen, J. J. M. Standard methods for virus research in Apis mellifera. J Apicult Res 2015, 52, (4), 1-56.

de Miranda, J.R.; Cornman, R.S.; Evans, J.D.; Semberg, E.; Haddad, N.; Neumann, P.; Gauthier, L. Genome Characterization, Prevalence and Distribution of a Macula-Like Virus from Apis mellifera and Varroa destructor. Viruses 2015, 7, 3586-3602, doi:10.3390/v7072789.

de Miranda, J.R.; Drebot, M.; Tyler, S.; Shen, M.; Cameron, C.E.; Stoltz, D.B.; Camazine, S.M. Complete nucleotide sequence of Kashmir bee virus and comparison with acute bee paralysis virus. J. Gen. Virol. 2004, 85, 2263–2270, doi:10.1099/vir.0.79990-0.

de Miranda, J.R.; Fries, I. Venereal and vertical transmission of deformed wing virus in honeybees (Apis mellifera L.). J. Invertebr. Pathol. 2008, 98, 184–189, doi:10.1016/j.jip.2008.02.004.

de Miranda, J.R.; Genersch, E. Deformed wing virus. J. Invertebr. Pathol. 2010, 103, S48–S61, doi:10.1016/j.jip.2009.06.012.

Dearing, S.C.; Scotti, P.D.; Wigley, P.J.; Dhana, S.D. A small RNA virus isolated from the grass grub, Costelytra zealandica (Coleoptera: Scarabaeidae). N. Z. J. Zool. 1980, 7, 267–269, doi:10.1080/03014223.1980.10423785.

Deboutte, W.; Beller, L.; Yinda, C.K.; Shi, C.; Smets, L.; Vanmechelen, B.; Conceição-Neto, N.; Dallmeier, K.; Maes, P.; de Graaf, D.C.; et al. Hymenoptera associated eukaryotic virome lacks host specificity. bioRxiv 2020, 2020.2009.2015.298042, doi:10.1101/2020.09.15.298042.

Deddouche, S.; Matt, N.; Budd, A.; Mueller, S.; Kemp, C.; Galiana-Arnoux, D.; Dostert, C.; Antoniewski, C.; Hoffmann, J.A.; Imler, J.-L. The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in drosophila. Nature Immunology 2008, 9, 1425-1432, doi:10.1038/ni.1664.

DeGrandi-Hoffman G, Chen Y, Huang E, Huang MH: The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (Apis mellifera L.). J. Insect Physiol. 2010, 56:1184–1191.

DeGrandi-Hoffman, G.; Chen, Y. Nutrition, immunity and viral infections in honey bees. Curr Opin Insect Sci 2015, 10, 170-176.

Delaplane KS, Pietravalle S, Brown MA, Budge GE: Honey bee colonies headed by hyperpolyandrous queens have improved brood rearing efficiency and lower infestation rates of parasitic Varroa mites. PLoS One 2015, 10:e0142985.

Delplane, K.S.; Mayer, D.F. Crop Pollination by Bees; CABI Publishing: Wallingford, UK, 2000.

Desai, S.D.; Eu, Y.J.; Whyard, S.; Currie, R.W. Reduction in deformed wing virus infection in larval and adult honey bees (Apis mellifera L.) by double-stranded RNA ingestion. Insect Molecular Biology 2012, 21, 446-455, doi:10.1111/j.1365-2583.2012.01150.x.

Di Prisco G, Annoscia D, Margiotta M, Ferrara R, Varricchio P, Zanni V, Caprio E, Nazzi F, Pennacchio F: A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. Proc. Natl. Acad. Sci. 2016, 113:3203–3208.

Di Prisco G, Pennacchio F, Caprio E, Boncristiani HF, Evans JD, Chen Y: Varroa destructor is an effective vector of Israeli acute paralysis virus in the honeybee, Apis mellifera. J. Gen. Virol. 2011, 92:151–155.

Di Prisco, G.; Annoscia, D.; Margiotta, M.; Ferrara, R.; Varricchio, P.; Zanni, V.; Caprio, E.; Nazzi, F.; Pennacchio, F. A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. Proceedings of the National Academy of Sciences 2016, 113, 201523515, doi:10.1073/pnas.1523515113.

Di Prisco, G.; Cavaliere, V.; Annoscia, D.; Varricchio, P.; Caprio, E.; Nazzi, F.; Gargiulo, G.; Pennacchio, F. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. Proc Natl Acad Sci USA 2013, 110, (46), 18466-18471.

Di Prisco, G.; Cavaliere, V.; Annoscia, D.; Varricchio, P.; Caprio, E.; Nazzi, F.; Gargiulo, G.; Pennacchio, F. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. Proceedings of the National Academy of Sciences of the United States of America 2013, 110, 18466-18471, doi:10.1073/pnas.1314923110.

Diao, Q.; Sun, L.; Zheng, H.; Zeng, Z.; Wang, S.; Xu, S.; Zheng, H.; Chen, Y.; Shi, Y.; Wang, Y.; Meng, F.; Sang, Q.; Cao, L.; Liu, F.; Zhu, Y.; Li, W.; Li, Z.; Dai, C.; Yang, M.; Chen, S.; Chen, R.; Zhang, S.; Evans, J. D.; Huang, Q.; Liu, J.; Hu, F.; Su, S.; Wu, J. Genomic and transcriptomic analysis of the Asian honeybee Apis cerana provides novel insights into honeybee biology. Sci Rep 2018, 8, (1), 822.

Ding, S.-W. RNA-based antiviral immunity. Nat Rev Immunol 2010, 10, (9), 632-644.

Dolezal, A.G.; Hendrix, S.D.; Scavo, N.A.; Carrillo-tripp, J.; Harris, A.; Wheelock, M.J.; Neal, M.E.O.; Toth, A.L. Honey Bee Viruses in Wild Bees : Viral Prevalence , Loads , and Experimental Inoculation. PLoS ONE 2016, 11, e0166190, doi:10.1371/journal.pone.0166190.

Dorner, S.; Lum, L.; Kim, M.; Paro, R.; Beachy, P.A.; Green, R. A genomewide screen for components of the RNAi pathway in Drosophila cultured cells. Proceedings of the National Academy of Sciences 2006, 103, 11880–11885.

Dostert, C.; Jouanguy, E.; Irving, P.; Troxler, L.; Galiana-Arnoux, D.; Hetru, C.; Hoffmann, J.A.; Imler, J.-L. The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. Nature Immunology 2005, 6, 946-953, doi:10.1038/ni1237.

Doublet, V.; Poeschl, Y.; Gogol-Döring, A.; Alaux, C.; Annoscia, D.; Aurori, C.; Barribeau, S. M.; Bedoya-Reina, O. C.; Brown, M. J. F.; Bull, J. C.; Flenniken, M. L.; Galbraith, D. A.; Genersch, E.; Gisder, S.; Grosse, I.; Holt, H. L.; Hultmark, D.; Lattorff, H. M. G.; Le Conte, Y.; Manfredini, F.; McMahon, D. P.; Moritz, R. F. A.; Nazzi, F.; Nino, E. L.; Nowick, K.; van Rij, R. P.; Paxton, R. J.; Grozinger, C. M. Unity in defence: honeybee workers exhibit conserved molecular responses to diverse pathogens. Bmc Genomics 2017, 18, (1), 207.

Drier, E.A.; Steward, R. The dorsoventral signal transduction pathway and the Rel-like transcription factors inDrosophila. Seminars in Cancer Biology 1997, 8, 83-92, doi:https://doi.org/10.1006/scbi.1997.0059.

Dunker, A.K.; Cortese, M.S.; Romero, P.; Iakoucheva, L.M.; Uversky, V.N. Flexible nets. The roles of intrinsic disorder in protein interaction networks. Febs j 2005, 272, 5129-5148, doi:10.1111/j.1742-4658.2005.04948.x.

Dunnett, C.W. A Multiple Comparison Procedure for Comparing Several Treatments with a Control. J. Am. Stat. Assoc. 1955, 50, 1096–1121, doi:10.2307/2281208.

Dyson, H.J.; Wright, P.E. Coupling of folding and binding for unstructured proteins. Curr Opin Struct Biol 2002, 12, 54-60, doi:10.1016/s0959-440x(02)00289-0.

Eckerle, L.D.; Albariño, C.G.; Ball, L.A. Flock House virus subgenomic RNA3 is replicated and its replication correlates with transactivation of RNA2. Virology 2003, 317, 95–108, doi:10.1016/j.virol.2003.08.029.

Edelman, A.M.; Blumenthal, D.K.; Krebs, E.G. Protein serine/threonine kinases. Annu Rev Biochem 1987, 56, 567-613, doi:10.1146/annurev.bi.56.070187.003031.

Edgar RC: MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004, 32:1792–1797.

Eilers, E.J.; Kremen, C.; Smith Greenleaf, S.; Garber, A.K.; Klein, A.-M. Contribution of Pollinator-Mediated Crops to Nutrients in the Human Food Supply. PLOS ONE 2011, 6, e21363, doi:10.1371/journal.pone.0021363.

Eleftherianos, I.; Won, S.; Chtarbanova, S.; Squiban, B.; Ocorr, K.; Bodmer, R.; Beutler, B.; Hoffmann, J. A.; Imler, J. L. ATP-sensitive potassium channel (KATP)-dependent regulation of cardiotropic viral infections. Proc Natl Acad Sci USA 2011, 108, (29), 12024-12029.

Elekonich, M.M. Extreme thermotolerance and behavioral induction of 70-kDa heat shock proteins and their encoding genes in honey bees. Cell Stress and Chaperones 2009, 14, 219-226, doi: https://doi.org/10.1007/s12192-008-0063-z.

Ellis, J.D.; Munn, P.A. The worldwide health status of honey bees. Bee World 2005, 86, 88–101, doi:10.1080/0005772X.2005.11417323.

Elsik, C.G.; Worley, K.C.; Bennett, A.K.; Beye, M.; Camara, F.; Childers, C.P.; de Graaf, D.C.; Debyser, G.; Deng, J.; Devreese, B.; et al. Finding the missing honey bee genes: lessons learned from a genome upgrade. BMC Genomics 2014, 15, 86, doi:10.1186/1471-2164-15-86.

Erez, T.; Chejanovsky, N. Infection of a Lepidopteran Cell Line with Deformed Wing Virus. Viruses 2020, 12, 739, doi:10.3390/v12070739.

Evans JD, Schwarz RS: Bees brought to their knees: Microbes affecting honey bee health. Trends Microbiol. 2011, 19:614–620.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L., Jiang, H., Kanost, M., Thompson, G.J., Zou, Z., Hultmark, D., J D. Immune pathways and defence mechanisms in honey bees Apis mellifera. Insect Molecular Biology 2006, 15, 645-656, doi:10.1111/j.1365-2583.2006.00682.x.

Evans, J.D.; Chen, Y.P.; Prisco, G.d.; Pettis, J.; Williams, V. Bee cups: single-use cages for honey bee experiments. Journal of Apicultural Research 2009, 48, 300-302, doi:10.1080/00218839.2009.11101548.

Evans, J.D.; Spivak, M. Socialized medicine: Individual and communal disease barriers in honey bees. Journal of Invertebrate Pathology 2010, 103, S62-S72, doi:https://doi.org/10.1016/j.jip.2009.06.019.

Fürst, M. A.; McMahon, D. P.; Osborne, J. L.; Paxton, R. J.; Brown, M. J. F. Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature 2014, 506, (7488), 364-366.

Fan, Y.; Sanyal, S.; Bruzzone, R. Breaking Bad: How Viruses Subvert the Cell Cycle. Front Cell Infect Microbiol 2018, 8, 396-396, doi:10.3389/fcimb.2018.00396.

Faurot-Daniels, C.; Glenny, W.; Daughenbaugh, K.F.; McMenamin, A.J.; Burkle, L.A.; Flenniken, M.L. Longitudinal monitoring of honey bee colonies reveals dynamic nature of virus abundance and indicates a negative impact of Lake Sinai virus 2 on colony health. PLoS ONE 2020, 15, e0237544, doi:10.1371/journal.pone.0237544.

Felsenstein J: Confidence limits on phylogenies: An approach using the bootstrap. Evolution (N. Y). 1985, 39:783–791.

Feng Y, Krueger EN, Liu S, Dorman K, Bonning BC, Miller WA: Discovery of known and novel viral genomes in soybean aphid by deep sequencing. Phytobiomes 2017, 1:36–45.

Fernandes, J.C.R.; Acuña, S.M.; Aoki, J.I.; Floeter-Winter, L.M.; Muxel, S.M. Long Non-Coding RNAs in the Regulation of Gene Expression: Physiology and Disease. Noncoding RNA 2019, 5, doi:10.3390/ncrna5010017.

Ferrandon, D.; Imler, J.L.; Hetru, C.; Hoffmann, J.A. The Drosophila systemic immune response: Sensing and signalling during bacterial and fungal infections. Nature Reviews Immunology 2007, 7, 862-874, doi:10.1038/nri2194.

Ferreira, Á.G.; Naylor, H.; Esteves, S.S.; Pais, I.S.; Martins, N.E.; Teixeira, L. The Toll-Dorsal Pathway Is Required for Resistance to Viral Oral Infection in Drosophila. PLOS Pathogens 2014, 10, e1004507, doi:10.1371/journal.ppat.1004507.

Fire, A. RNA-triggered gene silencing. Trends Genet 1999, 15, (9), 358-363.

Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998, 391, (6669), 806-811.

Flenniken, M. L.; Kunitomi, M.; Tassetto, M.; Andino, R., The Antiviral Role of RNA Interference. In Insect Virology, Asgari, S.; Johnson, K. N., Eds. Caister Academic Press: Norfolk, UK, 2010; pp 367-388.

Flenniken, M.L.; Andino, R. Non-Specific dsRNA-Mediated Antiviral Response in the Honey Bee. PLoS ONE 2013, 8, e77263, doi:10.1371/journal.pone.0077263.

Forsgren, E. European foulbrood in honey bees. Journal of Invertebrate Pathology 2010, 103, S5-S9, doi:10.1016/j.jip.2009.06.016.

Francis, R.M.; Nielsen, S.L.; Kryger, P. Patterns of viral infection in honey bee queens. Journal of General Virology 2013, 94, 668-676, doi:https://doi.org/10.1099/vir.0.047019-0.

Fujiyuki, T.; Takeuchi, H.; Ono, M.; Ohka, S.; Sasaki, T.; Nomoto, A.; Kubo, T. Novel Insect Picorna-Like Virus Identified in the Brains of Aggressive Worker Honeybees. J. Virol. 2004, 78, 1093–1100, doi:10.1128/JVI.78.3.1093-1100.2004.

Gábor, E.; Cinege, G.; Csordás, G.; Török, T.; Folkl-Medzihradszky, K.; Darula, Z.; Andó, I.; Kurucz, É. Hemolectin expression reveals functional heterogeneity in honey bee (Apis mellifera) hemocytes. Dev. Comp. Immunol. 2017, 76, 403–411, doi:10.1016/j.dci.2017.07.013.

Gabler, F.; Nam, S.Z.; Till, S.; Mirdita, M.; Steinegger, M.; Söding, J.; Lupas, A.N.; Alva, V. Protein Sequence Analysis Using the MPI Bioinformatics Toolkit. Curr Protoc Bioinformatics 2020, 72, e108, doi:10.1002/cpbi.108.

Galbraith, D.A.; Fuller, Z.L.; Ray, A.M.; Brockmann, A.; Frazier, M.; Gikungu, M.W.; Martinez, J.F.I.; Kapheim, K.M.; Kerby, J.T.; Kocher, S.D.; et al. Investigating the viral ecology of global bee communities with high-throughput metagenomics. Scientific Reports 2018, 8, 8879, doi:10.1038/s41598-018-27164-z.

Galbraith, D.A.; Yang, X.; Nino, E.L.; Yi, S.; Grozinger, C. Parallel epigenomic and transcriptomic responses to viral infection in honey bees (Apis mellifera). PLoS Pathog. 2015, 11, e1004713, doi:10.1371/journal.ppat.1004713.

Galiana-Arnoux, D.; Dostert, C.; Schneemann, A.; Hoffmann, J.A.; Imler, J.-L. Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. Nature Immunology 2006, 7, 590-597, doi:10.1038/ni1335.

Gallagher, T.M.; Rueckert, R.R. Assembly-dependent maturation cleavage in provirions of a small icosahedral insect ribovirus. J. Virol. 1988, 62, 3399–3406, doi:10.1128/jvi.62.9.3399-3406.1988.

Gallai, N.; Salles, J.-M.; Settele, J.; Vaissière, B.E. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological Economics 2009, 68, 810-821, doi:10.1016/j.ecolecon.2008.06.014.

Gammon, D.B.; Mello, C.C. RNA interference-mediated antiviral defense in insects. Current Opinion in Insect Science 2015, 8, 111-120, doi:10.1016/j.cois.2015.01.006.

García, M.A.; Meurs, E.F.; Esteban, M. The dsRNA protein kinase PKR: Virus and cell control. Biochimie 2007, 89, 799-811, doi:https://doi.org/10.1016/j.biochi.2007.03.001.

Garibaldi, L. A.; Steffan-Dewenter, I.; Winfree, R.; Aizen, M. A.; Bommarco, R.; Cunningham, S. A.; Kremen, C.; Carvalheiro, L. G.; Harder, L. D.; Afik, O.; Bartomeus, I.; Benjamin, F.; Boreux, V.; Cariveau, D.; Chacoff, N. P.; Dudenhoffer, J. H.; Freitas, B. M.; Ghazoul, J.; Greenleaf, S.; Hipolito, J.; Holzschuh, A.; Howlett, B.; Isaacs, R.; Javorek, S. K.; Kennedy, C. M.; Krewenka, K. M.; Krishnan, S.; Mandelik, Y.; Mayfield, M. M.; Motzke, I.; Munyuli, T.; Nault, B. A.; Otieno, M.; Petersen, J.; Pisanty, G.; Potts, S. G.; Rader, R.; Ricketts, T. H.; Rundlof, M.; Seymour, C. L.; Schuepp, C.; Szentgyorgyi, H.; Taki, H.; Tscharntke, T.; Vergara, C. H.; Viana, B. F.; Wanger, T. C.; Westphal, C.; Williams, N.; Klein, A. M. Wild Pollinators Enhance Fruit Set of Crops Regardless of Honey Bee Abundance. Science 2013, 339, (6127), 1608-1611.

Gauthier, L.; Cornman, S.; Hartmann, U.; Cousserans, F.; Evans, J.; de Miranda, J.; Neumann, P. The Apis mellifera Filamentous Virus Genome. Viruses 2015, 7, (7), 3798-3815.

Genersch E, Yue C, Fries I, de Miranda JR: Detection of Deformed wing virus, a honey bee viral pathogen, in bumble bees (Bombus terrestris and Bombus pascuorum) with wing deformities. J. Invertebr. Pathol. 2006, 91:61–63.

Genersch, E. American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. Journal of Invertebrate Pathology 2010, 103, S10-S19, doi:10.1016/j.jip.2009.06.015.

Genersch, E.; Aubert, M. Emerging and re-emerging viruses of the honey bee (Apis mellifera L.). Vet Res 2010, 41, (6), 54.

Genersch, E.; Gisder, S.; Hedtke, K.; Hunter, W.B.; Möckel, N.; Müller, U. Standard methods for cell cultures in Apis mellifera research. Journal of Apicultural Research 2013, 52, 1-8, doi:10.3896/IBRA.1.52.1.02.

Genersch, E.; von der Ohe, W.; Kaatz, H.; Schroeder, A.; Otten, C.; Büchler, R.; Berg, S.; Ritter, W.; Mühlen, W.; Gisder, S.; Meixner, M.; Liebig, G.; Rosenkranz, P. The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 2010, 41, (3), 332-352.

Genersch, E.; Yue, C.; Fries, I.; de Miranda, J.R. Detection of Deformed wing virus, a honey bee viral pathogen, in bumble bees (Bombus terrestris and Bombus pascuorum) with wing deformities. J. Invertebr. Pathol. 2006, 91, 61–63, doi:10.1016/j.jip.2005.10.002.

Genzel, Y.; Reichl, U. Vaccine Production. In Animal Cell Biotechnology: Methods and Protocols; Pörtner, R., Ed.; Humana Press: Totowa, NJ, USA, 2007; pp. 457–473.

Gertz, E. BLAST Scoring Parameters. 2005.

Ghosh, R.C.; Ball, B.V.; Willcocks, M.M.; Carter, M.J. The nucleotide sequence of sacbrood virus of the honey bee: An insect picorna-like virus. J. Gen. Virol. 1999, 80, 1541–1549, doi:10.1099/0022-1317-80-6-1541.

Gil, N.; Ulitsky, I. Regulation of gene expression by cis-acting long non-coding RNAs. Nature Reviews Genetics 2020, 21, 102-117, doi:10.1038/s41576-019-0184-5.

Ginzinger, D.G. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. Exp. Hematol. 2002, 30, 503–512, doi:10.1016/s0301-472x(02)00806-8.

Gisder, S.; Aumeier, P.; Genersch, E. Deformed wing virus: Replication and viral load in mites (Varroa destructor). J. Gen. Virol. 2009, 90, 463–467, doi:10.1099/vir.0.005579-0.

Gisder, S.; Genersch, E.; Pfeiffer Julie, K. Direct Evidence for Infection of Varroa destructor Mites with the Bee-Pathogenic Deformed Wing Virus Variant B, but Not Variant A, via Fluorescence In Situ Hybridization Analysis. J. Virol. 2021, 95, e01786–01720, doi:10.1128/JVI.01786-20.

Glenny, W.; Cavigli, I.; Daughenbaugh, K.F.; Radford, R.; Kegley, S.E.; Flenniken, M.L. Honey bee (Apis mellifera) colony health and pathogen composition in migratory beekeeping operations involved in California almond pollination. PLoS ONE 2017, 12, e0182814, doi:10.1371/journal.pone.0182814.

Glotzer, J.B.; Saltik, M.; Chiocca, S.; Michou, A.-I.; Moseley, P.; Cotten, M. Activation of heat-shock response by an adenovirus is essential for virus replication. Nature 2000, 407, 207-211, doi:10.1038/35025102.

Goblirsch, M.J.; Spivak, M.S.; Kurtti, T.J. A Cell Line Resource Derived from Honey Bee (Apis mellifera) Embryonic Tissues. PLoS ONE 2013, 8, e09831, doi:10.1371/journal.pone.0069831.

Goic, B.; Vodovar, N.; Mondotte, J. A.; Monot, C.; Frangeul, L.; Blanc, H.; Gausson, V.; Vera-Otarola, J.; Cristofari, G.; Saleh, M.-C. RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model Drosophila. Nat Immunol 2013, 14, (4), 396-403.

Goodwin S, McPherson JD, McCombie WR: Coming of age: Ten years of next-generation sequencing technologies. Nat. Rev. Genet. 2016, 17:333–351.

Goulson D, Hanley ME, Darvill B, Ellis JS: Biotope associations and the decline of bumblebees (Bombus spp.). J. Insect Conserv. 2006, 10:95–103.

Goulson D, Lye GC, Darvill B: Decline and conservation of bumble bees. Annu. Rev. Entomol. 2008, 53:191–208.

Goulson D, Nicholls E, Botías C, Rotheray EL: Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 2015, 347:1255957.

Goulson, D. Bumblebees: Their Behaviour and Ecology. Oxford University Press: Oxford, UK and New York, USA, 2003.

Goulson, D. Effects of Introduced Bees on Native Ecosystems. Annu Rev Ecol Evol S 2003, 34, (1), 1-26.

Goulson, D.; Hanley, M.E.; Darvill, B.; Ellis, J.S. Biotope associations and the decline of bumblebees (Bombus spp.). Journal of Insect Conservation 2006, 10, 95-103, doi:10.1007/s10841-006-6286-3.

Goulson, D.; Nicholls, E.; Botias, C.; Rotheray, E.L. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 2015, 347, 1255957, doi:10.1126/science.1255957.

Gourbal, B.; Pinaud, S.; Beckers, G. J. M.; Van Der Meer, J. W. M.; Conrath, U.; Netea, M. G. Innate immune memory: An evolutionary perspective. Immunol Rev 2018, 283, (1), 21-40.

Govan, V.A.; Leat, N.; Allsopp, M.; Davison, S. Analysis of the Complete Genome Sequence of Acute Bee Paralysis Virus Shows That It Belongs to the Novel Group of Insect-Infecting RNA Viruses. Virology 2000, 277, 457–463, doi:10.1006/viro.2000.0616.

Government, T. A. Status of the Asian honey bee in mainland Australia http://www.agriculture.gov.au/pests-diseases-weeds/bees/the-asian-honey-bee-in-australia (July 2, 2018),

Granberg, F.; Vicente-Rubiano, M.; Rubio-Guerri, C.; Karlsson, O.E.; Kukielka, D.; Belák, S.; Sánchez-Vizcaíno, J.M. Metagenomic Detection of Viral Pathogens in Spanish Honeybees: Co-Infection by Aphid Lethal Paralysis, Israel Acute Paralysis and Lake Sinai Viruses. PLoS ONE 2013, 8, e57459, doi:10.1371/journal.pone.0057459.

Graystock P, Goulson D, Hughes WOH: The relationship between managed bees and the prevalence of parasites in bumblebees. Peer J 2014, 2:e522.

Graystock P, Meeus I, Smagghe G, Goulson D, Hughes WOH: The effects of single and mixed infections of Apicystis bombi and Deformed wing virus in Bombus terrestris. Parasitology 2016, 143:358–365.

Graystock P, Yates K, Darvill B, Goulson D, Hughes WOH: Emerging dangers: Deadly effects of an emergent parasite in a new pollinator host. J. Invertebr. Pathol. 2013, 114:114–119.

Graystock P, Yates K, Evison SE, Darvill B, Goulson D, Hughes WOH: The Trojan hives: Pollinator pathogens, imported and distributed in bumblebee colonies. J. Appl. Ecol. 2013, 50:1207–1215.

Green, T. J.; Speck, P. Antiviral Defense and Innate Immune Memory in the Oyster. Viruses 2018, 10, (3), 133.

Greenleaf, S. S.; Kremen, C. Wild bees enhance honey bees' pollination of hybrid sunflower. Proc Natl Acad Sci USA 2006, 103, (37), 13890-13895.

Grozinger, C.M.; Flenniken, M.L. Bee Viruses: Ecology, Pathogenicity, and Impacts. Annual Review of Entomology 2019, 64, 205-226, doi:10.1146/annurev-ento-011118-111942.

Guo, Y.; Goodman, C.L.; Stanley, D.W.; Bonning, B.C. Cell Lines for Honey Bee Virus Research. Viruses 2020, 12, 236, doi:10.3390/v12020236.

Gusachenko, O.N.; Woodford, L.; Balbirnie-Cumming, K.; Campbell, E.M.; Christie, C.R.; Bowman, A.S.; Evans, D.J. Green Bees: Reverse Genetic Analysis of Deformed Wing Virus Transmission, Replication, and Tropism. Viruses 2020, 12, doi:10.3390/v12050532.

Guzman-Novoa E, Hamiduzzaman M, Anguiano-Baez R, Correa-Benítez A, Castañeda-Cervantes E, Arnold NI: First detection of honey bee viruses in stingless bees in North America. J. Apic. Res. 2016, 54:93–95.

Haddad, N.; Mahmud Batainh, A.; Suleiman Migdadi, O.; Saini, D.; Krishnamurthy, V.; Parameswaran, S.; Alhamuri, Z. Next generation sequencing of Apis mellifera syriaca identifies genes for Varroa resistance and beneficial bee keeping traits. Insect Science 2016, 23, 579-590, doi:https://doi.org/10.1111/1744-7917.12205.

Hahn, C.S.; Hahn, Y.S.; Braciale, T.J.; Rice, C.M. Infectious Sindbis virus transient expression vectors for studying antigen processing and presentation. Proceedings of the National Academy of Sciences 1992, 89, 2679, doi:10.1073/pnas.89.7.2679.

Hammond, S. M. Argonaute2, a Link Between Genetic and Biochemical Analyses of RNAi. Science 2001, 293, (5532), 1146-1150.

Han, F.; Wallberg, A.; Webster, M. T. From where did the Western honeybee (Apis mellifera) originate? Ecol Evol 2012, 2, (8), 1949-1957.

Harschnitz, O.; Studer, L. Human stem cell models to study host–virus interactions in the central nervous system. Nature Reviews Immunology 2021, doi:10.1038/s41577-020-00474-y.

Hartmann U, Forsgren E, Charrière J-D, Neumann P, Gauthier L: Dynamics of Apis mellifera filamentous virus (AmFV) infections in honey bees and relationships with other parasites. Viruses 2015, 7:2654–2667.

Hatten, T. D.; Looney, C.; Strange, J. P.; Bosque-Perez, N. A. Bumble bee fauna of Palouse Prairie: Survey of native bee pollinators in a fragmented ecosystem. Journal of Insect Science 2013, 13.

Healthy Bees, Healthy People, Healthy Planet. Tools for Varroa Management: A Guide to Effective Varroa Sampling and Control, 1st ed.; The Honey Bee Health Coalition: Keystone, United States. 2015; p. 8.

Highfield AC, Nagar A El, Mackinder LCM, Noël LMJ, Hall MJ, Martin SJ, Schroeder DC: Deformed wing virus implicated in overwintering honeybee colony losses. Appl. Environ. Microbiol. 2009, 75:7212–7220.

Higurashi, M.; Ishida, T.; Kinoshita, K. Identification of transient hub proteins and the possible structural basis for their multiple interactions. Protein Sci 2008, 17, 72-78, doi:10.1110/ps.073196308.

Hoffmann, J.A. The immune response of Drosophila. Nature 2003, 426, 33-38, doi:10.1038/nature02021.

Hou C, Li B, Deng S, Chu Y, Diao Q: Diagnosis and distribution of the Apis mellifera filamentous virus (AmFV) in honey bees (Apis mellifera) in China. Insectes Soc. 2017, 64:597–603.

Hou C, Rivkin H, Slabezki Y, Chejanovsky N: Dynamics of the presence of Israeli acute paralysis virus in honey bee colonies with colony collapse disorder. Viruses 2014, 6:2012–2027.

Hsieh, E.M.; Carrillo-Tripp, J.; Dolezal, A.G. Preparation of Virus-Enriched Inoculum for Oral Infection of Honey Bees (Apis Mellifera). J. Vis. Exp. 2020, 162, e61725, doi:10.3791/61725.

Hudu, S.A.; Alshrari, A.S.; Syahida, A.; Sekawi, Z. Cell Culture, Technology: Enhancing the Culture of Diagnosing Human Diseases. J. Clin. Diagn. Res. 2016, 10, DE01–DE05, doi:10.7860/JCDR/2016/15837.7460.

Huelsenbeck, J.P.; Ronquist, F. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 2001, 17, 754-755, doi:10.1093/bioinformatics/17.8.754.

Hunter, W.; Ellis, J.; vanEngelsdorp, D.; Hayes, J.; Westervelt, D.; Glick, E.; Williams, M.; Sela, I.; Maori, E.; Pettis, J.; Cox-Foster, D.; Paldi, N. Large-Scale Field Application of RNAi Technology Reducing Israeli Acute Paralysis Virus Disease in Honey Bees (Apis mellifera, Hymenoptera: Apidae). Plos Pathog 2010, 6, (12), e1001160.

Hunter, W.B. Medium for development of bee cell cultures (Apis mellifera: Hymenoptera: Apidae). In Vitro Cellular & Developmental Biology - Animal 2010, 46, 83-86, doi:10.1007/s11626-009-9246-x.

Ihle, K. E.; Fondrk, M. K.; Page, R. E.; Amdam, G. V. Genotype effect on lifespan following vitellogenin knockdown. Exp. Gerontol. 2015, 61, 113-122.

Imhoof, B.; Schmid-Hempel, P. Colony success of the bumble bee, Bombus terrestris, in relation to infections by two protozoan parasites, Crithidia bombi and Nosema bombi. Insect Soc 2014, 46, (3), 233-238.

Imler, J. L.; Hoffmann, J. A. Toll receptors in innate immunity. Trends Cell Biol. 11, (7), 304-311.

Imler, J.-L.; Bulet, P., Antimicrobial Peptides in Drosophila: Structures,Activities and Gene Regulation. In Mechanisms of Epithelial Defense, KARGER: Basel, 2005; Vol. 86, pp 1-21.

Iqbal, J.; Mueller, U. Virus infection causes specific learning deficits in honeybee foragers. Proc. Biol. Sci. 2007, 274, 1517–1521, doi:10.1098/rspb.2007.0022.

Ishida, T.; Kinoshita, K. PrDOS: prediction of disordered protein regions from amino acid sequence. Nucleic Acids Res 2007, 35, W460-464, doi:10.1093/nar/gkm363.

Iuchi, S. Three classes of C2H2 zinc finger proteins. Cellular and Molecular Life Sciences CMLS 2001, 58, 625-635, doi:10.1007/PL00000885.

Iwasaki, S.; Kobayashi, M.; Yoda, M.; Sakaguchi, Y.; Katsuma, S.; Suzuki, T.; Tomari, Y. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. Mol. Cell. 2010, 39, 292–299, doi:10.1016/j.molcel.2010.05.015.

Jackson, R.J.; Standart, N. How Do MicroRNAs Regulate Gene Expression? Science's STKE 2007, 2007, re1, doi:10.1126/stke.3672007re1.

Jarosch, A.; Moritz, R.F.A. RNA interference in honeybees: off-target effects caused by dsRNA. Apidologie 2012, 43, 128-138, doi:10.1007/s13592-011-0092-y.

Johnson RM, Evans JD, Robinson GE, Berenbaum MR: Changes in transcript abundance relating to colony collapse disorder in honey bees (Apis mellifera). Proc. Natl. Acad. Sci. 2009, 106:14790–14795.

Johnson, L.S.; Eddy, S.R.; Portugaly, E. Hidden Markov model speed heuristic and iterative HMM search procedure. BMC Bioinformatics 2010, 11, 431, doi:10.1186/1471-2105-11-431.

Johnson, R. M. Honey Bee Toxicology. Annu Rev Entomol 2015, 60, (1), 415-434.

Johnson, R. M.; Ellis, M. D.; Mullin, C. A.; Frazier, M. Pesticides and honey bee toxicity – USA. Apidologie 2010, 41, (3), 312-331.

Johnston, B.A.; Hooks, K.B.; McKinstry, M.; Snow, J.W. Divergent forms of endoplasmic reticulum stress trigger a robust unfolded protein response in honey bees. Journal of Insect Physiology 2016, 86, 1-10, doi:https://doi.org/10.1016/j.jinsphys.2015.12.004.

Kampmueller, K.M.; Miller, D.J. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells. Journal of virology 2005, 79, 6827-6837, doi:10.1128/JVI.79.11.6827-6837.2005.

Kapp, K.; Schrempf, S.; Lemberg, M.; Bernhard, D. Post-Targeting Functions of Signal Peptides. In Protein Transport into the Endoplasmic Reticulum; Landes Bioscience: Madame Curie Bioscience Database [Internet], 2009.

Katsuma, S.; Tanaka, S.; Omuro, N.; Takabuchi, L.; Daimon, T.; Imanishi, S.; Yamashita, S.; Iwanaga, M.; Mita, K.; Maeda, S.; et al. Novel Macula-Like Virus Identified in Bombyx mori Cultured Cells. J. Virol. 2005, 79, 5577–5584, doi:10.1128/JVI.79.9.5577-5584.2005.

Kawamura, Y.; Saito, K.; Kin, T.; Ono, Y.; Asai, K.; Sunohara, T.; Okada, T.N.; Siomi, M.C.; Siomi, H. Drosophila endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature 2008, 453, 793-797, doi:10.1038/nature06938.

Kemp, C.; Imler, J.-L. Antiviral immunity in drosophila. Current Opinion in Immunology 2009, 21, 3-9, doi:https://doi.org/10.1016/j.coi.2009.01.007.

Kemp, C.; Mueller, S.; Goto, A.; Barbier, V.; Paro, S.; Bonnay, F.; Dostert, C.; Troxler, L.; Hetru, C.; Meignin, C., et al. Broad RNA Interference–Mediated Antiviral Immunity and Virus-Specific Inducible Responses in Drosophila. The Journal of Immunology 2013, 190, 650, doi:10.4049/jimmunol.1102486.

Kevill, J.L.; Highfield, A.; Mordecai, G.J.; Martin, S.J.; Schroeder, D.C. ABC Assay: Method Development and Application to Quantify the Role of Three DWV Master Variants in Overwinter Colony Losses of European Honey Bees. Viruses 2017, 9, 314.

Kim, K.; Lee, Y.S.; Carthew, R.W. Conversion of pre-RISC to holo-RISC by Ago2 during assembly of RNAi complexes. RNA 2007, 13, 22-29, doi:10.1261/rna.283207.

Kim, K.; Lee, Y.S.; Harris, D.; Nakahara, K.; Carthew, R.W. The RNAi pathway initiated by Dicer-2 in Drosophila. Cold Spring Harbor symposia on quantitative biology 2006, 71, 39-44, doi:10.1101/sqb.2006.71.008.

Kim, P.M.; Sboner, A.; Xia, Y.; Gerstein, M. The role of disorder in interaction networks: a structural analysis. Mol Syst Biol 2008, 4, 179-179, doi:10.1038/msb.2008.16.

Kim, S. ppcor: An R Package for a Fast Calculation to Semi-partial Correlation Coefficients. Commun Stat Appl Methods 2015, 22, 665-674, doi:10.5351/CSAM.2015.22.6.665.

Kingsolver, M.B.; Hardy, R.W. Making connections in insect innate immunity. Proceedings of the National Academy of Sciences of the United States of America 2012, 109, 18639-18640, doi:10.1073/pnas.1216736109.

Kingsolver, M.B.; Huang, Z.; Hardy, R.W. Insect antiviral innate immunity: Pathways, effectors, and connections. Journal of Molecular Biology 2013, 425, 4921-4936, doi:10.1016/j.jmb.2013.10.006.

Klein, A.M.; Vaissiere, B.E.; Cane, J.H.; Steffan-Dewenter, I.; Cunningham, S.A.; Kremen, C.; Tscharntke, T. Importance of pollinators in changing landscapes for world crops. Proc Biol Sci 2007, 274, 303-313, doi:10.1098/rspb.2006.3721.

Koch, H.; Schmid-Hempel, P. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proc Natl Acad Sci USA 2011, 108, (48), 19288-19292.

Koleske, A.J.; Young, R.A. The RNA polymerase II holoenzyme and its implications for gene regulation. Trends Biochem Sci 1995, 20, 113-116, doi:10.1016/s0968-0004(00)88977-x.

Kovac, H.; Käfer, H.; Stabentheiner, A.; Costa, C. Metabolism and upper thermal limits of Apis mellifera carnica and A. m. ligustica. Apidologie 2014, 45, 664-677, doi:10.1007/s13592-014-0284-3.

Kovac, H.; Stabentheiner, A.; Schmaranzer, S. Thermoregulation of water foraging honeybees—Balancing of endothermic activity with radiative heat gain and functional requirements. Journal of Insect Physiology 2010, 56, 1834-1845, doi:https://doi.org/10.1016/j.jinsphys.2010.08.002.

Kulhanek, K.; Steinhauer, N.; Rennich, K.; Caron, D.M.; Sagili, R.R.; Pettis, J.S.; Ellis, J.D.; Wilson, M.E.; Wilkes, J.T.; Tarpy, D.R., et al. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. Journal of Apicultural Research 2017, 56, 328-340, doi:10.1080/00218839.2017.1344496.

Kumar S, Stecher G, Tamura K: MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 2016, 33:1870–1874.

Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 2018, 35, 1547-1549, doi:10.1093/molbev/msy096.

Kuster, R. D.; Boncristiani, H. F.; Rueppell, O. Immunogene and viral transcript dynamics during parasitic Varroa destructor mite infection of developing honey bee (Apis mellifera) pupae. J. Exp. Biol. 2014, 217, (10), 1710-1718.

Lah, Grace J.-e.; Li, Joshua Shing S.; Millard, S.S. Cell-Specific Alternative Splicing of Drosophila Dscam2 Is Crucial for Proper Neuronal Wiring. Neuron 2014, 83, 1376-1388, doi:https://doi.org/10.1016/j.neuron.2014.08.002.

Lamiable, O.; Arnold, J.; de Faria, I.; Olmo, R.P.; Bergami, F.; Meignin, C.; Hoffmann, J.A.; Marques, J.T.; Imler, J.L. Analysis of the Contribution of Hemocytes and Autophagy to Drosophila Antiviral Immunity. J. Virol. 2016, 90, 5415–5426, doi:10.1128/jvi.00238-16.

Lamiable, O.; Imler, J.-L. Induced antiviral innate immunity in Drosophila. Current Opinion in Microbiology 2014, 20, 62-68.

Lamp, B.; Url, A.; Seitz, K.; Eichhorn, J.; Riedel, C.; Sinn, L.J.; Indik, S.; Koglberger, H.; Rumenapf, T. Construction and Rescue of a Molecular Clone of Deformed Wing Virus (DWV). PLoS One 2016, 11, e0164639, doi:10.1371/journal.pone.0164639.

Langfelder, P.; Horvath, S. Fast R Functions for Robust Correlations and Hierarchical Clustering. 2012 2012, 46, 17, doi:10.18637/jss.v046.i11.

Langfelder, P.; Horvath, S. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 2008, 9, 559, doi:10.1186/1471-2105-9-559.

Lanot, R.; Zachary, D.; Holder, F.; Meister, M. Postembryonic Hematopoiesis in Drosophila. Dev. Biol. 2001, 230, 243–257, doi:10.1006/dbio.2000.0123.

Lanzi, G.; de Miranda, J.R.; Boniotti, M.B.; Cameron, C.E.; Lavazza, A.; Capucci, L.; Camazine, S.M.; Rossi, C. Molecular and biological characterization of deformed wing virus of honeybees (Apis mellifera L.). J. Virol. 2006, 80, 4998–5009, doi:10.1128/JVI.80.10.4998-5009.2006.

Lauring AS, Andino R: Quasispecies theory and the behavior of RNA viruses. PLoS Pathog. 2010, 6:e1001005.

Lavine, M.D.; Strand, M.R. Insect hemocytes and their role in immunity. Insect Biochemistry and Molecular Biology 2002, 32, 1295-1309, doi:https://doi.org/10.1016/S0965-1748(02)00092-9.

Leat, N.; Ball, B.; Govan, V.; Davison, S. Analysis of the complete genome sequence of black queen-cell virus, a picorna-like virus of honey bees. J. Gen. Virol. 2000, 81, 2111–2119, doi:10.1099/0022-1317-81-8-2111.

Lee, K.; Steinhauer, N.; Travis, D. A.; Meixner, M. D.; Deen, J.; vanEngelsdorp, D. Honey bee surveillance: a tool for understanding and improving honey bee health. Current Opinion in Insect Science 2015, 10, 37-44.

Lee, K.V.; Steinhauer, N.; Rennich, K.; Wilson, M.E.; Tarpy, D.R.; Caron, D.M.; Rose, R.; Delaplane, K.S.; Baylis, K.; Lengerich, E.J., et al. A national survey of managed honey bee 2013-2014 annual colony losses in the USA. Apidologie 2015, 46, 292-305, doi:10.1007/s13592-015-0356-z.

Lemaitre, B.; Hoffmann, J. The Host Defense of Drosophila melanogaster. Annu. Rev. Immunol. 2007, 25, (1), 697-743.

Leonard, S.P.; Powell, J.E.; Perutka, J.; Geng, P.; Heckmann, L.C.; Horak, R.D.; Davies, B.W.; Ellington, A.D.; Barrick, J.E.; Moran, N.A. Engineered symbionts activate honey bee immunity and limit pathogens. Science 2020, 367, 573, doi:10.1126/science.aax9039.

Levin, S.; Galbraith, D.; Sela, N.; Erez, T.; Grozinger, C.; Chejanovsky, N. Identification of a novel Rhabdovirus in populations of pollinators and their parasites from two continents (in preparation). 2017.

Levin, S.; Galbraith, D.; Sela, N.; Erez, T.; Grozinger, C.M.; Chejanovsky, N. Presence of Apis Rhabdovirus-1 in Populations of Pollinators and Their Parasites from Two Continents. Frontiers in Microbiology 2017, 8, doi:10.3389/fmicb.2017.02482.

Levin, S.; Sela, N.; Chejanovsky, N. Two novel viruses associated with the Apis mellifera pathogenic mite Varroa destructor. Sci. Rep. 2016, 6, 37710, doi:10.1038/srep37710.

Levitt, A. L.; Singh, R.; Cox-Foster, D. L.; Rajotte, E.; Hoover, K.; Ostiguy, N.; Holmes, E. C. Cross-species transmission of honey bee viruses in associated arthropods. Virus Res 2013, 176, (1-2), 232-240.

Li J, Peng W, Wu J, Strange JP, Boncristiani H, Chen Y: Cross-species infection of Deformed wing virus poses a new threat to pollinator conservation. J. Econ. Entomol. 2011, 104:732–739.

Li JL, Scott Cornman R, Evans JD, Pettis JS, Zhao Y, Murphy C, Peng WJ, Wu J, Hamilton M, Boncristiani HF, et al.: Systemic spread and propagation of a plant-pathogenic virus in European honeybees, Apis mellifera. MBio 2014, 5:e00898-13.

Li, H.; Li, W.X.; Ding, S.W. Induction and Suppression of RNA Silencing by an Animal Virus. Science 2002, 296, 1319, doi:10.1126/science.1070948.

Li, T.-C.; Scotti Paul, D.; Miyamura, T.; Takeda, N. Latent Infection of a New Alphanodavirus in an Insect Cell Line. J. Virol. 2007, 81, 10890–10896, doi:10.1128/JVI.00807-07.

Lin, Y.S.; Hsu, W.L.; Hwang, J.K.; Li, W.H. Proportion of solvent-exposed amino acids in a protein and rate of protein evolution. Mol Biol Evol 2007, 24, 1005-1011, doi:10.1093/molbev/msm019.

Lindquist, S. Regulation of protein synthesis during heat shock. Nature 1981, 293, 311-314, doi:10.1038/293311a0.

Lingel, A.; Simon, B.; Izaurralde, E.; Sattler, M. The structure of the flock house virus B2 protein, a viral suppressor of RNA interference, shows a novel mode of double-stranded RNA recognition. EMBO Rep. 2005, 6, 1149–1155, doi:10.1038/sj.embor.7400583.

Liu, X.; Zhang, Y.; Yan, X.; Han, R. Prevention of chinese sacbrood virus infection in apis cerana using rna interference. Current Microbiology 2010, 61, 422-428, doi:10.1007/s00284-010-9633-2.

Liu, Y.; Ye, X.; Jiang, F.; Liang, C.; Chen, D.; Peng, J.; Kinch, L. N.; Grishin, N. V.; Liu, Q. C3PO, an Endoribonuclease That Promotes RNAi by Facilitating RISC Activation. Science 2009, 325, (5941), 750-753.

Longdon B, Murray GGR, Palmer WJ, Day JP, Parker DJ, Welch JJ, Obbard DJ, Jiggins FM: The evolution, diversity, and host associations of rhabdoviruses. Virus Evol. 2015, 1:vev014.

Lourenço, A.P.; Florecki, M.M.; Simões, Z.L.P.; Evans, J.D. Silencing of Apis mellifera dorsal genes reveals their role in expression of the antimicrobial peptide defensin-1. Insect Molecular Biology 2018, 27, 577-589, doi:https://doi.org/10.1111/imb.12498.

Lourenco, A. P.; Florecki, M. M.; Simões, Z. L. P.; Evans, J. D. Silencing of Apis mellifera dorsal genes reveals their role in expression of the antimicrobial peptide defensin-1. Insect Mol Biol 2018.

Lozano, J.; Gomez-Orte, E.; Lee, H.-J.; Belles, X. Super-induction of Dicer-2 expression by alien double-stranded RNAs: an evolutionary ancient response to viral infection? Development Genes and Evolution 2012, 222, 229-235, doi:10.1007/s00427-012-0404-x.

Lu, R.; Maduro, M.; Li, F.; Li, H. W.; Broitman-Maduro, G.; Li, W. X.; Ding, S. W. Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. Nature 2005, 436, (7053), 1040-1043.

Lucia M, Reynaldi FJ, Sguazza GH, Abrahamovich AH: First detection of deformed wing virus in Xylocopa augusti larvae (Hymenoptera: Apidae) in Argentina. J. Apic. Res. 2014, 53:466–468.

Lundgren, J. G.; Duan, J. J. RNAi-Based Insecticidal Crops: Potential Effects on Nontarget Species. Bioscience 2013, 63, (8), 657-665.

Müller, W.E.G.; Müller, I.M. Origin of the metazoan immune system: identification of the molecules and their functions in sponges. Integrative and comparative biology 2003, 43, 281-292, doi:10.1093/icb/43.2.281.

Ma, H.; Galvin, T.A.; Glasner, D.R.; Shaheduzzaman, S.; Khan, A.S. Identification of a novel rhabdovirus in Spodoptera frugiperda cell lines. J. Virol. 2014, 88, 6576–6585, doi:10.1128/jvi.00780-14.

Mahat, Dig B.; Salamanca, H.H.; Duarte, Fabiana M.; Danko, Charles G.; Lis, John T. Mammalian Heat Shock Response and Mechanisms Underlying Its Genome-wide Transcriptional Regulation. Molecular Cell 2016, 62, 63-78, doi:https://doi.org/10.1016/j.molcel.2016.02.025.

Malfroy SF, Roberts JMK, Perrone S, Maynard G, Chapman N: A pest and disease survey of the isolated Norfolk Island honey bee (Apis mellifera) population. J. Apic. Res. 2016, 55:202–211.

Manley R, Boots M, Wilfert L: Condition-dependent virulence of Slow bee paralysis virus in Bombus terrestris: are the impacts of honeybee viruses in wild pollinators underestimated ? Oecologia 2017, 184:305–315.

Manley R, Boots M, Wilfert L: Emerging viral disease risk to pollinating insects: Ecological, evolutionary and anthropogenic factors. J. Appl. Ecol. 2015, 52:331–340.

Maori, E.; Lavi, S.; Mozes-Koch, R.; Gantman, Y.; Peretz, Y.; Edelbaum, O.; Tanne, E.; Sela, I. Isolation and characterization of Israeli acute paralysis virus, a dicistrovirus affecting honeybees in Israel: Evidence for diversity due to intra- and inter-species recombination. J. Gen. Virol. 2007, 88, 3428–3438, doi:10.1099/vir.0.83284-0.

Maori, E.; Paldi, N.; Shafir, S.; Kalev, H.; Tsur, E.; Glick, E.; Sela, I. IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. Insect Mol Biol 2009, 18, (1), 55-60.

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

Maringer, K.; Yousuf, A.; Heesom, K.J.; Fan, J.; Lee, D.; Fernandez-Sesma, A.; Bessant, C.; Matthews, D.A.; Davidson, A.D. Proteomics informed by transcriptomics for characterising active transposable elements and genome annotation in Aedes aegypti. BMC Genom. 2017, 18, 101, doi:10.1186/s12864-016-3432-5.

Marringa, W.J.; Krueger, M.J.; Burritt, N.L.; Burritt, J.B. Honey Bee Hemocyte Profiling by Flow Cytometry. PLoS ONE 2014, 9, e108486, doi:10.1371/journal.pone.0108486.

Marshall, D.; Schneemann, A. Specific packaging of nodaviral RNA2 requires the N-terminus of the capsid protein. Virology 2001, 285, 165–175, doi:10.1006/viro.2001.0951.

Marston HD, Folkers GK, Morens DM, Fauci AS: Emerging viral diseases: Confronting threats with new technologies. Sci. Transl. Med. 2014, 6:253ps10.

Martin, S. J.; Highfield, A. C.; Brettell, L.; Villalobos, E. M.; Budge, G. E.; Powell, M.; Nikaido, S.; Schroeder, D. C. Global Honey Bee Viral Landscape Altered by a Parasitic Mite. Science 2012, 336, (6086), 1304-1306.

Martins-da-Silva, A.; Telleria, E.; Batista, M.; Marchini, F.; Traub-Csekö, Y.; Tempone, A. Identification of Secreted Proteins Involved in Nonspecific dsRNA-Mediated Lutzomyia longipalpis LL5 Cell Antiviral Response. Viruses 2018, 10, (1), 43.

Mattick, J.S. Non-coding RNAs: the architects of eukaryotic complexity. EMBO reports 2001, 2, 986-991, doi:10.1093/embo-reports/kve230.

Mazzei, M.; Carrozza, M.L.; Luisi, E.; Forzan, M.; Giusti, M.; Sagona, S.; Tolari, F.; Felicioli, A. Infectivity of DWV associated to flower pollen: experimental evidence of a horizontal transmission route. PLoS One 2014, 9, e113448, doi:10.1371/journal.pone.0113448.

McKinstry, M.; Chung, C.; Truong, H.; Johnston, B.A.; Snow, J.W. The heat shock response and humoral immune response are mutually antagonistic in honey bees. Scientific Reports 2017, 7, 8850, doi:10.1038/s41598-017-09159-4.

McMahon, D. P.; Fürst, M. A.; Caspar, J.; Theodorou, P.; Brown, M. J. F.; Paxton, R. J. A sting in the spit: widespread cross-infection of multiple RNA viruses across wild and managed bees. J Anim Ecol 2015, 84, (3), 615-624.

McMahon, D. P.; Natsopoulou, M. E.; Doublet, V.; Fürst, M.; Weging, S.; Brown, M. J. F.; Gogol-Döring, A.; Paxton, R. J. Elevated virulence of an emerging viral genotype as a driver of honeybee loss. P R Soc B 2016, 283, (1833), 20160811.

McMenamin, A.J.; Brutscher, L.M.; Daughenbaugh, K.F.; Flenniken, M.L. The honey bee gene, bee antiviral protein-1, formerly MF116383, is a taxonomically restricted antiviral immune gene. Front. Insect Sci. 2021, in prep.

McMenamin, A.J.; Brutscher, L.M.; Glenny, W.; Flenniken, M.L. Abiotic and biotic factors affecting the replication and pathogenicity of bee viruses. Current Opinion in Insect Science 2016, 16, 14-21, doi:10.1016/j.cois.2016.04.009.

McMenamin, A.J.; Daughenbaugh, K.F.; Flenniken, M.L. The Heat Shock Response in the Western Honey Bee (Apis mellifera) is Antiviral. Viruses 2020, 12, 245, doi:10.3390/v12020245.

McMenamin, A.J.; Daughenbaugh, K.F.; Parekh, F.; Pizzorno, M.C.; Flenniken, M.L. Honey Bee and Bumble Bee Antiviral Defense. Viruses 2018, 10, 395, doi:10.3390/v10080395.

McMenamin, A.J.; Flenniken, M.L. Recently identified bee viruses and their impact on bee pollinators. Current Opinion in Insect Science 2018, 26, 120-129, doi:https://doi.org/10.1016/j.cois.2018.02.009.

McMenamin, A.J.; Genersch, E. Honey bee colony losses and associated viruses. Current Opinion in Insect Science 2015, 8, 121-129, doi:10.1016/j.cois.2015.01.015.

Meeus, I.; Brown, M. J. F.; de Graaf, D. C.; Smagghe, G. Effects of Invasive Parasites on Bumble Bee Declines. Conservation Biology 2011, 25, (4), 662-671.

Meeus, I.; de Miranda, J. R.; de Graaf, D. C.; Wäckers, F.; Smagghe, G. Effect of oral infection with Kashmir bee virus and Israeli acute paralysis virus on bumblebee (Bombus terrestris) reproductive success. J Invertebr Pathol 2014, 121, 64-69.

Meeus, I.; Pisman, M.; Smagghe, G.; Piot, N. Interaction effects of different drivers of wild bee decline and their influence on host-pathogen dynamics. Current Opinion in Insect Science 2018, 26, 136-141.

Memmott, J.; Craze, P. G.; Waser, N. M.; Price, M. V. Global warming and the disruption of plant-pollinator interactions. Ecol Lett 2007, 10, (8), 710-7.

Meng, X.; Khanuja, B.S.; Ip, Y.T. Toll receptor-mediated Drosophila immune response requires Dif, an NF-kappaB factor. Genes Dev 1999, 13, 792-797, doi:10.1101/gad.13.7.792.

Merkling, S. H.; van Rij, R. P. Analysis of resistance and tolerance to virus infection in Drosophila. Nat Protoc 2015, 10, (7), 1084-1097.

Merkling, S. H.; van Rij, R. P. Beyond RNAi: Antiviral defense strategies in Drosophila and mosquito. J Insect Physiol 2013, 59, (2), 159-170.

Merkling, S.H.; Overheul, G.J.; Mierlo, J.T.V.; Arends, D.; Gilissen, C.; Rij, R.P.V. The heat shock response restricts virus infection in Drosophila. Nature Scientific Reports 2015, 5, 1-15, doi:10.1038/srep12758.

Meyer, D.I. Protein translocation into the endoplasmic reticulum: a light at the end of the tunnel. Trends in Cell Biology 1991, 1, 154-159, doi:https://doi.org/10.1016/0962-8924(91)90016-3.

Michener, C. D. The Bees of the World. 2nd ed.; John Hopkins University Press: Baltimore and London, 2007.

Milbrath, M. Varroa Mite Monitoring: Using a Sugar Roll to Identify Populations of Varroa Destructor in Honey Bee Colonies. Available online: https://pollinators.msu.edu/resources/beekeepers/varroa-mite-monitoring1/ (accessed on 27 June 2020).

Millard, S.S.; Flanagan, J.J.; Pappu, K.S.; Wu, W.; Zipursky, S.L. Dscam2 mediates axonal tiling in the Drosophila visual system. Nature 2007, 447, 720-724, doi:10.1038/nature05855.

Miller, D.J.; Ahlquist, P. Flock house virus RNA polymerase is a transmembrane protein with amino-terminal sequences sufficient for mitochondrial localization and membrane insertion. J. Virol. 2002, 76, 9856–9867, doi:10.1128/jvi.76.19.9856-9867.2002.

Miller, D.J.; Schwartz, M.D.; Ahlquist, P. Flock house virus RNA replicates on outer mitochondrial membranes in Drosophila cells. J. Virol. 2001, 75, 11664–11676, doi:10.1128/JVI.75.23.11664-11676.2001.

Misof, B.; Liu, S.; Meusemann, K.; Peters, R.S.; Donath, A.; Mayer, C.; Frandsen, P.B.; Ware, J.; Flouri, T.; Beutel, R.G.; et al. Phylogenomics resolves the timing and pattern of insect evolution. Science 2014, 346, 763, doi:10.1126/science.1257570.

Miyoshi, T.; Takeuchi, A.; Siomi, H.; Siomi, M. C. A direct role for Hsp90 in pre-RISC formation in Drosophila. Nat Struct Mol Biol 2010, 17, (8), 1024-1026.

Moeckel, N.; Gisder, S.; Genersch, E. Horizontal transmission of deformed wing virus: Pathological consequences in adult bees (Apis mellifera) depend on the transmission route. J. Gen. Virol. 2011, 92, 370–377, doi:10.1099/vir.0.025940-0.

Mondet F, de Miranda JR, Kretzschmar A, Le Conte Y, Mercer AR: On the front line: Quantitative virus dynamics in honeybee (Apis mellifera L.) colonies along a new expansion front of the parasite Varroa destructor. PLoS Pathog. 2014, 10:e1004323.

Montagnon, B.J.; Fanget, B.; Vincent-Falquet, J.C. Industrial-scale production of inactivated poliovirus vaccine prepared by culture of Vero cells on microcarrier. Rev. Infect. Dis. 1984, 6 (Suppl. 2), S341–S344, doi:10.1093/clinids/6.supplement_2.s341.

Moore, J.; Jironkin, A.; Chandler, D.; Burroughs, N.; Evans, D.J.; Ryabov, E.V. Recombinants between Deformed wing virus and Varroa destructor virus-1 may prevail in Varroa destructor-infested honeybee colonies. J. Gen. Virol. 2011, 92, 156–161, doi:10.1099/vir.0.025965-0.

Mordecai GJ, Brettell LE, Martin SJ, Dixon D, Jones IM, Schroeder DC: Superinfection exclusion and the long-term survival of honey bees in Varroa-infested colonies. ISME J. 2016, 10:1182–1191.

Mordecai GJ, Brettell LE, Pachori P, Villalobos EM, Martin SJ, Jones IM, Schroeder DC: Moku virus; a new Iflavirus found in wasps, honey bees and Varroa. Sci. Rep. 2016, 6:34983.

Mordecai, G.J.; Wilfert, L.; Martin, S.J.; Jones, I.M.; Schroeder, D.C. Diversity in a honey bee pathogen: First report of a third master variant of the Deformed Wing Virus quasispecies. ISME J. 2016, 10, 1264–1273, doi:10.1038/ismej.2015.178.

Munoz-Torres, M. C.; Reese, J. T.; Childers, C. P.; Bennett, A. K.; Sundaram, J. P.; Childs, K. L.; Anzola, J. M.; Milshina, N.; Elsik, C. G. Hymenoptera Genome Database: integrated community resources for insect species of the order Hymenoptera. Nucleic Acids Res 2010, 39, (Database), D658-D662.

Mussen, E.C.; Furgala, B. Replication of sacbrood virus in larval and adult honeybees, Apis mellifera. J. Invertebr. Pathol. 1977, 30, 20–34, doi:10.1016/0022-2011(77)90033-7.

Myles, K.M.; Wiley, M.R.; Morazzani, E.M.; Adelman, Z.N. Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. Proceedings of the National Academy of Sciences 2008, 105, 19938, doi:10.1073/pnas.0803408105.

Natsopoulou ME, McMahon DP, Doublet V, Frey E, Rosenkranz P, Paxton RJ: The virulent, emerging genotype B of Deformed wing virus is closely linked to overwinter honeybee worker loss. Sci. Rep. 2017, 7:5242.

Nayak, A.; Berry, B.; Tassetto, M.; Kunitomi, M.; Acevedo, A.; Deng, C.; Krutchinsky, A.; Gross, J.; Antoniewski, C.; Andino, R. Cricket paralysis virus antagonizes Argonaute 2 to modulate antiviral defense in Drosophila. Nat. Struct. Mol. Biol. 2010, 17, 547–554, doi:10.1038/nsmb.1810.

Nayak, A.; Kim, D.Y.; Trnka, M.J.; Kerr, C.H.; Lidsky, P.V.; Stanley, D.J.; Rivera, B.M.; Li, K.H.; Burlingame, A.L.; Jan, E.; et al. A Viral Protein Restricts Drosophila RNAi Immunity by Regulating Argonaute Activity and Stability. Cell Host Microbe 2018, 24, 542–557.e549, doi:10.1016/j.chom.2018.09.006.

Nazzi, F.; Brown, S.P.; Annoscia, D.; Del Piccolo, F.; Di Prisco, G.; Varricchio, P.; Vedova, G.D.; Cattonaro, F.; Caprio, E.; Pennacchio, F. Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. PLoS Pathog. 2012, 8, e1002735, doi:10.1371/journal.ppat.1002735.

Nazzi, F.; Le Conte, Y. Ecology of Varroa destructor, the Major Ectoparasite of the Western Honey Bee, Apis mellifera. Annual Review of Entomology 2016, 61, 417-432, doi:10.1146/annurev-ento-010715-023731.

Nazzi, F.; Pennacchio, F. Disentangling multiple interactions in the hive ecosystem. Trends Parasitol. 2014, 30, 556–561, doi:10.1016/j.pt.2014.09.006.

Nazzi, F.; Pennacchio, F. Honey Bee Antiviral Immune Barriers as Affected by Multiple Stress Factors: A Novel Paradigm to Interpret Colony Health Decline and Collapse. Viruses 2018, 10, (4).

NCBI Research Coordinators: Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 2016, 44:D7–D19.

Nei M, Kumar S: Molecular Evolution and Phylogenetics. Oxford University Press; 2000.

Nelson, C. M.; Ihle, K. E.; Fondrk, M. K.; Page, R. E.; Amdam, G. V. The Gene vitellogenin Has Multiple Coordinating Effects on Social Organization. Plos Biol 2007, 5, (3), e62-677.

Nguyen, B. K.; Mignon, J.; Laget, D.; de Graaf, D. C.; Jacobs, F. J.; vanEngelsdorp, D.; Brostaux, Y.; Saegerman, C.; Haubruge, E. Honey bee colony losses in Belgium during the 2008–9 winter. J Apicult Res 2015, 49, (4), 337-339.

Nguyen, B. K.; Ribière, M.; vanEngelsdorp, D.; Snoeck, C.; Saegerman, C.; Kalkstein, A. L.; Schurr, F.; Brostaux, Y.; Faucon, J.-P.; Haubruge, E. Effects of honey bee virus prevalence, Varroa destructor load and queen condition on honey bee colony survival over the winter in Belgium. J Apicult Res 2015, 50, (3), 195-202.

Nilsen, K. A.; Ihle, K. E.; Frederick, K.; Fondrk, M. K.; Smedal, B.; Hartfelder, K.; Amdam, G. V. Insulin-like peptide genes in honey bee fat body respond differently to manipulation of social behavioral physiology. J. Exp. Biol. 2011, 214, (9), 1488-1497.

Niu, J.; Cappelle, K.; de Miranda, J. R.; Smagghe, G.; Meeus, I. Analysis of reference gene stability after Israeli acute paralysis virus infection in bumblebees Bombus terrestris. J Invertebr Pathol 2014, 115, 76-79.

Niu, J.; Meeus, I.; Cappelle, K.; Piot, N.; Smagghe, G. The immune response of the small interfering RNA pathway in the defense against bee viruses. Current Opinion in Insect Science 2014, 6, 22-27, doi:10.1016/j.cois.2014.09.014.

Niu, J.; Meeus, I.; Coninck, D.I.M.D.; Deforce, D.; Etebari, K.; Asgari, S.; Smagghe, G. Infections of virulent and avirulent viruses differentially influenced the microRNAs in Bombus terrestris. Nature Publishing Group 2017, 1-11, doi:10.1038/srep45620.

Niu, J.; Meeus, I.; Smagghe, G. Differential expression pattern of Vago in bumblebee (Bombus terrestris), induced by virulent and avirulent virus infections. Scientific Reports 2016, 6, 34200, doi:10.1038/srep34200.

Niu, J.; Smagghe, G.; De Coninck, D.I.; Van Nieuwerburgh, F.; Deforce, D.; Meeus, I. In vivo study of Dicer-2-mediated immune response of the small interfering RNA pathway upon

systemic infections of virulent and avirulent viruses in Bombus terrestris. Insect Biochem Mol Biol 2016, 70, 127-137, doi:10.1016/j.ibmb.2015.12.006.

Nothwehr, S.F.; Gordon, J.I. Targeting of proteins into the eukaryotic secretory pathway: signal peptide structure/function relationships. Bioessays 1990, 12, 479-484, doi:10.1002/bies.950121005.

Nunes, F.; Aleixo, A.; Barchuk, A.; Bomtorin, A.; Grozinger, C.; Simões, Z. Non-Target Effects of Green Fluorescent Protein (GFP)-Derived Double-Stranded RNA (dsRNA-GFP) Used in Honey Bee RNA Interference (RNAi) Assays. Insects 2012, Vol. 3, Pages 601-615 2013, 4, (1), 90-103.

O'Neal, S. T.; Brewster, C. C.; Bloomquist, J. R.; Anderson, T. D. Amitraz and its metabolite modulate honey bee cardiac function and tolerance to viral infection. J Invertebr Pathol 2017, 149, 119-126.

O'Neal, S. T.; Swale, D. R.; Anderson, T. D. ATP-sensitive inwardly rectifying potassium channel regulation of viral infections in honey bees. Sci Rep 2017, 7, (1), 614.

O'Neal, S.T.; Brewster, C.C.; Bloomquist, J.R.; Anderson, T.D. Amitraz and its metabolite modulate honey bee cardiac function and tolerance to viral infection. J. Invertebr. Pathol. 2017, 149, 119–126, doi:10.1016/j.jip.2017.08.005.

O'Neal, S.T.; Swale, D.R.; Anderson, T.D. ATP-sensitive inwardly rectifying potassium channel regulation of viral infections in honey bees. Sci. Rep. 2017, 7, 8668–8668, doi:10.1038/s41598-017-09448-y.

Obbard, D.J.; Jiggins, F.M.; Halligan, D.L.; Little, T.J. Natural Selection Drives Extremely Rapid Evolution in Antiviral RNAi Genes. Current Biology 2006, 16, 580-585, doi:https://doi.org/10.1016/j.cub.2006.01.065.

Olivier, V.; Blanchard, P.; Chaouch, S.; Lallemand, P.; Schurr, F.; Celle, O.; Dubois, E.; Tordo, N.; Thiéry, R.; Houlgatte, R., et al. Molecular characterisation and phylogenetic analysis of Chronic bee paralysis virus, a honey bee virus. Virus Research 2008, 132, 59-68, doi:https://doi.org/10.1016/j.virusres.2007.10.014.

Ongus, J.R.; Peters, D.; Bonmatin, J.M.; Bengsch, E.; Vlak, J.M.; van Oers, M.M. Complete sequence of a picorna-like virus of the genus Iflavirus replicating in the mite Varroa destructor. J. Gen. Virol. 2004, 85, 3747–3755, doi:10.1099/vir.0.80470-0.

Organtini, L. J.; Shingler, K. L.; Ashley, R. E.; Capaldi, E. A.; Durrani, K.; Dryden, K. A.; Makhov, A. M.; Conway, J. F.; Pizzorno, M. C.; Hafenstein, S. Honey Bee Deformed Wing Virus Structures Reveal that Conformational Changes Accompany Genome Release. J Virol 2017, 91, (2).

Owen, R. E.; Otterstatter, M. C.; Carter, R. V.; Farmer, A.; Colla, S. R.; O'Toole, N. Significant expansion of the distribution of the bumble bee Bombus moderatus (Hymenoptera:

Apidae) in Alberta over 20 years. Canadian Journal of Zoology-Revue Canadienne De Zoologie 2012, 90, (1), 133-138.

Palmer, W. H.; Varghese, F. S.; van Rij, R. P. Natural Variation in Resistance to Virus Infection in Dipteran Insects. Viruses 2018, 10, (3), 118.

Palmer, W.H.; Hadfield, J.D.; Obbard, D.J. RNA-Interference Pathways Display High Rates of Adaptive Protein Evolution in Multiple Invertebrates. Genetics 2018, 208, 1585-1599, doi:10.1534/genetics.117.300567.

Paradkar, P. N.; Duchemin, J. B.; Voysey, R.; Walker, P. J. Dicer-2-dependent activation of Culex Vago occurs via the TRAF-Rel2 signaling pathway. PLoS Negl Trop Dis 2014, 8, (4), e2823.

Paradkar, P. N.; Trinidad, L.; Voysey, R.; Duchemin, J. B.; Walker, P. J. Secreted Vago restricts West Nile virus infection in Culex mosquito cells by activating the Jak-STAT pathway. Proc Natl Acad Sci USA 2012, 109, (46), 18915-18920.

Paradkar, P.N.; Duchemin, J.-B.; Voysey, R.; Walker, P.J. Dicer-2-Dependent Activation of Culex Vago Occurs via the TRAF-Rel2 Signaling Pathway. PLOS Neglected Tropical Diseases 2014, 8, e2823, doi:10.1371/journal.pntd.0002823.

Paradkar, P.N.; Trinidad, L.; Voysey, R.; Duchemin, J.B.; Walker, P.J. Secreted Vago restricts West Nile virus infection in Culex mosquito cells by activating the Jak-STAT pathway. Proc Natl Acad Sci U S A 2012, 109, 18915-18920, doi:10.1073/pnas.1205231109.

Parmentier, L.; Smagghe, G.; de Graaf, D. C.; Meeus, I. Varroa destructor Macula-like virus, Lake Sinai virus and other new RNA viruses in wild bumblebee hosts (Bombus pascuorum, Bombus lapidarius, and Bombus pratorum). J Invertebr Pathol 2016, 134, (134), 6-11.

Patel, A.; Fondrk, M. K.; Kaftanoglu, O.; Emore, C.; Hunt, G.; Frederick, K.; Amdam, G. V. The Making of a Queen: TOR Pathway Is a Key Player in Diphenic Caste Development. PLoS ONE 2007, 2, (6), e509.

Paterson, S.; Vogwill, T.; Buckling, A.; Benmayor, R.; Spiers, A.J.; Thomson, N.R.; Quail, M.; Smith, F.; Walker, D.; Libberton, B.; et al. Antagonistic coevolution accelerates molecular evolution. Nature 2010, 464, 275-278, doi:10.1038/nature08798.

Pellmyr, O. Evolution of insect pollination and angiosperm diversification. Trends in Ecology & Evolution 1992, 7, 46-49, doi:https://doi.org/10.1016/0169-5347(92)90105-K.

Peng W, Li J, Boncristiani H, Strange JP, Hamilton M, Chen Y: Host range expansion of honey bee Black Queen Cell Virus in the bumble bee, Bombus huntii. Apidologie 2011, 42:650–658.

Peters, R.S.; Krogmann, L.; Mayer, C.; Donath, A.; Gunkel, S.; Meusemann, K.; Kozlov, A.; Podsiadlowski, L.; Petersen, M.; Lanfear, R.; et al. Evolutionary History of the

Hymenoptera. Current Biology 2017, 27, 1013-1018, doi:https://doi.org/10.1016/j.cub.2017.01.027.

Pettis, J. S.; Rice, N.; Joselow, K.; vanEngelsdorp, D.; Chaimanee, V. Colony Failure Linked to Low Sperm Viability in Honey Bee (Apis mellifera) Queens and an Exploration of Potential Causative Factors. PLoS ONE 2016, 11, (2), e0147220.

Pichlmair, A.; Schulz, O.; Tan, C. P.; Rehwinkel, J.; Kato, H.; Takeuchi, O.; Akira, S.; Way, M.; Schiavo, G.; Reis e Sousa, C. Activation of MDA5 Requires Higher-Order RNA Structures Generated during Virus Infection. J Virol 2009, 83, (20), 10761-10769.

Pincus, D.; Chevalier, M.W.; Aragón, T.; van Anken, E.; Vidal, S.E.; El-Samad, H.; Walter, P. BiP Binding to the ER-Stress Sensor Ire1 Tunes the Homeostatic Behavior of the Unfolded Protein Response. PLOS Biology 2010, 8, e1000415, doi:10.1371/journal.pbio.1000415.

Piot N, Snoeck S, Vanlede M, Smagghe G, Meeus I: The effect of oral administration of dsRNA on viral replication and mortality in Bombus terrestris. Viruses 2015, 7:3172–3185.

Piot, N.; Snoeck, S.; Vanlede, M.; Smagghe, G.; Meeus, I. The effect of oral administration of dsRNA on viral replication and mortality in Bombus terrestris. Viruses 2015, 7, 3172-3185, doi:10.3390/v7062765.

Pitaluga, A.N.; Mason, P.W.; Traub-Cseko, Y.M. Non-specific antiviral response detected in RNA-treated cultured cells of the sandfly, Lutzomyia longipalpis. Developmental & Comparative Immunology 2008, 32, 191-197, doi:https://doi.org/10.1016/j.dci.2007.06.008.

Pizzorno, M.C.; Field, K.; Kobokovich, A.L.; Martin, P.L.; Gupta, R.A.; Mammone, R.; Rovnyak, D.; Capaldi, E.A. Transcriptomic Responses of the Honey Bee Brain to Infection with Deformed Wing Virus. Viruses 2021, 13, 287, doi:10.3390/v13020287.

Plaskon NE, Adelman ZN, Myles KM: Accurate strand-specific quantification of viral RNA. PLoS One 2009, 4:e7468.

Poirier, E. Z.; Goic, B.; Tome-Poderti, L.; Frangeul, L.; Boussier, J.; Gausson, V.; Blanc, H.; Vallet, T.; Loyd, H.; Levi, L. I.; Lanciano, S.; Baron, C.; Merkling, S. H.; Lambrechts, L.; Mirouze, M.; Carpenter, S.; Vignuzzi, M.; Saleh, M. C. Dicer-2-Dependent Generation of Viral DNA from Defective Genomes of RNA Viruses Modulates Antiviral Immunity in Insects. Cell Host Microbe 2018, 23, (3), 353-365 e8.

Porrini, C.; Mutinelli, F.; Bortolotti, L.; Granato, A.; Laurenson, L.; Roberts, K.; Gallina, A.; Silvester, N.; Medrzycki, P.; Renzi, T., et al. The Status of Honey Bee Health in Italy: Results from the Nationwide Bee Monitoring Network. PLOS ONE 2016, 11, e0155411, doi:10.1371/journal.pone.0155411.

Posada-Florez, F.; Childers, A.K.; Heerman, M.C.; Egekwu, N.I.; Cook, S.C.; Chen, Y.; Evans, J.D.; Ryabov, E.V. Deformed wing virus type A, a major honey bee pathogen, is vectored by the mite Varroa destructor in a non-propagative manner. Sci. Rep. 2019, 9, 12445, doi:10.1038/s41598-019-47447-3.

Posada-Florez, F.; Lamas, Z.S.; Hawthorne, D.J.; Chen, Y.; Evans, J.D.; Ryabov, E.V. Pupal cannibalism by worker honey bees contributes to the spread of deformed wing virus. Scientific Reports 2021, 11, 8989, doi:10.1038/s41598-021-88649-y.

Posada-Florez, F.; Ryabov, E.V.; Heerman, M.C.; Chen, Y.; Evans, J.D.; Sonenshine, D.E.; Cook, S.C. Varroa destructor mites vector and transmit pathogenic honey bee viruses acquired from an artificial diet. PLoS ONE 2020, 15, e0242688, doi:10.1371/journal.pone.0242688.

Potts SG, Biesmeijer K, Bommarco R, Breeze TD, Carvalheiro L, Franzén M, González-Varo JP, Holzschuh A, Kleijn D, Klein A, et al.: Status and trends of European pollinators. key findings of the STEP project. Pensoft Publishers; 2015.

Prithika, U.; Deepa, V.; Balamurugan, K. External induction of heat shock stimulates the immune response and longevity of Caenorhabditis elegans towards pathogen exposure. Innate Immunity 2016, 22, 466-478, doi:10.1177/1753425916654557.

Provost, K.; Dancho, B.A.; Ozbay, G.; Anderson, R.S.; Richards, G.P.; Kingsley, D.H. Hemocytes are sites of enteric virus persistence within oysters. Appl. Environ. Microbiol. 2011, 77, 8360–8369, doi:10.1128/aem.06887-11.

Rückert, C.; Bell-Sakyi, L.; Fazakerley, J. K.; Fragkoudis, R. Antiviral responses of arthropod vectors: an update on recent advances. VirusDis. 2014, 25, (3), 249-260.

Radzevičiūtė R, Theodorou P, Husemann M, Japoshvili G, Kirkitadze G, Zhusupbaeva A, Paxton RJ: Replication of honey bee-associated RNA viruses across multiple bee species in apple orchards of Georgia, Germany and Kyrgyzstan. J. Invertebr. Pathol. 2017, 146:14–23.

Rambaut, A.; Drummond, A. FigTree version 1.4.0, 2021.

Ramsey, S.D.; Ochoa, R.; Bauchan, G.; Gulbronson, C.; Mowery, J.D.; Cohen, A.; Lim, D.; Joklik, J.; Cicero, J.M.; Ellis, J.D.; et al. Varroa destructor feeds primarily on honey bee fat body tissue and not hemolymph. Proc. Natl. Acad. Sci. USA 2019, 116, 1792–1801, doi:10.1073/pnas.1818371116.

Rand, T.A.; Ginalski, K.; Grishin, N.V.; Wang, X. Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity. Proc Natl Acad Sci U S A 2004, 101, 14385-14389, doi:10.1073/pnas.0405913101.

Ravindran, M.S.; Bagchi, P.; Cunningham, C.N.; Tsai, B. Opportunistic intruders: how viruses orchestrate ER functions to infect cells. Nat Rev Microbiol 2016, 14, 407-420, doi:10.1038/nrmicro.2016.60.

Ravindran, M.S.; Bagchi, P.; Cunningham, C.N.; Tsai, B. Opportunistic intruders: How viruses orchestrate ER functions to infect cells. Nat. Rev. Microbiol. 2016, 14, 407–420, doi:10.1038/nrmicro.2016.60.

Ravoet J, De Smet L, Meeus I, Smagghe G, Wenseleers T, de Graaf DC: Widespread occurrence of honey bee pathogens in solitary bees. J. Invertebr. Pathol. 2014, 122:55–58.

Ravoet J, De Smet L, Wenseleers T, de Graaf DC: Genome sequence heterogeneity of Lake Sinai virus found in honey bees and Orf1/RdRP-based polymorphisms in a single host. Virus Res. 2015, 201:67–72.

Ravoet J, Maharramov J, Meeus I, De Smet L, Wenseleers T, Smagghe G, de Graaf DC: Comprehensive bee pathogen screening in Belgium reveals Crithidia mellificae as a new contributory factor to winter mortality. PLoS One 2013, 8:e72443.

Ravoet, J.; Schwarz, R.S.; Descamps, T.; Yañez, O.; Tozkar, C.O.; Martin-Hernandez, R.; Bartolomé, C.; De Smet, L.; Higes, M.; Wenseleers, T.; et al. Differential diagnosis of the honey bee trypanosomatids Crithidia mellificae and Lotmaria passim. Journal of Invertebrate Pathology 2015, 130, 21-27, doi:https://doi.org/10.1016/j.jip.2015.06.007.

Regal, P.J. Ecology and Evolution of Flowering Plant Dominance. Science 1977, 196, 622, doi:10.1126/science.196.4290.622.

Remnant, E.J.; Shi, M.; Buchmann, G.; Blacquiere, T.; Holmes, E.C.; Beekman, M.; Ashe, A. A Diverse Range of Novel RNA Viruses in Geographically Distinct Honey Bee Populations. J. Virol. 2017, 91, e00158-17, doi:10.1128/JVI.00158-17.

Retschnig G, Williams GR, Mehmann MM, Yañez O, de Miranda JR, Neumann P: Sex-specific differences in pathogen susceptibility in honey bees (Apis mellifera). PLoS One 2014, 9:e85261.

Ribière, M.; Olivier, V.; Blanchard, P. Chronic bee paralysis: A disease and a virus like no other? J. Invertebr. Pathol. 2010, 103, S120–S131, doi:10.1016/j.jip.2009.06.013.

Richardson, R.T.; Ballinger, M.N.; Qian, F.; Christman, J.W.; Johnson, R.M. Morphological and functional characterization of honey bee, Apis mellifera, hemocyte cell communities. Apidologie 2018, 49, 397–410, doi:10.1007/s13592-018-0566-2.

Riddell, C.; Adams, S.; Schmid-Hempel, P.; Mallon, E. B. Differential Expression of Immune Defences Is Associated with Specific Host-Parasite Interactions in Insects. PLoS ONE 2009, 4, (10), e7621.

Robalino, J.; Bartlett, T. C.; Chapman, R. W.; Gross, P. S.; Browdy, C. L.; Warr, G. W. Double-stranded RNA and antiviral immunity in marine shrimp: Inducible host mechanisms and

evidence for the evolution of viral counter-responses. Dev Comp Immunol 2007, 31, (6), 539-547.

Roberts JMK, Anderson DL, Durr PA: Absence of Deformed wing virus and Varroa destructor in Australia provides unique perspectives on honeybee viral landscapes and colony losses. Sci. Rep. 2017, 7:6925.

Ronald, P. C.; Beutler, B. Plant and Animal Sensors of Conserved Microbial Signatures. Science 2010, 330, (6007), 1061-1064.

Ronald, P.C.; Beutler, B. Plant and Animal Sensors of Conserved Microbial Signatures. Science 2010, 330, 1061-1064, doi:10.1126/science.1189468.

Ronquist, F.; Huelsenbeck, J.P. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 2003, 19, 1572-1574, doi:10.1093/bioinformatics/btg180.

Runckel, C.; DeRisi, J.; Flenniken, M.L. A draft genome of the honey bee trypanosomatid parasite crithidia mellificae. PLoS ONE 2014, 9, e95057, doi:10.1371/journal.pone.0095057.

Runckel, C.; Flenniken, M.L.; Engel, J.C.; Ruby, J.G.; Ganem, D.; Andino, R.; DeRisi, J.L. Temporal Analysis of the Honey Bee Microbiome Reveals Four Novel Viruses and Seasonal Prevalence of Known Viruses, Nosema, and Crithidia. PLoS ONE 2011, 6, e20656, doi:10.1371/journal.pone.0020656.

Ryabov, E.V.; Christmon, K.; Heerman, M.C.; Posada-Florez, F.; Harrison, R.L.; Chen, Y.; Evans, J.D. Development of a Honey Bee RNA Virus Vector Based on the Genome of a Deformed Wing Virus. Viruses 2020, 12, doi:10.3390/v12040374.

Ryabov, E.V.; Fannon, J.M.; Moore, J.D.; Wood, G.R.; Evans, D.J. The Iflaviruses Sacbrood virus and Deformed wing virus evoke different transcriptional responses in the honeybee which may facilitate their horizontal or vertical transmission. PeerJ 2016, 4, e1591, doi:10.7717/peerj.1591.

Ryabov, E.V.; Wood, G.R.; Fannon, J.M.; Moore, J.D.; Bull, J.C.; Chandler, D.; Mead, A.; Burroughs, N.; Evans, D.J. A Virulent Strain of Deformed Wing Virus (DWV) of Honeybees (Apis mellifera) Prevails after Varroa destructor-Mediated, or In Vitro, Transmission. PLoS Pathog. 2014, 10, e1004230, doi:10.1371/journal.ppat.1004230.

Sébastien A, Lester PJ, Hall RJ, Wang J, Moore NE, Gruber MAM: Invasive ants carry novel viruses in their new range and form reservoirs for a honeybee pathogen. Biol. Lett. 2015, 11:20150610.

Sadd, B. M.; Barribeau, S. M.; Bloch, G.; de Graaf, D. C.; Dearden, P.; Elsik, C. G.; Gadau, J.; Grimmelikhuijzen, C. J. P.; Hasselmann, M.; Lozier, J. D.; Robertson, H. M.; Smagghe, G.; Stolle, E.; Van Vaerenbergh, M.; Waterhouse, R. M.; Bornberg-Bauer, E.; Klasberg, S.; Bennett, A. K.; Camara, F.; Guigo, R.; Hoff, K.; Mariotti, M.; Munoz-Torres, M.;

Murphy, T.; Santesmasses, D.; Amdam, G. V.; Beckers, M.; Beye, M.; Biewer, M.; Bitondi, M. M. G.; Blaxter, M. L.; Bourke, A. F. G.; Brown, M. J.; Buechel, S. D.; Cameron, R.; Cappelle, K.; Carolan, J. C.; Christiaens, O.; Ciborowski, K. L.; Clarke, D. F.; Colgan, T. J.; Collins, D. H.; Cridge, A. G.; Dalmay, T.; Dreier, S.; du Plessis, L.; Duncan, E.; Erler, S.; Evans, J.; Falcon, T.; Flores, K.; Freitas, F. C. P.; Fuchikawa, T.; Gempe, T.; Hartfelder, K.; Hauser, F.; Helbing, S.; Humann, F. C.; Irvine, F.; Jermiin, L. S.; Johnson, C. E.; Johnson, R. M.; Jones, A. K.; Kadowaki, T.; Kidner, J. H.; Koch, V.; Köhler, A.; Kraus, F. B.; Lattorff, H. M. G.; Leask, M.; Lockett, G. A.; Mallon, E. B.; Antonio, D. S. M.; Marxer, M.; Meeus, I.; Moritz, R. F. A.; Nair, A.; Näpflin, K.; Nissen, I.; Niu, J.; Nunes, F. M. F.; Oakeshott, J. G.; Osborne, A.; Otte, M.; Pinheiro, D. G.; Rossié, N.; Rueppell, O.; Santos, C. G.; Schmid-Hempel, R.; Schmitt, B. D.; Schulte, C.; Simões, Z. L. P.; Soares, M. P. M.; Swevers, L.; Winnebeck, E. C.; Wolschin, F.; Yu, N.; Zdobnov, E. M.; Aqrawi, P. K.; Blankenburg, K. P.; Coyle, M.; Francisco, L.; Hernandez, A. G.; Holder, M.; Hudson, M. E.; Jackson, L.; Jayaseelan, J.; Joshi, V.; Kovar, C.; Lee, S. L.; Mata, R.; Mathew, T.; Newsham, I. F.; Ngo, R.; Okwuonu, G.; Pham, C.; Pu, L.-L.; Saada, N.; Santibanez, J.; Simmons, D.; Thornton, R.; Venkat, A.; Walden, K. K. O.; Wu, Y.-Q.; Debyser, G.; Devreese, B.; Asher, C.; Blommaert, J.; Chipman, A. D.; Chittka, L.; Fouks, B.; Liu, J.; O'Neill, M. P.; Sumner, S.; Puiu, D.; Qu, J.; Salzberg, S. L.; Scherer, S. E.; Muzny, D. M.; Richards, S.; Robinson, G. E.; Gibbs, R. A.; Schmid-Hempel, P.; Worley, K. C. The genomes of two key bumblebee species with primitive eusocial organization. Genome Biol. 2015, 16, (1), 227.

Sagili, R. R.; Burgett, D. M. Evaluating honey bee colonies for pollination: a guide for commercial growers and beekeepers; Oregon State University Extension Service, 2011; pp 1-8.

Saleh, M.-C.; van Rij, R. P.; Hekele, A.; Gillis, A.; Foley, E.; O'Farrell, P. H.; Andino, R. The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nat. Cell Biol. 2006, 8, (8), 793-802.

Saleh, M.C.; Tassetto, M.; van Rij, R.P.; Goic, B.; Gausson, V.; Berry, B.; Jacquier, C.; Antoniewski, C.; Andino, R. Antiviral immunity in Drosophila requires systemic RNA interference spread. Nature 2009, 458, 346-350, doi:10.1038/nature07712.

Samuel, G. H.; Adelman, Z. N.; Myles, K. M. Antiviral Immunity and Virus-Mediated Antagonism in Disease Vector Mosquitoes. Trends Microbiol 2018, 26, (5), 447-461.

Sanchez-Vargas, I.; Travanty, E. A.; Keene, K. M.; Franz, A. W. E.; Beaty, B. J.; Blair, C. D.; Olson, K. E. RNA interference, arthropod-borne viruses, and mosquitoes. Virus Res 2004, 102, (1), 65-74.

Scadden, A.D.J. The RISC subunit Tudor-SN binds to hyper-edited double-stranded RNA and promotes its cleavage. Nature Structural & Molecular Biology 2005, 12, 489-496, doi:10.1038/nsmb936.

Schlüns, H.; Crozier, R. H. Relish regulates expression of antimicrobial peptide genes in the honeybee, Apis mellifera, shown by RNA interference. Insect Mol Biol 2007, 16, (6), 753-759.

Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 2012, 9, 671-675, doi:10.1038/nmeth.2089.

Schnettler, E.; Tykalová, H.; Watson, M.; Sharma, M.; Sterken, M. G.; Obbard, D. J.; Lewis, S. H.; McFarlane, M.; Bell-Sakyi, L.; Barry, G.; Weisheit, S.; Best, S. M.; Kuhn, R. J.; Pijlman, G. P.; Chase-Topping, M. E.; Gould, E. A.; Grubhoffer, L.; Fazakerley, J. K.; Kohl, A. Induction and suppression of tick cell antiviral RNAi responses by tick-borne flaviviruses. Nucleic Acids Res 2014, 42, (14), 9436-9446.

Schoggins, J.W.; Rice, C.M. Interferon-stimulated genes and their antiviral effector functions. Current Opinion in Virology 2011, 1, 519-525, doi:10.1016/j.coviro.2011.10.008.

Schoonvaere, K.; Smagghe, G.; Francis, F.; de Graaf, D.C. Study of the Metatranscriptome of Eight Social and Solitary Wild Bee Species Reveals Novel Viruses and Bee Parasites. Frontiers in Microbiology 2018, 9, doi:10.3389/fmicb.2018.00177.

Schwarz RS, Bauchan GR, Murphy CA, Ravoet J, de Graaf DC, Evans JD: Characterization of two species of Trypanosomatidae from the honey bee Apis mellifera: Crithidia mellificae Langridge and McGhee, and Lotmaria passim n. gen., n. sp. J. Eukaryot. Microbiol. 2015, 62:567–583.

Schwarz, D.S.; Tomari, Y.; Zamore, P.D. The RNA-induced silencing complex is a Mg2+-dependent endonuclease. Curr Biol 2004, 14, 787-791, doi:10.1016/j.cub.2004.03.008.

Schwarz, R.S.; Bauchan, G.R.; Murphy, C.A.; Ravoet, J.; de Graaf, D.C.; Evans, J.D. Characterization of two species of trypanosomatidae from the honey bee apis mellifera: Crithidia mellificae langridge and mcghee, and lotmaria passim n. gen., n. sp. Journal of Eukaryotic Microbiology 2015, 1-17, doi:10.1111/jeu.12209.

Scotti, P.D.; Dearing, S.; Mossop, D.W. Flock house virus: A Nodavirus isolated fromCostelytra zealandica (White) (Coleoptera: Scarabaeida). Arch. Virol. 1983, 75, 181–189, doi:10.1007/BF01315272.

Seitz, K.; Buczolich, K.; Dikunová, A.; Plevka, P.; Power, K.; Rümenapf, T.; Lamp, B. A molecular clone of Chronic Bee Paralysis Virus (CBPV) causes mortality in honey bee pupae (Apis mellifera). Scientific Reports 2019, 9, 16274, doi:10.1038/s41598-019-52822-1.

Seitz, N.; Traynor, K.S.; Steinhauer, N.; Rennich, K.; Wilson, M.E.; Ellis, J.D.; Rose, R.; Tarpy, D.R.; Sagili, R.R.; Caron, D.M., et al. A national survey of managed honey bee 2014–2015 annual colony losses in the USA. Journal of Apicultural Research 2015, 54, 292-304, doi:10.1080/00218839.2016.1153294.

Selling, B.H.; Rueckert, R.R. Plaque assay for black beetle virus. J. Virol. 1984, 51, 251–253, doi:10.1128/JVI.51.1.251-253.1984.

Severson, D.W.; Erickson, E.H.; Williamson, J.L.; Aiken, J.M. Heat stress induced enhancement of heat shock protein gene activity in the honey bee (Apis mellifera). Experientia 1990, 46, 737-739, doi:10.1007/BF01939951.

Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N.S.; Wang, J.T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003, 13, 2498-2504, doi:10.1101/gr.1239303.

Shen M, Cui L, Ostiguy N, Cox-Foster D: Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and Sacbrood virus) with the honeybee host and the parasitic Varroa mite. J. Gen. Virol. 2005, 86:2281–2289.

Shen, M.; Cui, L.; Ostiguy, N.; Cox-Foster, D. Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite. J. Gen. Virol. 2005, 86, 2281–2289, doi:10.1099/vir.0.80824-0.

Shen, M.; Yang, X.; Cox-Foster, D.; Cui, L. The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. Virology 2005, 342, (1), 141-149.

Sheppard, W. S. A History of the Introduction of Honey Bee Races Into the United-States .2. Am Bee J 1989, 129, (10), 664-667.

Shokal, U.; Eleftherianos, I. Evolution and Function of Thioester-Containing Proteins and the Complement System in the Innate Immune Response. Frontiers in immunology 2017, 8, 759-759, doi:10.3389/fimmu.2017.00759.

Sim, C.; Hong, Y. S.; Tsetsarkin, K. A.; Vanlandingham, D. L.; Higgs, S.; Collins, F. H. Anopheles gambiae heat shock protein cognate 70B impedes o'nyong-nyong virus replication. Bmc Genomics 2007, 8, 231.

Simone-Finstrom, M. Social Immunity and the Superorganism: Behavioral Defenses Protecting Honey Bee Colonies from Pathogens and Parasites. Bee World 2017, 94, 21-29, doi:10.1080/0005772X.2017.1307800.

Simone-Finstrom, M.; Foo, B.; Tarpy, D.R.; Starks, P.T. Impact of Food Availability, Pathogen Exposure, and Genetic Diversity on Thermoregulation in Honey Bees (Apis mellifera). Journal of Insect Behavior 2014, 27, 527-539, doi:10.1007/s10905-014-9447-3.

Simone-Finstrom, M.; Li-Byarlay, H.; Huang, M. H.; Strand, M. K.; Rueppell, O.; Tarpy, D. R. Migratory management and environmental conditions affect lifespan and oxidative stress in honey bees. Sci Rep 2016, 6, (1), 810.

Simone-Finstrom, M.D.; Spivak, M. Increased resin collection after parasite challenge: a case of self-medication in honey bees? PloS one 2012, 7, e34601, doi:10.1371/journal.pone.0034601.

Simone, M.; Evans, J.D.; Spivak, M. Resin collection and social immunity in honey bees. Evolution; international journal of organic evolution 2009, 63, 3016-3022, doi:10.1111/j.1558-5646.2009.00772.x.

Singh, R.; Levitt, A.L.; Rajotte, E.G.; Holmes, E.C.; Ostiguy, N.; Vanengelsdorp, D.; Lipkin, W.I.; Depamphilis, C.W.; Toth, A.L.; Cox-Foster, D.L. RNA viruses in hymenopteran pollinators: evidence of inter-Taxa virus transmission via pollen and potential impact on non-Apis hymenopteran species. PloS one 2010, 5, e14357, doi:10.1371/journal.pone.0014357.

Singh, V.; Aballay, A. Heat Shock and Genetic Activation of HSF-1 Enhance Immunity to Bacteria. Cell Cycle 2006, 5, 2443-2446, doi:10.4161/cc.5.21.3434.

Singh, V.; Aballay, A. Heat-shock transcription factor (HSF)-1 pathway required for Caenorhabditis elegans immunity. Proceedings of the National Academy of Sciences 2006, 103, 13092, doi:10.1073/pnas.0604050103.

Skubnik, K.; Novacek, J.; Fuzik, T.; Pridal, A.; Paxton, R. J.; Plevka, P. Structure of deformed wing virus, a major honey bee pathogen. Proc Natl Acad Sci U S A 2017, 114, (12), 3210-3215.

Solís, Eric J.; Pandey, Jai P.; Zheng, X.; Jin, Dexter X.; Gupta, Piyush B.; Airoldi, Edoardo M.; Pincus, D.; Denic, V. Defining the Essential Function of Yeast Hsf1 Reveals a Compact Transcriptional Program for Maintaining Eukaryotic Proteostasis. Molecular Cell 2016, 63, 60-71, doi:https://doi.org/10.1016/j.molcel.2016.05.014.

Soltis, P.S.; Soltis, D.E. The origin and diversification of angiosperms. American Journal of Botany 2004, 91, 1614-1626, doi:https://doi.org/10.3732/ajb.91.10.1614.

Spain Ministry of Agriculture - Ministerio de Agricultura y Pesca, A. y. M. A. Informe de resultados del Programa de vigilancia sobre las pérdidas de colonias de abejas 2012 - 2015

Spivak, M.; Goblirsch, M.; Simone-Finstrom, M. Social-medication in bees: the line between individual and social regulation. Current Opinion in Insect Science 2019, 33, 49-55, doi:https://doi.org/10.1016/j.cois.2019.02.009.

Spleen, A. M.; Lengerich, E. J.; Rennich, K.; Caron, D.; Rose, R.; Pettis, J. S.; Henson, M.; Wilkes, J. T.; Wilson, M.; Stitzinger, J.; Lee, K.; Andree, M.; Snyder, R.; vanEngelsdorp, D.; for the Bee Informed, P. A national survey of managed honey bee 2011–12 winter colony losses in the United States: results from the Bee Informed Partnership. J Apicult Res 2015, 52, (2), 44-53.

Spleen, A.M.; Lengerich, E.J.; Rennich, K.; Caron, D.; Rose, R.; Pettis, J.S.; Henson, M.; Wilkes, J.T.; Wilson, M.; Stitzinger, J.; et al. A national survey of managed honey bee 2011–12 winter colony losses in the United States: Results from the Bee Informed Partnership. J. Apic. Res. 2013, 52, 44–53, doi:10.3896/IBRA.1.52.2.07.

Spolar, R.S.; Record, M.T., Jr. Coupling of local folding to site-specific binding of proteins to DNA. Science 1994, 263, 777-784, doi:10.1126/science.8303294.

Stabentheiner, A.; Kovac, H.; Schmaranzer, S. Honeybee nestmate recognition: the thermal behaviour of guards and their examinees. Journal of Experimental Biology 2002, 205, 2637.

Stabentheiner, A.; Kovac, H.; Schmaranzer, S. Thermal Behaviour of Honeybees During Aggressive Interactions. Ethology 2007, 113, 995-1006, doi:10.1111/j.1439-0310.2007.01403.x.

Stahlschmidt, Z.R.; Adamo, S.A. Context dependency and generality of fever in insects. Naturwissenschaften 2013, 100, 691-696, doi:10.1007/s00114-013-1057-y.

Starks, P.T.; Blackie, C.A.; Seeley, T.D. Fever in honeybee colonies. Naturwissenschaften 2000, 87, 229-231, doi:10.1007/s001140050709.

Steinhauer, N. A.; Rennich, K.; Wilson, M. E.; Caron, D. M.; Lengerich, E. J.; Pettis, J. S.; Rose, R.; Skinner, J. A.; Tarpy, D. R.; Wilkes, J. T.; vanEngelsdorp, D.; for the Bee Informed, P. A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. J Apicult Res 2015, 53, (1), 1-18.

Steinhauer, N.; Rennich, K.; Caron, D.M.; Ellis, J.D.; Koenig, P.; Kulhanek, K.; Klepps, J.; Lee, K.; Milbrath, M.; Rangel, J.R.; et al. Honey Bee Colony Losses 2016–2017: Preliminary Results; Bee Informed Partnership, 2017. Available online: https://beeinformed.org/2017/05/25/2016-2017-loss-results-thank-you-to-all-survey-participants/ (accessed on 27 June 2021).

Steinhauer, N.A.; Rennich, K.; Wilson, M.E.; Caron, D.M.; Lengerich, E.J.; Pettis, J.S.; Rose, R.; Skinner, J.A.; Tarpy, D.R.; Wilkes, J.T.; et al. A national survey of managed honey bee 2012–2013 annual colony losses in the USA: Results from the Bee Informed Partnership. J. Apic. Res. 2014, 53, 1–18, doi:10.3896/IBRA.1.53.1.01.

Steinmann, N.; Corona, M.; Neumann, P.; Dainat, B. Overwintering Is Associated with Reduced Expression of Immune Genes and Higher Susceptibility to Virus Infection in Honey Bees. PLoS ONE 2015, 10, (6), e0129956.

Stollar, V.; Thomas, V.L. An agent in the Aedes aegypti cell line (Peleg) which causes fusion of Aedes albopictus cells. Virology 1975, 64, 367–377, doi:10.1016/0042-6822(75)90113-0.

Stubbs, L.; Sun, Y.; Caetano-Anolles, D. Function and Evolution of C2H2 Zinc Finger Arrays. In A Handbook of Transcription Factors.; Springer, Dordrecht: 2011; Volume 52.

Suzuki, Y.; Baidaliuk, A.; Miesen, P.; Frangeul, L.; Crist, A.B.; Merkling, S.H.; Fontaine, A.; Lequime, S.; Moltini-Conclois, I.; Blanc, H.; et al. Non-retroviral Endogenous Viral Element Limits Cognate Virus Replication in Aedes aegypti Ovaries. Current Biology 2020, 30, 3495-3506.e3496, doi:https://doi.org/10.1016/j.cub.2020.06.057.

Taiyun Wei [cre, a.; [aut], V.S.; [ctb], M.L.; [ctb], Y.X.; [ctb], Y.J.; [ctb], J.Z. Package 'corrplot', 0.84; CRAN:Vienna, Austria, 2017.

Takeuchi, O.; Akira, S. RIG-I-like antiviral protein in flies. Nat Immunol 2008, 9, (12), 1327-1328.

Tassetto, M.; Kunitomi, M.; Andino, R. Circulating Immune Cells Mediate a Systemic RNAi-Based Adaptive Antiviral Response in Drosophila. Cell 2017, 169, 314-325.e313, doi:10.1016/j.cell.2017.03.033.

Taylor, R.C.; Berendzen, K.M.; Dillin, A. Systemic stress signalling: understanding the cell non-autonomous control of proteostasis. Nature Reviews Molecular Cell Biology 2014, 15, 211-217, doi:10.1038/nrm3752.

Team, R. RStudio: Integrated Development Environment for R, 1.1.456; RStudio, Inc.: Vienna, Austria, 2016.

Team, R.C. R: A language and environment for statistical computing., R Foundation for Statistical Computing: 2019.

Tehel, A.; Brown, M. J.; Paxton, R. J. Impact of managed honey bee viruses on wild bees. Curr Opin Virol 2016, 19, 16-22.

Tentcheva, D.; Gauthier, L.; Zappulla, N.; Dainat, B.; Cousserans, F.; Colin, M.E.; Bergoin, M. Prevalence and seasonal variations of six bee viruses in Apis mellifera L. and Varroa destructor mite populations in France. Appl. Environ. Microbiol. 2004, 70, 7185–7191, doi:10.1128/AEM.70.12.7185-7191.2004.

Terenius, O.; Papanicolaou, A.; Garbutt, J. S.; Eleftherianos, I.; Huvenne, H.; Kanginakudru, S.; Albrechtsen, M.; An, C.; Aymeric, J.-L.; Barthel, A.; Bebas, P.; Bitra, K.; Bravo, A.; Chevalier, F.; Collinge, D. P.; Crava, C. M.; de Maagd, R. A.; Duvic, B.; Erlandson, M.; Faye, I.; Felföldi, G.; Fujiwara, H.; Futahashi, R.; Gandhe, A. S.; Gatehouse, H. S.; Gatehouse, L. N.; Giebultowicz, J. M.; Gómez, I.; Grimmelikhuijzen, C. J. P.; Groot, A. T.; Hauser, F.; Heckel, D. G.; Hegedus, D. D.; Hrycaj, S.; Huang, L.; Hull, J. J.; Iatrou, K.; Iga, M.; Kanost, M. R.; Kotwica, J.; Li, C.; Li, J.; Liu, J.; Lundmark, M.; Matsumoto, S.; Meyering-Vos, M.; Millichap, P. J.; Monteiro, A.; Mrinal, N.; Niimi, T.; Nowara, D.; Ohnishi, A.; Oostra, V.; Ozaki, K.; Papakonstantinou, M.; Popadic, A.; Rajam, M. V.; Saenko, S.; Simpson, R. M.; Soberón, M.; Strand, M. R.; Tomita, S.; Toprak, U.; Wang, P.; Wee, C. W.; Whyard, S.; Zhang, W.; Nagaraju, J.; Ffrench-Constant, R. H.; Herrero, S.; Gordon, K.; Swevers, L.; Smagghe, G. RNA interference in Lepidoptera: An

overview of successful and unsuccessful studies and implications for experimental design. J Insect Physiol 2011, 57, (2), 231-245.

Thoetkiattikul, H.; Beck, M.H.; Strand, M.R. Inhibitor κB-like proteins from a polydnavirus inhibit NF-κB activation and suppress the insect immune response. Proceedings of the National Academy of Sciences of the United States of America 2005, 102, 11426, doi:10.1073/pnas.0505240102.

Tidbury, H. J.; Pedersen, A. B.; Boots, M. Within and transgenerational immune priming in an insect to a DNA virus. Proc Biol Sci 2011, 278, (1707), 871-6.

Tokarev, A.A.; Alfonso, A.; Segev, N. Intracellular Compartments and Trafficking Pathways. In Trafficking Inside Cells: Pathways, Mechanisms and Regulation; Madame Curie Bioscience Database [Internet], 2009.

Tomari, Y.; Matranga, C.; Haley, B.; Martinez, N.; Zamore, P.D. A Protein Sensor for siRNA Asymmetry. Science 2004, 306, 1377, doi:10.1126/science.1102755.

Tools for Varroa Management: A Guide to Effective Varroa Sampling and Control. 2015, 8. Available online: https://honeybeehealthcoalition.org/wp-content/uploads/2015/08/HBHC-Guide_Varroa-Interactive-PDF.pdf (accessed 27 June 2021).

Traniello, I.M.; Bukhari, S.A.; Kevill, J.; Ahmed, A.C.; Hamilton, A.R.; Naeger, N.L.; Schroeder, D.C.; Robinson, G.E. Meta-analysis of honey bee neurogenomic response links Deformed wing virus type A to precocious behavioral maturation. Sci. Rep. 2020, 10, 3101, doi:10.1038/s41598-020-59808-4.

Trapnell, C.; Hendrickson, D.G.; Sauvageau, M.; Goff, L.; Rinn, J.L.; Pachter, L. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nature Biotechnology 2013, 31, 46-53, doi:10.1038/nbt.2450.

Trapnell, C.; Roberts, A.; Goff, L.; Pertea, G.; Kim, D.; Kelley, D.R.; Pimentel, H.; Salzberg, S.L.; Rinn, J.L.; Pachter, L. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature Protocols 2012, 7, 562-578, doi:10.1038/nprot.2012.016.

Travanty, E.; Zhou, B.; Zhang, H.; Di, Y.P.; Alcorn, J.F.; Wentworth, D.E.; Mason, R.; Wang, J. Differential Susceptibilities of Human Lung Primary Cells to H1N1 Influenza Viruses. J. Virol. 2015, 89, 11935–11944, doi:10.1128/JVI.01792-15.

Traynor, K.S.; Rennich, K.; Forsgren, E.; Rose, R.; Pettis, J.; Kunkel, G.; Madella, S.; Evans, J.; Lopez, D.; vanEngelsdorp, D. Multiyear survey targeting disease incidence in US honey bees. Apidologie 2016, 47, 325-347, doi:10.1007/s13592-016-0431-0.

Ueira-Veira C, Almeida LO, de Almeida FC, Amaral IMR, Brandeburgo MAM, Bonetti AM: Scientific note on the first molecular detection of the acute bee paralysis virus in Brazilian stingless bees. Apidologie 2015, 46:628–630.

van Cleef, K.W.R.; van Mierlo, J.T.; van den Beek, M.; van Rij, R.P. Identification of Viral Suppressors of RNAi by a Reporter Assay in Drosophila S2 Cell Culture. In Antiviral RNAi: Concepts, Methods, and Applications, van Rij, R.P., Ed.; Humana Press: Totowa, NJ, 2011; pp. 201-213.

van der Zee, R.; Gray, A.; Pisa, L.; de Rijk, T. An Observational Study of Honey Bee Colony Winter Losses and Their Association with Varroa destructor, Neonicotinoids and Other Risk Factors. PLoS ONE 2015, 10, (7), e0131611.

van der Zee, R.; Pisa, L.; Andonov, S.; Brodschneider, R.; Charriere, J.-D.; Chlebo, R.; Coffey, M. F.; Crailsheim, K.; Dahle, B.; Gajda, A.; Gray, A.; Drazic, M. M.; Higes, M.; Kauko, L.; Kence, A.; Kence, M.; Kezic, N.; Kiprijanovska, H.; Kralj, J.; Kristiansen, P.; Hernandez, R. M.; Mutinelli, F.; Nguyen, B. K.; Otten, C.; Özkırım, A.; Pernal, S. F.; Peterson, M.; Ramsay, G.; Santrac, V.; Soroker, V.; Topolska, G.; Uzunov, A.; Vejsnaes, F.; Wei, S.; Wilkins, S. Managed honey bee colony losses in Canada, China, Europe, Israel and Turkey, for the winters of 2008–9 and 2009–10. J Apicult Res 2015, 51, (1), 100-114.

van Mierlo, J. T.; van Cleef, K. W. R.; van Rij, R. P., Defense and Counterdefense in the RNAi-Based Antiviral Immune System in Insects. In Drosophila, Springer New York

van Rij, R.P.; Saleh, M.-C.; Berry, B.; Foo, C.; Houk, A.; Antoniewski, C.; Andino, R. The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes & Development 2006, 20, 2985-2995, doi:10.1101/gad.1482006.

vanEngelsdorp, D.; Caron, D.; Hayes, J.; Underwood, R.; Henson, M.; Rennich, K.; Spleen, A.; Andree, M.; Snyder, R.; Lee, K.; Roccasecca, K.; Wilson, M.; Wilkes, J.; Lengerich, E.; Pettis, J.; for the Bee Informed, P. A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. J Apicult Res 2015, 51, (1), 115-124.

vanEngelsdorp, D.; Evans, J. D.; Saegerman, C.; Mullin, C.; Haubruge, E.; Nguyen, B. K.; Frazier, M.; Frazier, J.; Cox-Foster, D.; Chen, Y.; Underwood, R.; Tarpy, D. R.; Pettis, J. S. Colony Collapse Disorder: A Descriptive Study. PLoS ONE 2009, 4, (8), e6481.

vanEngelsdorp, D.; Hayes, J.; Underwood, R. M.; Pettis, J. A Survey of Honey Bee Colony Losses in the U.S., Fall 2007 to Spring 2008. PLoS ONE 2008, 3, (12), e4071.

vanEngelsdorp, D.; Hayes, J.; Underwood, R.; Pettis, J. A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. Journal of Apicultural Research 2010, 49, 7, doi:10.3896/IBRA.1.49.1.03.

vanEngelsdorp, D.; Meixner, M. D. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J Invertebr Pathol 2010, 103, S80-S95.

Vaudo, A. D.; Tooker, J. F.; Grozinger, C. M.; Patch, H. M. Bee nutrition and floral resource restoration. Current Opinion in Insect Science 2015, 10, 133-141.

Velthuis, H. H. W.; van Doorn, A. A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie 2006, 37, (4), 421-451.

Venter, P.A.; Schneemann, A. Recent insights into the biology and biomedical applications of Flock House virus. Cell. Mol. Life Sci. 2008, 65, 2675–2687, doi:10.1007/s00018-008-8037-y.

Venticinque, L.; Meruelo, D. Sindbis viral vector induced apoptosis requires translational inhibition and signaling through Mcl-1 and Bak. Molecular Cancer 2010, 9, 37, doi:10.1186/1476-4598-9-37.

von Heijne, G. The signal peptide. J Membr Biol 1990, 115, 195-201, doi:10.1007/bf01868635.

Wallberg, A.; Bunikis, I.; Pettersson, O.V.; Mosbech, M.-B.; Childers, A.K.; Evans, J.D.; Mikheyev, A.S.; Robertson, H.M.; Robinson, G.E.; Webster, M.T. A hybrid de novo genome assembly of the honeybee, Apis mellifera, with chromosome-length scaffolds. BMC Genomics 2019, 20, 275, doi:10.1186/s12864-019-5642-0.

Walter, P.; Ron, D. The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation. Science 2011, 334, 1081, doi:10.1126/science.1209038.

Wang, G.; Zheng, C. Zinc finger proteins in the host-virus interplay: multifaceted functions based on their nucleic acid-binding property. FEMS Microbiol Rev 2021, 45, doi:10.1093/femsre/fuaa059.

Wang, H.; Meeus, I.; Smagghe, G. Israeli acute paralysis virus associated paralysis symptoms, viral tissue distribution and Dicer-2 induction in bumblebee workers (Bombus terrestris). Journal of General Virology 2016, 97, 1981-1989, doi:https://doi.org/10.1099/jgv.0.000516.

Wang, H.; Smagghe, G.; Meeus, I. The role of a single gene encoding the Single von Willebrand factor C-domain protein (SVC) in bumblebee immunity extends beyond antiviral defense. Insect Biochemistry and Molecular Biology 2017, 91, 10-20, doi:https://doi.org/10.1016/j.ibmb.2017.10.002.

Wang, X.-H.; Aliyari, R.; Li, W.-X.; Li, H.-W.; Kim, K.; Carthew, R.; Atkinson, P.; Ding, S.-W. RNA Interference Directs Innate Immunity Against Viruses in Adult Drosophila. Science 2006, 312, 452-454, doi:10.1126/science.1125694.

Wang, Y.; Baker, N.; Amdam, G. V. RNAi-mediated double gene knockdown and gustatory perception measurement in honey bees (Apis mellifera). J Vis Exp 2013, (77).

Wang, Y.; Jorda, M.; Jones, P. L.; Maleszka, R.; Ling, X.; Robertson, H. M.; Mizzen, C. A.; Peinado, M. A.; Robinson, G. E. Functional CpG Methylation System in a Social Insect. Science 2006, 314, (5799), 645-647.

Wang, Y.T.; Liu, W.; Seah, J.N.; Lam, C.S.; Xiang, J.H.; Korzh, V.; Kwang, J. White spot syndrome virus (WSSV) infects specific hemocytes of the shrimp Penaeus merguiensis. Dis. Aquat. Organ. 2002, 52, 249–259, doi:10.3354/dao052249.

Weeks, S.A.; Shield, W.P.; Sahi, C.; Craig, E.A.; Rospert, S.; Miller, D.J. A Targeted Analysis of Cellular Chaperones Reveals Contrasting Roles for Heat Shock Protein 70 in Flock House Virus RNA Replication. Journal of Virology 2010, 84, 330, doi:10.1128/JVI.01808-09.

Weinstock, G.M.; Robinson, G.E.; Gibbs, R.A.; Weinstock, G.M.; Weinstock, G.M.; Robinson, G.E.; Worley, K.C.; Evans, J.D.; Maleszka, R.; Robertson, H.M., et al. Insights into social insects from the genome of the honeybee Apis mellifera. Nature 2006, 443, 931-949, doi:10.1038/nature05260.

Wells, T.; Wolf, S.; Nicholls, E.; Groll, H.; Lim, K.S.; Clark, S.J.; Swain, J.; Osborne, J.L.; Haughton, A.J. Flight performance of actively foraging honey bees is reduced by a common pathogen. Environ. Microbiol. Rep. 2016, 8, 728–737, doi:10.1111/1758-2229.12434.

Wilfert L, Long G, Legget HC, Schmid-Hempel P, Butlin R, Martin SJ, Boots M: Deformed wing virus is a recent global epidemic in honeybees driven by Varroa mites. Science 2016, 351:594–597.

Wilkins, C.; Dishongh, R.; Moore, S. C.; Whitt, M. A.; Chow, M.; Machaca, K. RNA interference is an antiviral defence mechanism in Caenorhabditis elegans. Nature 2005, 436, (7053), 1044-7.

Winfree, R.; Williams, N. M.; Dushoff, J.; Kremen, C. Native bees provide insurance against ongoing honey bee losses. Ecol. Lett. 2007, 10, (11), 1105-1113.

Winston, M. L. The biology of the honey bee. Harvard University Press: Cambridge, USA, 1991.

Woodard, S. H. Bumble bee ecophysiology: integrating the changing environment and the organism. Current Opinion in Insect Science 2017, 22, 101-108.

Woodcock BA, Bullock JM, Shore RF, Heard MS, Pereira MG, Redhead J, Ridding L, Dean H, Sleep D, Henrys P, et al.: Country-specific effects of neonicotinoid pesticides on honey bees and wild bees. Science 2017, 356:1393–1395.

Wright, P.E.; Dyson, H.J. Intrinsically disordered proteins in cellular signalling and regulation. Nature Reviews Molecular Cell Biology 2014, 16, 18, doi:10.1038/nrm3920.

Wu, Q.; Luo, Y.; Lu, R.; Lau, N.; Lai, E.C.; Li, W.-X.; Ding, S.-W. Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. Proc. Natl. Acad. Sci. USA 2010, 107, 1606, doi:10.1073/pnas.0911353107.

Wu, Q.; Wang, X.; Ding, S.-W. Viral suppressors of RNA-based viral immunity: Host targets. Cell Host Microbe 2010, 8, 12–15, doi:10.1016/j.chom.2010.06.009.

Wu, S.X.; Kaesberg, P. Synthesis of template-sense, single-strand Flockhouse virus RNA in a cell-free replication system. Virology 1991, 183, 392–396, doi:10.1016/0042-6822(91)90153-3.

Xi, Z.; Ramirez, J.L.; Dimopoulos, G. The Aedes aegypti Toll Pathway Controls Dengue Virus Infection. PLOS Pathogens 2008, 4, e1000098, doi:10.1371/journal.ppat.1000098.

Xia, Y.; Franzosa, E.A.; Gerstein, M.B. Integrated Assessment of Genomic Correlates of Protein Evolutionary Rate. PLOS Computational Biology 2009, 5, e1000413, doi:10.1371/journal.pcbi.1000413.

Xiao, X.; Liu, Y.; Zhang, X.; Wang, J.; Li, Z.; Pang, X.; Wang, P.; Cheng, G. Complement-Related Proteins Control the Flavivirus Infection of Aedes aegypti by Inducing Antimicrobial Peptides. PLOS Pathogens 2014, 10, e1004027, doi:10.1371/journal.ppat.1004027.

Xu, P.; Shi, M.; Chen, X.-X. Antimicrobial Peptide Evolution in the Asiatic Honey Bee Apis cerana. PLoS ONE 2009, 4, (1), e4239.

Yañez, O.; Jaffé, R.; Jarosch, A.; Fries, I.; Moritz, R.F.A.; Paxton, R.J.; de Miranda, J.R. Deformed wing virus and drone mating flights in the honey bee (Apis mellifera): Implications for sexual transmission of a major honey bee virus. Apidologie 2012, 43, 17–30, doi:10.1007/s13592-011-0088-7.

Yañez, O.; Piot, N.; Dalmon, A.; de Miranda, J.R.; Chantawannakul, P.; Panziera, D.; Amiri, E.; Smagghe, G.; Schroeder, D.; Chejanovsky, N. Bee Viruses: Routes of Infection in Hymenoptera. Front. Microbiol. 2020, 11, 943–943, doi:10.3389/fmicb.2020.00943.

Yang X, Cox-Foster DL: Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proc. Natl. Acad. Sci. 2005, 102:7470–7475.

Yang, Z. PAML 4: Phylogenetic Analysis by Maximum Likelihood. Molecular Biology and Evolution 2007, 24, 1586-1591, doi:10.1093/molbev/msm088.

Yoneyama, M.; Kikuchi, M.; Natsukawa, T.; Shinobu, N.; Imaizumi, T.; Miyagishi, M.; Taira, K.; Akira, S.; Fujita, T. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nature Immunology 2004, 5, 730-737, doi:10.1038/ni1087.

Yue, C.; Genersch, E. RT-PCR analysis of Deformed wing virus in honeybees (Apis mellifera) and mites (Varroa destructor). J. Gen. Virol. 2005, 86, 3419–3424, doi:10.1099/vir.0.81401-0.

Yue, C.; Schroder, M.; Gisder, S.; Genersch, E. Vertical-transmission routes for deformed wing virus of honeybees (Apis mellifera). Journal of General Virology 2007, 88, (8), 2329-2336.

Zambon, R.A.; Nandakumar, M.; Vakharia, V.N.; Wu, L.P. The Toll pathway is important for an antiviral response in Drosophila. Proc Natl Acad Sci U S A 2005, 102, 7257-7262, doi:10.1073/pnas.0409181102.

Zambon, R.A.; Vakharia, V.N.; Wu, L.P. RNAi is an antiviral immune response against a dsRNA virus in Drosophila melanogaster. Cellular Microbiology 2006, 8, 880-889, doi:10.1111/j.1462-5822.2006.00688.x.

Zamore, P. D. Ancient pathways programmed by small RNAs. Science 2002, 296, (5571), 1265-9.

Zanni, V.; Galbraith, D. A.; Annoscia, D.; Grozinger, C. M.; Nazzi, F. Transcriptional signatures of parasitization and markers of colony decline in Varroa-infested honey bees (Apis mellifera). Insect Biochem Mol Biol 2017, 87, 1-13.

Zhao, M.; Tang, D.; Lechpammer, S.; Hoffman, A.; Asea, A.; Stevenson, M.A.; Calderwood, S.K. Double-stranded RNA-dependent Protein Kinase (pkr) Is Essential for Thermotolerance, Accumulation of HSP70, and Stabilization of ARE-containing HSP70 mRNA during Stress. Journal of Biological Chemistry 2002, 277, 44539-44547.

Zhao, P.; Xia, F.; Jiang, L.; Guo, H.; Xu, G.; Sun, Q.; Wang, B.; Wang, Y.; Lu, Z.; Xia, Q. Enhanced antiviral immunity against Bombyx mori cytoplasmic polyhedrosis virus via overexpression of peptidoglycan recognition protein S2 in transgenic silkworms. Developmental & Comparative Immunology 2018, 87, 84-89, doi:https://doi.org/10.1016/j.dci.2018.05.021.

Zhu, F.; Ding, H.; Zhu, B. Transcriptional profiling of Drosophila S2 cells in early response to Drosophila C virus. Virology Journal 2013, 10, 210, doi:10.1186/1743-422X-10-210.

Zimmermann, L.; Stephens, A.; Nam, S.Z.; Rau, D.; Kübler, J.; Lozajic, M.; Gabler, F.; Söding, J.; Lupas, A.N.; Alva, V. A Completely Reimplemented MPI Bioinformatics Toolkit with a New HHpred Server at its Core. J Mol Biol 2018, 430, 2237-2243, doi:10.1016/j.jmb.2017.12.007.

Zioni, N.; Soroker, V.; Chejanovsky, N. Replication of Varroa destructor virus 1 (VDV-1) and a Varroa destructor virus 1–deformed wing virus recombinant (VDV-1–DWV) in the head of the honey bee. Virology 2011, 417, 106–112, doi:10.1016/j.virol.2011.05.009.