

HONEY BEE ANTIVIRAL DEFENSE MECHANISMS
AT THE INDIVIDUAL AND CELLULAR LEVEL

by

Fenali Mukesh Parekh

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ABSTRACT

Honey bees are important pollinators of fruit, nut, and vegetable crops that constitute a large proportion of the human diet. Unfortunately, annual honey bee colony losses are high, averaging 38% from 2008-2018 in the United States. Honey bee colony losses are attributed to multiple factors, including pathogens and chemical exposure. Virus incidence and abundance have been associated with colony losses. The majority of honey bee viruses are positive-sense single stranded RNA viruses. Honey bees antiviral defense include RNA interference (RNAi), a double-stranded RNA (dsRNA) triggered sequence-specific post-transcriptional silencing mechanism and a non-sequence specific dsRNA-triggered pathway. In addition, signal transduction cascades include the Toll, Imd, and Jak/STAT pathways that promote the expression of honey bee immune response genes that are also induced in response to virus infections. To investigate the impact of chemical exposure on honey bee immune responses and virus infections, we infected bees with a panel of viruses including two model viruses (i.e., Flock House virus (FHV) and Sindbis-GFP) and a naturally infecting honey bee virus, deformed wing virus (DWV) and fed them sucrose syrup containing either thyme oil, a beekeeper applied fungicide Fumagilin-B[®], or the insecticide clothianidin. We determined that bees fed thyme oil augmented sucrose syrup exhibited greater expression of key immune genes, i.e., *ago2*, *dcr-like*, *abaecin*, *hymenoptaecin*, and *vitellogenin* and reduced virus abundance compared to virus-infected bees fed sucrose syrup. Whereas, virus-infected honey bees fed diets containing fumagillin or clothianidin exhibited reduced expression of key immune genes and higher virus abundance suggesting that chemical stressors act as immunosuppressors in honey bees. To understand the interplay of viruses and host cell gene expression more precisely, we cultured primary honey bee cells derived from larvae (i.e., hemocytes, immune cells) or pupae (i.e., mixed cell population including epithelial cells, adipocytes, muscle cells, hemocytes) and demonstrated that these cells supported replication of sacbrood virus, DWV, and FHV. Expression of select immune genes, including *bap1*, *ago2*, and *dcr-like*, in virus-infected honey bee cells was similar to expression in individual bees and varied for each virus. Together, these data further our understanding of the honey bee antiviral defense network and provide new tools for studying honey bee host-virus interactions.

CHAPTER ONE

INTRODUCTION TO HONEY BEE HOST-VIRUS INTERACTIONS

Impact Of Virus Infection on Honey Bee Health

Western honey bees (*Apis mellifera*) are eusocial insects in the order Hymenoptera which comprises wasps, ants, sawflies, and bees with over 150,000 described species (1). Honey bees typically live in colonies that consist of about 35,000 individuals, usually in hollowed out tree cavities in the wild, or are maintained by beekeepers in Langstroth hives, which are portable boxes with extractable frames that support honey combs and facilitate colony inspection. Each colony consists of one reproductive female bee (queen), hundreds of male bees (drones), and thousands of non-reproductive female worker bees. Honey bees are generalist pollinators that are amenable to management, and therefore commercial beekeepers maintain honey bee colonies which they transport to pollinate a wide variety of fruit, nut, and vegetable crops (2-4). Bee pollination is crucial to global food production, and pollination services are valued at \$14.6 billion annually in the United States (US) (3, 5). Unfortunately, US beekeeping operations have recently experienced high annual colony losses (i.e., averaging 38% from 2008-2018), compared to historic levels of 12-15% annual losses (6-12).

Honey bee health is threatened by multiple biotic and abiotic factors including insecticides, fungicides, lack of quality forage (i.e., nutritional resources), the *Varroa destructor* mite, and pathogens (reviewed in (13-15)). The largest group of honey bee infecting pathogens are viruses, specifically positive-sense single-strand RNA viruses (+ssRNA) including members of the *Dicistroviridae* family (e.g., Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), acute

bee paralysis virus (ABPV), and black queen cell virus (BQCV)), the *Iflaviridae* family (e.g., deformed wing virus (DWV), sacbrood virus (SBV)), chronic bee paralysis virus (CBPV), and the Lake Sinai viruses (LSVs) (reviewed in (13, 16, 17)). The advent of high throughput sequencing has led to the discovery of honey bee infecting viruses with negative-sense RNA genomes and enveloped virions (18-20). Honey bee virus transmission occurs horizontally via trophallaxis (mouth-to-mouth food transfer), close contact among ~30,000 bees in the colony, the ectoparasitic mite *Varroa destructor*, which feeds on developing and adult bees, and vertically from queen to eggs and via drone semen, and in turn offspring (16, 21-23). Viruses can either cause death, symptomatic infections that include deformity and/or paralysis, or asymptomatic infections (reviewed in (16, 17)) (24, 25). The outcome of virus infection is largely dependent on the honey bee hosts' ability to limit virus infections by eliciting a strong antiviral response.

Honey Bee Antiviral Defense Mechanisms

Honey bee immune mechanisms include the Toll and Imd (Immune Deficiency) pathways that are mediated by NF- κ B (Nuclear Factor κ B) transcription factor homologues (i.e., Dorsal and Relish, respectively), and eicosanoid signaling, JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription), JNK (c-Jun N-terminal kinase), MAPK (Mitogen-Activated Protein Kinases) pathways (26-30). Honey bee antiviral defense also includes double-stranded RNA (dsRNA) triggered pathways, since dsRNAs are a typical product of virus replication and are recognized as “non-self” by dsRNA binding proteins (31-33). The sequence-specific RNAi mediated response is triggered by Dicer recognition and cleavage of long double stranded dsRNA molecules which results in production of small interfering RNAs (siRNAs) that are 21-22 nucleotides in length (34, 35). These siRNAs are loaded onto the RNA induced silencing complex

(RISC); the passenger strand is released while the guide strand is retained for Argonaute-2 (Ago2) mediated gene silencing (36-38). The RISC complex surveils the cell, binds and degrades complementary sequences, which limits virus replication in a sequence specific manner (39, 40). In addition to the RNAi pathway, honey bees and bumble bees antiviral defense includes a non-sequence specific dsRNA-triggered response that is less well characterized (32, 41).

Studies aimed at better understanding honey bee immune defense networks have utilized naturally infected honey bee samples, or experimentally infected honey bees with virus stocks obtained from naturally infected bees which may result in mixed virus stocks (42, 43). In addition, the use of model viruses is important to investigate host defense mechanisms more precisely on encounter with a virus that has been extensively studied, easy to propagate in the lab and infect a wide host range including honey bees, fruit flies, and mosquitos, enabling comparative immunological studies. Sindbis virus tagged with green florescence protein (SINV-GFP) and Flock House virus (FHV) have been previously utilized to study the honey bee, fruit fly, and mosquito antiviral immune response (32, 41, 43-46). Their similarity to naturally infecting honey bee viruses in terms of genome structure and size make them an attractive alternative to utilize in studying the honey bee antiviral pathways (49, 50). In addition, the use of model virus system such as FHV circumvents the problem of analyzing data with varying levels of pre-existing or confounding infections since FHV does not naturally infect honey bees. However, a limitation of using FHV is that it has not naturally evolved with honey bees and thus, may not induce the virus-specific immune responses that occur in response to co-evolved viruses. Thus, it is important to carry out experiments using a panel of viruses, including naturally infecting honey bee viruses and model insect viruses, to gain insights into the antiviral immune responses and evaluate the host

immune response for specific virus-host pairs. Previous studies demonstrated that FHV can successfully establish infection in honey bees, suggesting that FHV can be utilized as a model virus to further understand the impact of virus infection and the interplay between viruses and other stressors on overall bee health (44, 46).

Honey bees are exposed to multiple chemicals within the hive, as well as outside the hive while foraging for nectar and pollen (51-56). These chemicals may positively or negatively impact bee health. For instance, plant derived phytochemicals including thymol and *p*-coumaric acid have been shown to prime the honey bee immune system and reduce levels of honey bee pathogens including *Crithidia bombi*, *Nosema ceranae*, and DWV, increase the expression of antimicrobial peptides such as *defensin* and *abaecin*, and increase longevity (23, 57-60). In addition to phytochemicals, honey bees are exposed to neonicotinoid insecticides such as clothianidin sprayed by growers for crop pest control (52, 53, 61, 62). Although, honey bees are not the target insects, bees come in contact with insecticides via consumption of contaminated pollen or nectar or via direct contact with spray droplets. Clothianidin is highly toxic to honey bees, with a median lethal dose (LD₅₀) of 3 ppb for oral exposure and 22 ppb for contact exposure (63-65). Detectable levels of clothianidin (0.3- 5.4 ppb) persist in hive products including pollen and honey (52, 66-69). Sublethal doses of clothianidin have been shown to negatively affect honey bee immunity, grooming and hygienic behavior, neural gene expression, flight activity and homing behavior (70-75). Furthermore, honey bees exposed to pesticides including chlorpyrifos, thiamethoxam, thiacloprid, and clothianidin have greater viral loads, indicating that chemical exposure in conjunction with virus infection have a negative synergistic impact (61, 76-79). Honey bees are also exposed to chemicals utilized by beekeepers to mitigate honey bee pathogens.

Specifically, beekeepers treat microsporidial infections such as *Nosema ceranae* and *Nosema apis* with fumagillin dicyclohexyl ammonium (i.e., Fumagilin-B®), a compound derived from *Aspergillus fumigatus* (80). While fumagillin is generally considered safe for honey bee colonies, and that potential negative impacts may be negligible in comparison to nosemosis, there are reports of higher mortality in honey bee queens and workers fed fumagillin containing sucrose syrup. The impact of fumagillin exposure on virus infected honey bees is not well understood. Thus, it is important to investigate the impact of multiple, simultaneous biotic and abiotic stressors and how their interactions may impact bee health and longevity.

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

The use of cell cultures is instrumental to virology, since they facilitate mechanistic studies. Immortalized cell lines have been utilized to study host pathogen interactions since they are easy to manipulate, well characterized, proliferate indefinitely, and are cost effective. Unfortunately, there are no widely utilized immortalized honey bee cell lines. Although, one immortalized honey bee cell line (i.e., AmE-711) was established from embryonic tissue, has been useful to the examination of honey bee host-virus interactions (43, 81). It is slow-growing, difficult to maintain in the laboratory and it is persistently infected with deformed wing virus (DWV). In addition, it is likely that the immune responses elicited by the embryonic tissue derived cells would not completely recapitulate responses of other cells, including, hemocytes which are the primary insect immune cells. In addition to the limited number of studies utilizing AmE-711 cells, some studies utilized primary cells derived from honey bee pupal tissues, which include a range of cell types (e.g., epithelial, fibroblast, and muscle cells) (82, 83). However, depending on the question, the

use of cell cultures derived from a single tissue may be required. Further development of honey bee cell culture techniques is instrumental to understanding host–virus interactions.

Hypotheses and Project Summaries

Intellectual Merit

Honey bees (*Apis mellifera*) are exposed to multiple stressors, including agrochemicals and pathogens, including viruses. Honey bee virus infections may remain asymptomatic, or cause symptoms including paralysis, or result in death. The outcome of virus infection is governed by host immune responses, which are governed by genetics and impacted by environmental factors, including phytochemical and agrochemical exposure. To better understand recent honey bee colony losses, it is important to better understand honey bee host-virus interactions, particularly the antiviral responses that limit virus infection. Honey bee virology is challenging due to the limited number of tools, and natural experimental models currently available. Therefore, the development and use of model insect viruses to investigate honey bee antiviral defense will advance our understanding and facilitate comparison in other insects (i.e., fruit flies and mosquitoes). In addition to investigations at the individual bee level, better understanding of honey bee immune responses at the cellular level will enable researchers to answer basic virology questions and in turn help elucidate novel honey bee antiviral defense pathways. In the long term, better understanding of honey bee host – virus interactions, may lead to the development of strategies that mitigate virus-associated honey bee colony losses.

Hypothesis Chapter 3

Chemical stimulants and stressors will impact honey bee immune gene expression and, in turn, the outcome of virus infection.

Research aims Chapter 3

Aim 1. Investigate the impact of thyme oil, fumagillin, and clothianidin on the outcome of virus infection in honey bees. Honey bees were infected with a panel of RNA viruses (FHV, SINV, DWV) and fed with sucrose syrup containing (a) thyme oil, a potential immune stimulant, (b) fumagillin, a beekeeper applied fungicide, or (c) clothianidin, a grower-applied insecticide. Virus abundance was lower in honey bees fed thyme oil augmented sucrose syrup, whereas bees fed fumagillin or clothianidin containing sucrose syrup generally harbored higher viral loads than virus-infected bees fed sucrose syrup only.

Aim 2. Evaluate the transcriptional regulation of immune genes in response to chemical containing diets. To investigate whether the changes in virus abundance were reflective of modulations in immune gene expression, the expression of eight immune genes was assessed. The expression of key immune genes, namely *dcr*, *ago2*, *abaecin*, *hymenoptaecin*, and *vitellogenin* was higher in virus-infected bees fed thyme oil augmented sucrose syrup. Conversely, the expression of these genes was lower in virus-infected honey bees fed sucrose syrup containing fumagillin or clothianidin. Together, these results suggest that thyme oil acts an immunostimulant that results in greater expression of immune genes and lower virus loads, whereas fumagillin and clothianidin suppress immune gene expression, which results in more severe virus infections.

Hypothesis Chapter 4

Primary honey bee cell cultures will support virus replication and recapitulate some of the virus-specific immune responses identified in individual bees, as well as provide more detail on cell-type specific immune function.

Research aims Chapter 4

Aim 1. Evaluate whether primary honey bee cell cultures support virus replication. Primary honey bee cell cultures were established from honey bee larvae or pupae and were infected with naturally occurring, bee-infecting viruses (i.e., SBV or DWV) or the insect model virus, FHV. Quantitative PCR and negative strand-specific PCR demonstrated that cultured honey bee cells supported replication of SBV, DWV, and FHV. The production of infectious virions was confirmed by passaging virus from infected cells to naïve cells.

Aim 2. Evaluate the expression of immune genes in response to virus infection in culture primary cells. The expression levels of honey bee immune genes (i.e., *bap1*, *ago2*, and *dcr-like*) were quantified in larval hemocyte cultures and mixed-cell cultures derived from pupae using qPCR. A virus specific immune response was exhibited by these cells in response to SBV, DWV, and FHV infection which recapitulated some of the virus-specific immune responses in adult honey bees.

Hypothesis Chapter 5

Adult honey bees will support Flock House virus replication and modulate the expression of key immune genes.

Research aims Chapter 5

Aim 1. Verify the ability of Flock House virus (FHV) to establish infection in honey bees. To verify whether adult honey bees support FHV infection, one-day old honey bees were inoculated with 3.5×10^8 RNA copies/bee FHV via intrathoracic injection. Quantitative PCR analyses determined that FHV abundance increases over time. The viral replicative intermediate was detected using negative strand specific PCR.

Aim 2. Assess the transcriptional regulation of honey bee immune genes in response to FHV infection. Quantitative PCR was used to measure the differential expression of six honey bee immune genes, *bee antiviral protein-1*, *vitellogenin*, *dicer-like*, *argonaute-2*, *heat shock protein 90*, and *lethal(2)essential for life like* in FHV-infected honey bees relative to buffer-infected bees. The expression of key RNAi genes (*dcr* and *ago-2*) and heat shock protein encoding genes (*hsp90* and *pl2*) was higher in FHV-infected bees compared to mock-infected bees. These results describe the transcriptional responses to FHV infection in honey bees.

Aim 3. Confirm the role of *dcr* and *ago2* in FHV-infected honey bees via RNAi-mediated gene knockdown. The expression of key RNAi genes *dcr* and *ago2* was reduced by co-injecting honey bees with FHV and gene specific dsRNA. Reduced expression of either *dcr* or *ago2* in FHV-infected honey bees did not impact the virus abundance compared to bees infected with FHV only or bees co-injected with FHV and ns-dsRNA in one experimental replicate. A potential explanation for these results that the Flock house virus encoded protein B2, which is an RNAi suppressor protein, that may limit the role of RNAi in FHV-infected bees. To test this hypothesis, future studies investigating the role of *dcr* and *ago2* in FHV infection should include the FHV $\Delta B2$ mutant.

REFERENCES CITED

1. Michener C. The bees of the world, The Johns Hopkins University Press: Baltimore, USA. 2007.
2. Gallai N, Salles J-M, Settele J, Vaissière BE. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*. 2009;68(3):810-21.
3. Eilers EJ, Kremen C, Smith Greenleaf S, Garber AK, Klein A-M. Contribution of Pollinator-Mediated Crops to Nutrients in the Human Food Supply. *PLOS ONE*. 2011;6(6):e21363.
4. Klein A-M, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, et al. Importance of pollinators in changing landscapes for world crops. *Proceedings of the royal society B: biological sciences*. 2007;274(1608):303-13.
5. Calderone NW. Insect Pollinated Crops, Insect Pollinators and US Agriculture: Trend Analysis of Aggregate Data for the Period 1992–2009. *PLOS ONE*. 2012;7(5):e37235.
6. Aizen MA, Garibaldi LA, Cunningham SA, Klein AM. Long-Term Global Trends in Crop Yield and Production Reveal No Current Pollination Shortage but Increasing Pollinator Dependency. *Current Biology*. 2008;18(20):1572-5.
7. Aizen MA, Harder LD. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. *Curr Biol*. 2009;19(11):915-8.
8. Kulhanek K, Steinhauer N, Rennich K, Caron DM, Sagili RR, Pettis JS, et al. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. *Journal of Apicultural Research*. 2017;56(4):328-40.
9. Spleen AM, Lengerich EJ, Rennich K, Caron D, Rose R, Pettis JS, et al. A national survey of managed honey bee 2011–12 winter colony losses in the United States: results from the Bee Informed Partnership. *Journal of Apicultural Research*. 2013;52(2):44-53.
10. vanEngelsdorp D, Caron D, Hayes J, Underwood R, Henson M, Rennich K, et al. A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. *Journal of Apicultural Research*. 2012;51(1):115-24.
11. Steinhauer NA, Rennich K, Wilson ME, Caron DM, Lengerich EJ, Pettis JS, et al. A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. *Journal of Apicultural Research*. 2014;53(1):1-18.
12. Traynor KS, Rennich K, Forsgren E, Rose R, Pettis J, Kunkel G, et al. Multiyear survey targeting disease incidence in US honey bees. *Apidologie*. 2016;47(3):325-47.

13. Brutscher LM, McMenamin AJ, Flenniken ML. The Buzz about Honey Bee Viruses. *PLOS Pathogens*. 2016;12(8):e1005757.
14. McMenamin AJ, Brutscher LM, Glenny W, Flenniken ML. Abiotic and biotic factors affecting the replication and pathogenicity of bee viruses. *Current Opinion in Insect Science*. 2016;16:14-21.
15. Cornman RS, Tarpy DR, Chen Y, Jeffreys L, Lopez D, Pettis JS, et al. Pathogen Webs in Collapsing Honey Bee Colonies. *PLOS ONE*. 2012;7(8):e43562.
16. Grozinger CM, Flenniken ML. Bee Viruses: Ecology, Pathogenicity, and Impacts. *Annual Review of Entomology*. 2019;64(1):205-26.
17. McMenamin AJ, Flenniken ML. Recently identified bee viruses and their impact on bee pollinators. *Current Opinion in Insect Science*. 2018;26:120-9.
18. Remnant EJ, Shi M, Buchmann G, Blacqui re T, Holmes EC, Beekman M, et al. A Diverse Range of Novel RNA Viruses in Geographically Distinct Honey Bee Populations. *J Virol*. 2017;91(16).
19. De Miranda JR, Cornman RS, Evans JD, Semberg E, Haddad N, Neumann P, et al. Genome Characterization, Prevalence and Distribution of a Macula-Like Virus from *Apis mellifera* and *Varroa destructor*. *Viruses*. 2015;7(7):3586-602.
20. Levin S, Galbraith D, Sela N, Erez T, Grozinger CM, Chejanovsky N. Presence of *Apis Rhabdovirus-1* in Populations of Pollinators and Their Parasites from Two Continents. *Frontiers in Microbiology*. 2017;8(2482).
21. Chen Y, Evans J, Feldlaufer M. Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. *J Invertebr Pathol*. 2006;92(3):152-9.
22. Yue C, Schr der M, Gisder S, Genersch E. Vertical-transmission routes for deformed wing virus of honeybees (*Apis mellifera*). *Journal of General Virology*. 2007;88(8):2329-36.
23. 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(1):417-32.
24. Daughenbaugh KF, Martin M, Brutscher LM, Cavigli I, Garcia E, Lavin M, et al. Honey Bee Infecting Lake Sinai Viruses. *Viruses*. 2015;7(6):3285-309.
25. Francis RM, Nielsen SL, Kryger P. Patterns of viral infection in honey bee queens. *J Gen Virol*. 2013;94(Pt 3):668-76.
26. McMenamin AJ, Daughenbaugh KF, Parekh F, Pizzorno MC, Flenniken ML. Honey Bee and Bumble Bee Antiviral Defense. *Viruses*. 2018;10(8):395.

27. Kingsolver MB, Huang Z, Hardy RW. Insect Antiviral Innate Immunity: Pathways, Effectors, and Connections. *Journal of Molecular Biology*. 2013;425(24):4921-36.
28. Ryabov EV, Fannon JM, Moore JD, Wood GR, Evans DJ. 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.
29. Evans J, Aronstein K, Chen YP, Hetru C, Imler JL, Jiang H, et al. Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect molecular biology*. 2006;15(5):645-56.
30. Brutscher LM, Daughenbaugh KF, Flenniken ML. Antiviral defense mechanisms in honey bees. *Current Opinion in Insect Science*. 2015;10:71-82.
31. Chen YP, Pettis JS, Corona M, Chen WP, Li CJ, Spivak M, et al. Israeli Acute Paralysis Virus: Epidemiology, Pathogenesis and Implications for Honey Bee Health. *PLOS Pathogens*. 2014;10(7):e1004261.
32. Brutscher LM, Daughenbaugh KF, Flenniken ML. Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. *Scientific reports*. 2017;7(1):1-15.
33. 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):3172-85.
34. Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, Imler J-L. Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. *Nature Immunology*. 2006;7(6):590-7.
35. Deddouche S, Matt N, Budd A, Mueller S, Kemp C, Galiana-Arnoux D, et al. The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in drosophila. *Nat Immunol*. 2008;9(12):1425-32.
36. Wang X-H, Aliyari R, Li W-X, Li H-W, Kim K, Carthew R, et al. RNA Interference Directs Innate Immunity Against Viruses in Adult *Drosophila*. *Science*. 2006;312(5772):452-4.
37. Brutscher LM, Flenniken ML. RNAi and Antiviral Defense in the Honey Bee. *Journal of Immunology Research*. 2015;2015:941897.
38. Parker JS, Barford D. Argonaute: a scaffold for the function of short regulatory RNAs. *Trends in Biochemical Sciences*. 2006;31(11):622-30.
39. Ding S-W. RNA-based antiviral immunity. *Nature Reviews Immunology*. 2010;10(9):632-44.
40. Kingsolver MB, Hardy RW. Making connections in insect innate immunity. *Proceedings of the National Academy of Sciences*. 2012;109(46):18639-40.

41. Flenniken ML, Andino R. Non-specific dsRNA-mediated antiviral response in the honey bee. *Plos one*. 2013;8(10):e77263.
42. Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E, et al. IAPV, a bee-affecting virus associated with colony collapse disorder can be silenced by dsRNA ingestion. *Insect molecular biology*. 2009;18(1):55-60.
43. Carrillo-Tripp J, Dolezal AG, Goblirsch MJ, Miller WA, Toth AL, Bonning BC. In vivo and in vitro infection dynamics of honey bee viruses. *Scientific Reports*. 2016;6(1):22265.
44. O'Neal ST, Swale DR, Anderson TD. ATP-sensitive inwardly rectifying potassium channel regulation of viral infections in honey bees. *Scientific reports*. 2017;7(1):1-9.
45. McMenamin AJ, Daughenbaugh KF, Flenniken ML. The heat shock response in the Western honey bee (*Apis Mellifera*) is antiviral. *Viruses*. 2020;12(2):245.
46. O'Neal ST, Brewster CC, Bloomquist JR, Anderson TD. Amitraz and its metabolite modulate honey bee cardiac function and tolerance to viral infection. *Journal of Invertebrate Pathology*. 2017;149:119-26.
47. Dasgupta R, Free HM, Zietlow SL, Paskewitz SM, Aksoy S, Shi L, et al. Replication of Flock House Virus in Three Genera of Medically Important Insects. *Journal of Medical Entomology*. 2007;44(1):102-10.
48. Dasgupta R, Cheng L-L, Bartholomay LC, Christensen BM. Flock house virus replicates and expresses green fluorescent protein in mosquitoes. *Journal of General Virology*. 2003;84(7):1789-97.
49. Dasgupta R, Selling B, Rueckert R, editors. *Flock house virus: a simple model for studying persistent infection in cultured Drosophila cells*1994; Vienna: Springer Vienna.
50. Scotti PD, Dearing S, Mossop DW. Flock house virus: A Nodavirus isolated from *Costelytra zealandica* (White) (Coleoptera: Scarabaeida). *Archives of Virology*. 1983;75(3):181-9.
51. Bernklau E, Bjostad L, Hogeboom A, Carlisle A, H. S. A. Dietary Phytochemicals, Honey Bee Longevity and Pathogen Tolerance. *Insects*. 2019;10(1):14.
52. Bonmatin JM, Giorio C, Girolami V, Goulson D, Kreutzweiser DP, Krupke C, et al. Environmental fate and exposure; neonicotinoids and fipronil. *Environmental Science and Pollution Research*. 2015;22(1):35-67.
53. Botías C, David A, Horwood J, Abdul-Sada A, Nicholls E, Hill E, et al. Neonicotinoid Residues in Wildflowers, a Potential Route of Chronic Exposure for Bees. *Environmental Science & Technology*. 2015;49(21):12731-40.

54. Drescher N, Klein A-M, Schmitt T, Leonhardt SD. A clue on bee glue: New insight into the sources and factors driving resin intake in honeybees (*Apis mellifera*). PLOS ONE. 2019;14(2):e0210594.
55. Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. Trends in Pharmacological Sciences. 2001;22(11):573-80.
56. Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, vanEngelsdorp D, et al. High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. PLOS ONE. 2010;5(3):e9754.
57. Mao W, Schuler MA, Berenbaum MR. A dietary phytochemical alters caste-associated gene expression in honey bees. Science Advances. 2015;1(7):e1500795.
58. Palmer-Young EC, Tozkar CÖ, Schwarz RS, Chen Y, Irwin RE, Adler LS, et al. Nectar and Pollen Phytochemicals Stimulate Honey Bee (Hymenoptera: Apidae) Immunity to Viral Infection. Journal of Economic Entomology. 2017;110(5):1959-72.
59. Strachecka A, Krauze M, Olszewski K, Borsuk G, Paleolog J, Merska M, et al. Unexpectedly strong effect of caffeine on the vitality of western honeybees (*Apis mellifera*). Biochemistry (Moscow). 2014;79(11):1192-201.
60. Negri P, Ramirez L, Quintana S, Szawarski N, Maggi MD, Eguaras MJ, et al. Immune-related gene expression of *Apis mellifera* larvae in response to cold stress and Absciscic Acid (ABA) dietary supplementation. Journal of Apicultural Research. 2020;59(4):669-76.
61. Alburaki M, Boutin S, Mercier P-L, Loublier Y, Chagnon M, Derome N. Neonicotinoid-Coated Zea mays Seeds Indirectly Affect Honeybee Performance and Pathogen Susceptibility in Field Trials. PLOS ONE. 2015;10(5):e0125790.
62. Blacqui re T, Smagghe G, van Gestel CAM, Mommaerts V. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology. 2012;21(4):973-92.
63. Laurino D, Manino A, Patetta A, Porporato M. Toxicity of neonicotinoid insecticides on different honey bee genotypes. 2013.
64. Iwasa T, Motoyama N, Ambrose JT, Roe RM. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. Crop Protection. 2004;23(5):371-8.
65. Decourtye A, Devillers J. Ecotoxicity of Neonicotinoid Insecticides to Bees. In: Thany SH, editor. Insect Nicotinic Acetylcholine Receptors. New York, NY: Springer New York; 2010. p. 85-95.

66. Rundlöf M, Andersson GKS, Bommarco R, Fries I, Hederström V, Herbertsson L, et al. Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature*. 2015;521(7550):77-80.
67. Sanchez-Bayo F, Goka K. Pesticide Residues and Bees – A Risk Assessment. *PLOS ONE*. 2014;9(4):e94482.
68. Johnson RM, Ellis MD, Mullin CA, Frazier M. Pesticides and honey bee toxicity – USA*. *Apidologie*. 2010;41(3):312-31.
69. Cutler CG, Scott-Dupree CD. Exposure to Clothianidin Seed-Treated Canola Has No Long-Term Impact on Honey Bees. *Journal of Economic Entomology*. 2007;100(3):765-72.
70. Morfin N, Goodwin PH, Correa-Benitez A, Guzman-Novoa E. Sublethal exposure to clothianidin during the larval stage causes long-term impairment of hygienic and foraging behaviours of honey bees. *Apidologie*. 2019;50(5):595-605.
71. Morfin N, Goodwin PH, Guzman-Novoa E. Interaction of field realistic doses of clothianidin and Varroa destructor parasitism on adult honey bee (*Apis mellifera* L.) health and neural gene expression, and antagonistic effects on differentially expressed genes. *PLOS ONE*. 2020;15(2):e0229030.
72. Morfin N, Goodwin PH, Hunt GJ, Guzman-Novoa E. Effects of sublethal doses of clothianidin and/or *V. destructor* on honey bee (*Apis mellifera*) self-grooming behavior and associated gene expression. *Scientific Reports*. 2019;9(1):5196.
73. Matsumoto T. Reduction in homing flights in the honey bee *Apis mellifera* after a sublethal dose of neonicotinoid insecticides. *Bulletin of Insectology*. 2013;66(1):1-9.
74. Samson-Robert O, Labrie G, Chagnon M, Fournier V. Planting of neonicotinoid-coated corn raises honey bee mortality and sets back colony development. *PeerJ*. 2017;5:e3670.
75. Tsvetkov N, Samson-Robert O, Sood K, Patel HS, Malena DA, Gajiwala PH, et al. Chronic exposure to neonicotinoids reduces honey bee health near corn crops. *Science*. 2017;356(6345):1395-7.
76. DeGrandi-Hoffman G, Chen Y, Simonds R. The Effects of Pesticides on Queen Rearing and Virus Titers in Honey Bees (*Apis mellifera* L.). *Insects*. 2013;4(1):71-89.
77. Doublet V, Labarussias M, de Miranda JR, Moritz RF, Paxton RJ. Bees under stress: sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle. *Environmental microbiology*. 2015;17(4):969-83.
78. Di Prisco G, Cavaliere V, Annoscia D, Varricchio P, Caprio E, Nazzi F, et al. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral

pathogen in honey bees. *Proceedings of the National Academy of Sciences*. 2013;110(46):18466-71.

79. Coulon M, Schurr F, Martel A-C, Cougoule N, Bégaud A, Mangoni P, et al. Influence of chronic exposure to thiamethoxam and chronic bee paralysis virus on winter honey bees. *PLOS ONE*. 2019;14(8):e0220703.

80. Guruceaga X, Perez-Cuesta U, Abad-Diaz de Cerio A, Gonzalez O, Alonso RM, Hernando FL, et al. Fumagillin, a Mycotoxin of *Aspergillus fumigatus*: Biosynthesis, Biological Activities, Detection, and Applications. *Toxins (Basel)*. 2019;12(1):7.

81. Goblirsch MJ, Spivak MS, Kurtti TJ. A Cell Line Resource Derived from Honey Bee (*Apis mellifera*) Embryonic Tissues. *PLOS ONE*. 2013;8(7):e69831.

82. Genersch E, Gisder S, Hedtke K, Hunter WB, Möckel N, Müller U. Standard methods for cell cultures in *Apis mellifera* research. *Journal of Apicultural Research*. 2013;52(1):1-8.

83. Hunter WB. Medium for development of bee cell cultures (*Apis mellifera*: Hymenoptera: Apidae). *In Vitro Cellular & Developmental Biology-Animal*. 2010;46(2):83-6.

CHAPTER TWO

HONEY BEE AND BUMBLE BEE ANTIVIRAL DEFENSE

Contributions of Authors and Co-authors

Manuscript in Chapter Two

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

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Honey Bee and Bumble Bee Antiviral Defense

Alexander J. McMenamin^{1,4}, Katie F. Daughenbaugh^{2,4}, Fenali Parekh^{1,4}, Marie Pizzorno³, and Michelle L. Flenniken^{2,4}

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

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

⁴Center 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- κ B 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- κ B (i.e., Dorsal / Relish) mediated Toll and Imd pathways, RNA-triggered 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- κ B 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- κ B 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- κ B 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].

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- κ B (Nuclear Factor κ B/Dorsal); Imd (Immune deficiency) via NF- κ 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 β -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 *DmDicer-2* cleavage of viral dsRNA into 21-22 bp siRNAs; *AmDicer-like* shares ~30% aa identity with *DmDicer-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, *DmDicer-2* serves as a **dsRNA sensor** that mediates a signal transduction cascade resulting in increased expression of *DmVago*, which suppresses viral replication. *AmDicer-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- κ 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 *Amel102*. 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 ($I\kappa$ B 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 *DmToll-7* and ~45% aa identity with *DmToll-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. Eicosanoid 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- κ B/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- κ B/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- κ B /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 *DmDicer-2*, *BtSVC-vago* expression is governed by *BtDicer-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 *BtSVC-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.

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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. *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.; Roccasacca, 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.; Tschamntke, 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.; Mougél, 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.; Salignac, 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.; Jermini, 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.; Aqrabi, 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.; Merklings, 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 THREE

CHEMICAL STIMULANTS AND STRESSORS IMPACT THE OUTCOME OF VIRUS
INFECTION AND IMMUNE GENE EXPRESSION IN HONEY BEES (*Apis mellifera*)

Contributions of Authors and Co-Authors

Manuscript in Chapter Three

Author: Fenali Parekh

Contributions: 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

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Fenali Parekh, Katie F. Daughenbaugh, Michelle L. Flenniken

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Chemical Stimulants and Stressors Impact the Outcome of Virus Infection and Immune Gene Expression in Honey Bees (*Apis mellifera*)

Fenali Parekh ^{1,2,3}, Katie F. Daughenbaugh ^{1,3} and Michelle L. Flenniken ^{1,2,3*}

¹ Department of Microbiology and Immunology, Montana State University, Bozeman, MT, United States,

² Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT, United States

³ Pollinator Health Center, Montana State University, Bozeman, MT, United States

*Correspondence: Michelle L. Flenniken, michelle.flenniken@montana.edu

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Abstract: Western honey bees (*Apis mellifera*) are ecologically, agriculturally, and economically important plant pollinators. High average annual losses of honey bee colonies in the US have been partially attributed to agrochemical exposure and virus infections. To examine the potential negative synergistic impacts of agrochemical exposure and virus infection, as well as the potential promise of phytochemicals to ameliorate the impact of pathogenic infections on honey bees, we infected bees with a panel of viruses (i.e., Flock House virus, deformed wing virus, or Sindbis virus) and exposed to one of three chemical compounds. Specifically, honey bees were fed sucrose syrup containing: (1) thyme oil, a phytochemical and putative immune stimulant, (2) fumagillin, a beekeeper applied fungicide, or (3) clothianidin, a grower-applied insecticide. We determined that virus abundance was lower in honey bees fed 0.16 ppm thyme oil augmented sucrose syrup, compared to bees fed sucrose syrup alone. Parallel analysis of honey bee gene expression revealed that honey bees fed thyme oil augmented sucrose syrup had higher expression of key RNAi genes (*argonaute-2* and *dicer-like*), antimicrobial peptide expressing genes (*abaecin* and *hymenoptaecin*), and *vitellogenin*, a putative honey bee health and age indicator, compared to bees

fed only sucrose syrup. Virus abundance was higher in bees fed fumagillin (25 ppm or 75 ppm) or 1 ppb clothianidin containing sucrose syrup relative to levels in bees fed only sucrose syrup. Whereas, honey bees fed 10 ppb clothianidin dose had lower virus levels, likely because consuming a near lethal dose of insecticide made them poor hosts for virus infection. The negative impact of fumagillin and clothianidin on honey bee health was indicated by the lower expression of *argonaute-2*, *dicer-like*, *abaecin*, and *hymenoptaecin*, and *vitellogenin*. Together, these results indicate that chemical stimulants and stressors impact the outcome of virus infection and immune gene expression in honey bees.

Keywords: honey bee, *Apis mellifera*, insect antiviral defense, honey bee viruses, deformed wing virus, thymol, fumagillin, clothianidin

1 Introduction

Honey bees (*Apis mellifera*) are important pollinators of fruit, nut, and vegetable crops that make up a large proportion of the human diet (1-3). The value of pollination services in the United States is \$14.6 billion annually (4). Maintaining the honey bee pollination force has been challenging in many parts of the world, including the US, where annual colony losses averaged 38% from 2008 to 2018 (5-10). A combination of biotic and abiotic factors impact colony health including lack of quality nutritional resources, exposure to beekeeper and/or grower applied chemicals, parasites, and pathogens [reviewed in (11-13)]. The ectoparasitic mite, *Varroa destructor*, is responsible for a large percentage of colony deaths in the US and Europe (14-16) reviewed in (17). Varroa mites feed on the bee fat bodies and hemolymph, kill brood, and vector viruses, including deformed wing

virus within and between colonies (17-21). Honey bee pathogens, including bacteria, microsporidia, fungi, trypanosomatids, and viruses also contribute to honey bee colony deaths.

The largest category of honey bee infecting pathogens are positive-sense single-stranded RNA (+ssRNA) viruses [reviewed in (22, 23)]. Honey bee infecting viruses include members of the Dicistroviridae family (e.g., Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), acute bee paralysis virus (ABPV), and black queen cell virus (BQCV)), the Iflaviridae family (e.g., deformed wing virus (DWV), sacbrood virus (SBV)), chronic bee paralysis virus (CBPV), and the Lake Sinai viruses (LSVs) [reviewed in (22, 24)]. DWV is one of the most well-characterized, globally distributed bee-infecting viruses (25, 26). In temperate climates, the prevalence and/or abundance of DWV typically increases throughout the beekeeping season (15, 27-29). High levels of DWV have been associated with honey bee colony deaths (15, 30-34). Honey bees infected with DWV during development may have wing deformities, whereas DWV infection in adult bees may result in asymptomatic infections or symptomatic infections characterized by shortened abdomen, cuticle discoloration, and reduced lifespan (15, 25, 35, 36). While other factors may influence the severity of virus infections, the effectiveness of honey bee antiviral defense mechanisms is the greatest determinant of the outcome of viral infection.

Honey bee immune defense mechanisms include NF- κ B mediated Toll and Imd (Immune Deficiency), 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 (22, 37)]. The antiviral role of NF- κ B mediated Toll and Imd pathways in response to specific viruses has, in part, been determined by examining the expression of antimicrobial peptide encoding genes, including *abaecin* and *hymenoptaecin*, in conjunction

with quantifying virus abundance (37-41). RNA viruses produce long dsRNA molecules as they replicate within cells. These viral associated molecule patterns (VAMPS) are recognized as non-self and trigger antiviral responses. In honey bees and bumble bees dsRNA-mediated antiviral responses include both sequence-specific RNA interference (RNAi) and an non-sequence specific virus reducing response (42-47). RNAi mediated antiviral defense is initiated by the recognition of long dsRNAs by the endoribonuclease, Dicer, which cleaves these dsRNAs into 21-23 nucleotide long small interfering RNAs (siRNAs) (48). These siRNAs are incorporated into the RNA-induced silencing complex (RISC), the non-target binding passenger strand is released, and the remaining strand in the RISC targets cognate RNAs, including viral genomes and transcripts, for Argonaute mediated cleavage, thereby lowering viral abundance (49-55). Although, the functions of these proteins have been best-characterized in other organisms, including *Drosophila melanogaster*, (52, 55, 56) the antiviral role of the RNAi pathway has been demonstrated in vivo in adult honey bees and larvae and transcriptome analyses of virus-infected honey bees revealed that the expression of dicer-like and ago2 was increased in response to SINV-GFP or IAPV infection, but not in response to DWV (34, 42, 43, 45, 57-59).

In addition to the immune pathways described above, the heat shock response is involved in antiviral defense in fruit flies and honey bees (60, 61). The heat shock response pathway is induced by various stressors including heat-stress (62). In the context of viral infection, the role of the heat shock response must be assessed for individual host-virus pairs since heat shock proteins can both positively and negatively affect viral replication (62-65). In honey bees, the expression of several *heat shock protein encoding genes including heat shock protein 90 (hsp90)*, and *heat shock 70-kDa protein cognate 4 (hsc70-4)* are induced by SINV-GFP infection and/or heat-shock, and the

expression of *protein lethal(2)essential for life-like (pl2)*, which encodes a protein with an Hsp20-domain, is modulated in honey bees infected with several viruses (i.e., IAPV, DWV, and SINV-GFP) (43, 57, 61, 66). Similar to the heat shock response genes, another honey bee gene that is involved in multiple functions include immunity, nutrition, stress resistance, behavioral development, ageing, oxidative stress, and longevity is *vitellogenin (vg)* (67-76). Therefore, vg expression is often utilized as a proxy for either or both honey bee age and health status [reviewed in (77)] (78-80).

Studies aimed at better characterizing the impact of viruses on honey bee health and the mechanisms of honey bee antiviral defense have involved the use of naturally infected bees, which often have varying levels of infections, or bees experimentally inoculated with honey bee viruses (e.g., DWV, IAPV, or mixed virus stocks) or model viruses (43, 45, 61, 81). In this study, we carried out experiments using a panel of viruses. Specifically, we utilized DWV, and two model viruses (i.e., Flock House virus (FHV) and Sindbis virus, tagged with green fluorescent protein (SINV-GFP)). While it is important to carry out studies with known honey bee infecting viruses, honey bee virus preparations obtained from pupae may include co-purifying viruses, as well as other proteins, and they are not a standardized source of infectious material. The recent development of infectious clones of honey bee viruses is promising, but they have not been utilized for studies in laboratories beyond those in which they were developed (82-84). The use of model viruses circumvents the problems associated with analyzing data obtained from studies impacted by pre-existing/confounding infections, enables preparation of standardized viral stocks from cultured cells, and also facilitates comparative studies across different insect species. Flock House virus (FHV) naturally infects grass grubs (*Costelytra zealandica*) and is extensively utilized model

virus that infects a wide range of other insects including mosquitoes, fruit flies, tsetse flies, and honey bees (85-91). FHV has evolved a mechanism to counter the host immune response; it encodes for protein B2, a viral suppressor of RNAi [reviewed in (92)]. SINV-GFP is another well-characterized model virus that has been extensively utilized to investigate antiviral defense mechanisms in a wide range of insects including fruit flies, mosquitos, and honey bees (42, 43, 61, 93). SINV-GFP is easily trackable both visually using microscopy, and at the protein level via Western blot analyses. Unlike FHV, Sindbis virus does not encode a viral suppressor of RNAi (94-96). Overall, the use of a panel of viruses to examine the impact of abiotic factors on the honey bee antiviral response and the outcome of virus infection is important to ensure that results are robust and potentially generalizable.

The honey bee immune system, and in turn the outcome of virus infections in individual bees is influenced by nutritional status and environmental factors (97-100). Honey bees gather pollen and nectar from flowering plants which provision the colony with nutrients including proteins, lipids, carbohydrates, vitamins, and phytochemicals (101). In addition, honey bees gather plant resins, which are the primary component of propolis (102). Recent research efforts have focused on interaction of phytochemicals and pathogens, since plant-derived compounds can either be toxic or medicinal depending on the administered dose (103). There is precedence in the literature that honey bee diets supplemented with phytochemicals increase longevity and prime the immune system by increasing the expression of antimicrobial peptide encoding genes, for example, in honey bees fed *p*-coumaric acid, the expression of antimicrobial peptides *defensin* and *abaecin* was two-fold higher compared to control bees (104-107). For the studies described herein, we investigated the impact of phytochemical containing thyme oil on the outcome of honey bee virus

infections. Honey bees forage on common thyme plants (*Thymus vulgaris*), which produce thymol, a phytochemical that has shown promise in reducing DWV loads in honey bees (104). Thymol, a terpene found in pollen and nectar of *Thymus vulgaris*, is a major component of thyme oil. *T. vulgaris* nectar contains 5.2 - 8.2 ppm thymol, while thymol concentrations in bee-collected pollen samples range between 0.0263 to 55.8 ppm (108-110). Thymol has also been detected in honey (i.e., 0.27 ppm, 4-weeks post colony treatment with 8 grams per week for 4 weeks) (111). Thyme oil contains 10% - 64% thymol depending on the plant species, geographical sources, and harvest season, which may affect the volatile composition of the plant (112-118). Thymol is widely used as an acaricide and is an active ingredient in commercial Varroa control formulations such as Apiguard® and ApiLife VAR®, which contain 25% and 74% thymol respectively (119, 120). Thymol treatments, ranging from 0.16 ppm - 120 ppm, have also shown promise in reducing levels of honey bee pathogens including *Crithidia bombi*, *Nosema ceranae*, a microsporidial/fungal pathogen, and DWV (104, 121-124). Previous research by Palmer-Young et al., determined that newly emerged honey bees that were fed a mixture DWV and 0.16 ppm thymol and released back in the colony, had 26-fold decrease in DWV levels 7 days post infection compared to bees exposed to DWV (104). However, in parallel studies the natural occurring DWV-levels in young bees that were maintained in laboratory and fed thymol (0.16 ppm) for 10 days had similar DWV levels to untreated bees. This study also determined that a higher concentration of thymol (16 ppm) was toxic to honey bees. Overall, the results described by Palmer-Young et al., were intriguing, but somewhat difficult to interpret due to the lack of quantification of DWV inoculum, which was hemolymph obtained from symptomatic bees; the high degree of variation in DWV abundance in individual bees (i.e., ΔC_t ranged from -20 to 12); and the results describing similar DWV levels in

virus-inoculated bees and control bees that were not fed thymol, indicating a high level of pre-existing DWV abundance in the honey bees utilized for their studies (104).

In addition to phytochemicals, honey bees are exposed to neonicotinoid insecticides, fungicides, acaricides, and herbicides (125, 126). Neonicotinoids, including clothianidin, are utilized by growers to reduce the number of insects including aphids, whiteflies, leafhoppers, and planthoppers that damage crops (e.g., canola, corn, canola, cotton, soybeans) (127). Neonicotinoids are neurotoxins that disrupt the insect nervous system by irreversibly binding to nicotinic acetylcholine receptors that transmit nerve signals. This prevents signal transduction from the neurotransmitter acetylcholine, leading to paralysis and death (128, 129). Although honey bees are not the target insect, clothianidin is highly toxic to honey bees at doses near the median lethal dose (LD₅₀) of 3 ppb for oral exposure and 22 ppb for contact exposure (130-134). Clothianidin concentrations of up to 2.6 ppb have been detected in pollen samples and concentrations ranging from 1 to 14 ppb, with most ranging between 0.3 to 5.4 ppb, in the nectar of treated crops (133, 135-140). In addition to potential lethal impact of clothianidin, sublethal doses have been shown to negatively affect honey bee immunity, grooming and hygienic behavior, neural gene expression, flight activity and homing behavior (141-152). Furthermore, honey bees exposed to pesticides including chlorpyrifos, thiamethoxam, thiacloprid, and clothianidin have greater viral loads, indicating that chemical exposure in conjunction with virus infection have a negative synergistic impact. (153-157). Higher virus abundance in individual bees is in part due to dampened immune responses (154). For example, young bees exposed to clothianidin had greater DWV loads due to negative modulation of the NF- κ B pathway (154).

Honey bee colonies are also exposed to chemicals utilized as in-hive treatments against honey bee pathogens, including *Nosema ceranae* and *Nosema apis*. *Nosema* infections are treated at the colony level with fumagillin dicyclohexyl ammonium (i.e., Fumagilin-B®), a compound derived from *Aspergillus fumigatus* (158-160). Beekeepers typically treat nosemosis with the manufacturer's recommended concentration of 25 ppm fumagillin, albeit field studies have recorded the use of higher concentrations (161-164). In fact, detectable levels of fumagillin residues (0.002 ppm- 0.066 ppm) persist inside the hives after treatment (159, 165). While fumagillin is generally considered safe for honey bee colonies, and that potential negative impacts may be negligible in comparison to nosemosis, reports of higher mortality in honey bee queens and workers fed fumagillin containing sucrose syrup led to the recommendations that bees should not be treated during the foraging season, and that treatments should be restricted to one time in the spring and fall (166-168).

To better understand the impact of a phytochemical (i.e., thymol) and agrochemicals, including a grower-applied insecticide (i.e., clothianidin) and a beekeeper applied fungicide (i.e., fumagillin), on the outcome of virus infection in honey bees, we carried out laboratory-based experiments on individual virus-infected honey bees that were fed chemical containing diets. For these studies, we utilized a panel of RNA viruses, including two model viruses FHV and SINV-GFP, and one honey bee virus, DWV. We hypothesized that thymol would act as an immunostimulant and reduce virus infection levels. In contrast, we hypothesized that virus-infected honey bees exposed to fumagillin or clothianidin would harbor more severe virus infections as a result of immunosuppressive effects. We determined that virus-infected honey bees fed thyme oil augmented sucrose syrup harbored lower virus levels, likely due to greater expression of key immune genes including *dcr-like*, *ago2*,

abaecin, *hymenoptaecin*, and *vitellogenin*. Conversely, we determined that fumagillin and clothianidin exposure had a negative impact on honey bee immunocompetence with reduced expression of key immune genes including *dcr-like*, *ago2*, *abaecin*, *hymenoptaecin*, and *vitellogenin*, which resulted in higher virus abundance in chemical-fed bees than virus levels in bees fed only sucrose syrup. Together our results indicate that thyme oil acts as an immune stimulant and thus reduces viral burden, whereas other chemicals (i.e., fumagillin or clothianidin) negatively impact honey bee immune gene expression, and in turn result in more severe virus infections.

2 Results

2.1 Impact of chemicals on virus infections in honey bees

2.1.1 Virus abundance reduced in honey bees fed thyme oil augmented sugar syrup

To further investigate the potential of thyme oil augmented diets to reduce viral burden, age-matched adult bees were infected with FHV, SINV-GFP or DWV. Viruses were administered via injection, which mimicked Varroa destructor mite-mediated virus transmission and ensured that individual bees received equivalent doses. In these assays, virus-inoculated honey bees were fed 0.16 ppm thyme oil (~ 60 ppb thymol) augmented sucrose syrup and virus abundance was assessed at 72 hpi in three independent experiments. The 72 hpi timepoint was selected since previous studies documented readily detectable levels of disseminated virus (i.e., SINV-GFP and DWV) and corresponding host immune gene expression changes at that time point (42, 43, 57, 61, 66). To ensure that 72 hpi was also appropriate time to assess FHV infection levels, honey bees were inoculated with FHV (3.5×10^8 FHV RNA copies per bee), and virus abundance was quantified by RT-qPCR at 6, 48, 72, and 96 hpi (Supplemental Figure S1, Supplemental Table S2). FHV

abundance increased from 0 hpi to 72 hpi and decreased from 72 hpi to 96 hpi. Since FHV abundance peaked at 72 hpi, this timepoint was selected to assay for FHV infection levels.

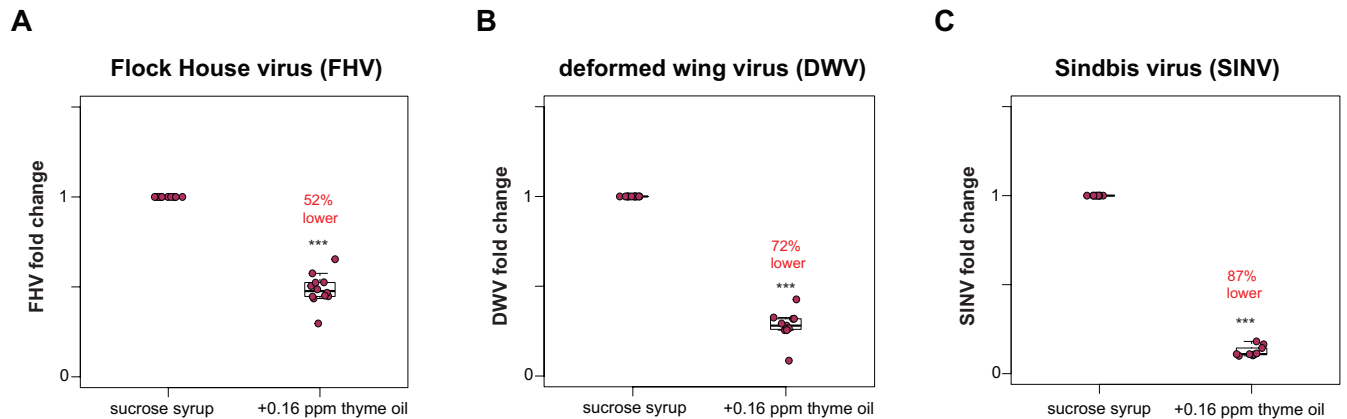


Figure 1. Lower virus abundance in honey bees fed thyme oil augmented sucrose syrup.

Virus abundance in individual honey bees that were either fed sucrose syrup only or sucrose syrup augmented with 0.16 ppm thyme oil was assessed at 72 h post-infection by qPCR (n = 9-12 per treatment group) and the relative virus abundance is presented as ranked fold-changes. Together, these data illustrate that virus-infected bees fed thyme oil augmented sucrose syrup harbored less virus than virus-infected bees fed only sucrose syrup. **(A)** Flock House virus (FHV)-infected bees fed sucrose syrup augmented with thyme oil (0.16 ppm) had 52% less FHV (0.48 mean fold change) than bees fed sucrose syrup alone ($p = 2.1 \times 10^{-5}$). **(B)** In deformed wing virus (DWV)-infected bees, virus abundance was 72% less in bees fed sucrose syrup augmented with thyme oil (0.28 median fold change) compared to bees fed only sucrose syrup ($p = 0.00016$). **(C)** Sindbis virus (SINV)-infected bees fed thyme oil augmented sucrose syrup had 87% less SINV (0.13 median fold change) than bees fed sucrose syrup. Data were analyzed by a pairwise Wilcoxon Rank Sums with a Benjamini–Hochberg correction for multiple comparisons. Asterisks indicate a significant difference in virus abundance; significance levels: *** $p < 0.0005$. Figure 1 includes results from one representative biological replicate for each virus (i.e., rep1). The data for all three biological replicates are presented in Supplemental Figure S2 and raw data are in Supplemental Tables S3 and S4.

Honey bees fed sucrose syrup augmented with thyme oil harbored less virus (i.e., FHV, DWV, SINV-GFP) compared to virus-infected bees fed sucrose syrup only (Figure 1, Supplemental Figure S2). Specifically, the total FHV abundance was 52% lower in honey bees fed thyme oil

(0.16 ppm) augmented sucrose syrup compared to bees fed sucrose syrup only ($n = 10$ bees per treatment) ($p < 0.001$, Wilcoxon Rank Sums test with a Benjamini–Hochberg correction for multiple comparisons) (Figure 1). Similar levels of reduction were observed in two additional experimental replicates with 48% and 35% reduction in FHV loads respectively (Supplemental Figure S2, $p < 0.001$). Similarly, DWV abundance was 72% lower in DWV-infected bees fed thyme oil (0.16 ppm) augmented sucrose syrup compared to bees fed sucrose syrup only (Figure 1, $p < 0.001$). DWV abundance in two additional biological replicates was reduced by 48% and 25% in DWV-infected bees fed thyme oil augmented sucrose syrup compared to bees fed sucrose syrup only (Supplemental Figure S2, $p < 0.01$). Lastly, SINV-infected bees fed thyme oil augmented sucrose syrup had 87% less virus relative to SINV-infected bees fed sucrose syrup only (Figure 1, $p < 0.001$). In two additional biological replicates of this experiment, SINV levels were reduced by 49% and 42% in SINV-infected bees fed thyme oil augmented sucrose syrup relative to bees fed sucrose syrup only (Supplemental Figure S2, $p < 0.001$). Together these results demonstrate that virus abundance was lower when virus-infected bees were fed 0.16 ppm thyme oil containing sucrose syrup compared to levels in bees fed non-augmented syrup and suggest that this dose of thyme oil is beneficial to honey bees.

2.1.2 Greater virus abundance in honey bees fed fumagillin or clothianidin containing sugar syrup

To examine the potential impact of other chemicals, including a beekeeper applied fungicide and a commonly used neonicotinoid insecticide on the outcome of virus infections, virus-infected honey bees were fed sucrose syrup containing fumagillin and clothianidin. Specifically, virus-infected honey bees were fed sucrose syrup containing 25 ppm or 75 ppm fumagillin, or a sublethal

(1 ppb) or a near lethal dose (10 ppb) of clothianidin, and virus abundance was quantified at 72 hpi. We hypothesized that virus abundance would be greater in bees exposed to virus infection and chemical exposure. We determined that, in general, virus abundance was greater in honey bees that were exposed to chemicals in their diet. Specifically, FHV abundance in bees fed sucrose syrup containing 25 ppm or 75 ppm fumagillin was 15% ($p = 0.006$) and 30% higher ($p < 0.001$), respectively (Figure 2). FHV-infected bees fed clothianidin (1 ppb) containing sucrose syrup had 94% more FHV relative to sucrose syrup fed bees ($p < 0.001$), whereas unexpectedly virus abundance was reduced by 15% in FHV-infected bees fed sucrose syrup containing 10 ppb clothianidin relative to FHV-infected bees fed sucrose syrup only ($p < 0.001$) (Figure 2). Similarly, in DWV-infected bees, virus abundance was 53% higher in bees fed sucrose syrup containing 25 ppm fumagillin ($p < 0.001$), 11% higher in bees fed 75 ppm fumagillin sucrose syrup ($p < 0.01$), and 291% higher in bees fed 1 ppb clothianidin containing sucrose syrup ($p < 0.001$) (Figure 2). DWV abundance was 21% lower in DWV-infected bees fed 10 ppb clothianidin containing sucrose syrup compared to DWV-infected bees fed sucrose syrup only ($p = 0.04$) (Figure 2).

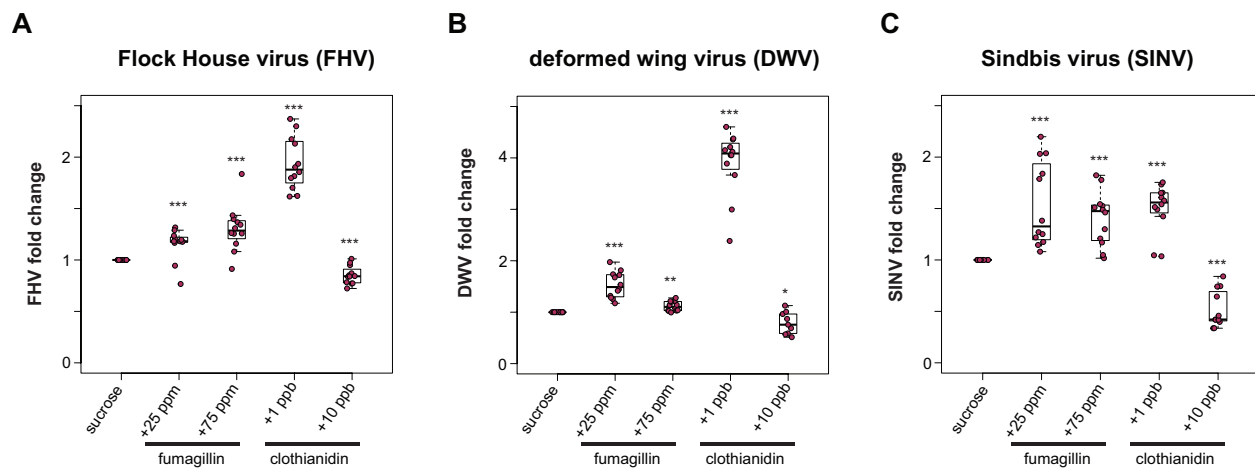


Figure 2. Honey bees fed sucrose syrup containing fumagillin or clothianidin had greater virus levels than bees feed only sucrose syrup.

Virus abundance in individual honey bees that were either fed sucrose syrup only or fungicide or insecticide containing sucrose syrup was assessed at 72 h post-infection by qPCR ($n = 9-12$ per treatment group) and the relative virus abundance is presented as ranked fold-changes. Data from virus infected honey bees fed sucrose syrup mixed with fumagillin (25 ppm or 75 ppm) or clothianidin (1 ppb or 10 ppb) illustrate that bees fed sucrose syrup containing 25 ppm fumagillin, 75 ppm fumagillin, and 1 ppb clothianidin harbored more virus than virus-infected bees fed sucrose syrup only, whereas bees fed sucrose syrup containing 10 ppb clothianidin had lower virus levels **(A)** Flock House virus (FHV)-infected bees fed 25 ppm fumagillin ($p = 0.006$), 75 ppm fumagillin ($p = 0.0009$) or 1 ppb clothianidin ($p = 8.2 \times 10^{-5}$) containing sucrose syrup had greater virus levels than bees fed sucrose only syrup. Whereas, FHV-infected bees fed sucrose syrup containing 10 ppb clothianidin had lower virus levels than bees fed only sucrose syrup ($p = 0.0009$). **(B)** Deformed wing virus (DWV)-infected bees fed sucrose syrup containing 25 ppm fumagillin ($p = 8.2 \times 10^{-5}$), 75 ppm fumagillin ($p = 0.0022$) or 1 ppb clothianidin ($p = 8.2 \times 10^{-5}$) harbored more virus than DWV-infected bees fed only sucrose syrup. DWV-infected bees fed 10 ppb clothianidin containing sucrose syrup had less virus compared to bees fed sucrose syrup ($p = 0.04$). **(C)** Sindbis virus (SINV) abundance was higher in bees fed sucrose syrup containing 25 ppm fumagillin ($p = 6.2 \times 10^{-5}$), 75 ppm fumagillin ($p = 6.2 \times 10^{-5}$) or 1 ppb clothianidin ($p = 6.2 \times 10^{-5}$) compared to SINV-infected bees fed only sucrose syrup. In contrast, SINV-infected bees fed sucrose syrup containing 10 ppb clothianidin had lower levels of virus infection than bees fed sucrose only syrup ($p = 6.8 \times 10^{-5}$). Data were analyzed by a pairwise Wilcoxon Rank Sums with a Benjamini–Hochberg correction for multiple comparisons. Asterisks indicate a significant change in virus abundance; significance levels: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$. Figure 2 includes results from one representative biological replicate for each virus (i.e., rep3). The data for all three biological replicates are presented in Figure S3. Raw data are included in Supplemental Tables S5 and S6.

Honey bees infected with SINV and fed 25 ppm fumagillin containing sucrose syrup harbored 53% more virus ($p < 0.001$), SINV-infected bees fed 75 ppm fumagillin containing sucrose syrup harbored 41% more virus ($p < 0.001$), while SINV infected bees fed 1 ppb clothianidin containing sucrose syrup had 50% more virus relative to sucrose only ($p < 0.001$). SINV-infected bees fed 10 ppb clothianidin containing sucrose syrup harbored 48% less SINV compared to bees fed sucrose syrup only ($p < 0.001$) (Figure 2). Data obtained from two additional biological replicates resulted in similar changes in virus abundance when virus-infected bees (i.e., FHV, DWV, or SINV-GFP)

were fed sucrose syrup containing fumagillin or clothianidin (Supplemental Figure S3, $p < 0.05$). Together these data indicate that virus-infected bees fed recommended (25 ppm) or higher (75 ppm) doses of fumagillin or 1 ppb clothianidin had greater viral loads, whereas bees fed 10 ppb clothianidin in sucrose syrup ad libitum had slightly lower virus levels at 72 hpi. This may be indicative of the poor overall health status of the host to support virus replication.

2.2 Impact of chemicals on honey bee immune gene expression

2.2.1 Expression of key RNAi genes is higher in thyme oil fed bees, reduced in fumagillin and clothianidin fed honey bees

To investigate whether the changes in virus abundance in honey bees fed sucrose syrup containing additives were reflective of modulations in the expression of key RNAi genes, the expression of *argonaute-2* (*ago2*) and *dicer-like* (*dcr*) were assessed at 72 hpi. In general, the expression of *dcr-like* and *ago2* were higher in virus-infected honey bees fed thyme oil containing sucrose syrup, and modestly reduced in virus-infected honey bees fed fumagillin or clothianidin containing sucrose syrup (Figure 3). Specifically, FHV-infected bees fed thyme oil exhibited 1.57 fold ($p < 0.001$) higher *dcr-like* expression and 1.23 fold higher *ago2* expression, compared to virus-infected bees fed sucrose syrup only ($p = 0.001$). Whereas FHV-infected bees fed sucrose syrup containing either 25 ppm or 75 ppm fumagillin exhibited reduced expression of *dcr-like* (i.e., 0.40 fold, $p = 0.006$, and 0.34 fold, $p < 0.001$, respectively) and *ago2* (i.e., 0.82 fold, $p = 0.002$ and 0.69 fold, $p < 0.001$, respectively). Similarly, FHV-infected bees fed clothianidin containing sucrose syrup expressed less *dcr-like* (i.e., 1 ppb - 0.81 fold, $p < 0.001$ and 10 ppb - 0.49 fold, $p = 0.0031$) and *ago2* (i.e., 1 ppb - 0.86 fold, $p = 0.023$ and 10 ppb - 0.74 fold, $p < 0.01$) (Figure 3). These results

were consistent in two additional biological replicates of this experiment (Supplemental Figure S4, $p < 0.05$) except, in rep2, *ago2* expression in FHV-infected bees fed 1 ppb clothianidin was not reduced.

DWV-infected bees fed thyme oil (0.16 ppm) augmented sucrose syrup had 1.66 fold *higher dcr-like* expression relative to DWV-infected bees fed sucrose syrup only ($p < 0.001$). However, *dcr-like* expression in DWV-infected bees fed fumagillin was reduced (i.e., 25 ppm - 0.58 fold, $p < 0.001$ and 75 ppm - 0.49 fold, $p < 0.001$). A reduction in *dcr-like* expression was also observed in DWV-infected bees fed 1 ppb clothianidin (0.67 fold, $p = 0.04$), but *dcr-like* expression levels were not changed in bees fed 10 ppb clothianidin containing sucrose syrup (Figure 3A). The expression of *ago2* in DWV-infected bees fed thyme oil containing sucrose followed a similar trend with 1.16 fold higher expression ($p = 0.035$), and reduced expression in bees fed sucrose syrup containing 25 ppm fumagillin (0.72 fold, $p = 0.024$), 75 ppm fumagillin (0.43 fold, $p < 0.001$), and 1 ppb clothianidin (0.81 fold, $p < 0.001$), whereas *ago2* expression was not changed in DWV-infected bees fed sucrose syrup containing 10 ppb clothianidin (Figure 3B). These results were consistent in two additional biological replicates of this experiment (Supplemental Figure S4 and S5, $p < 0.05$).

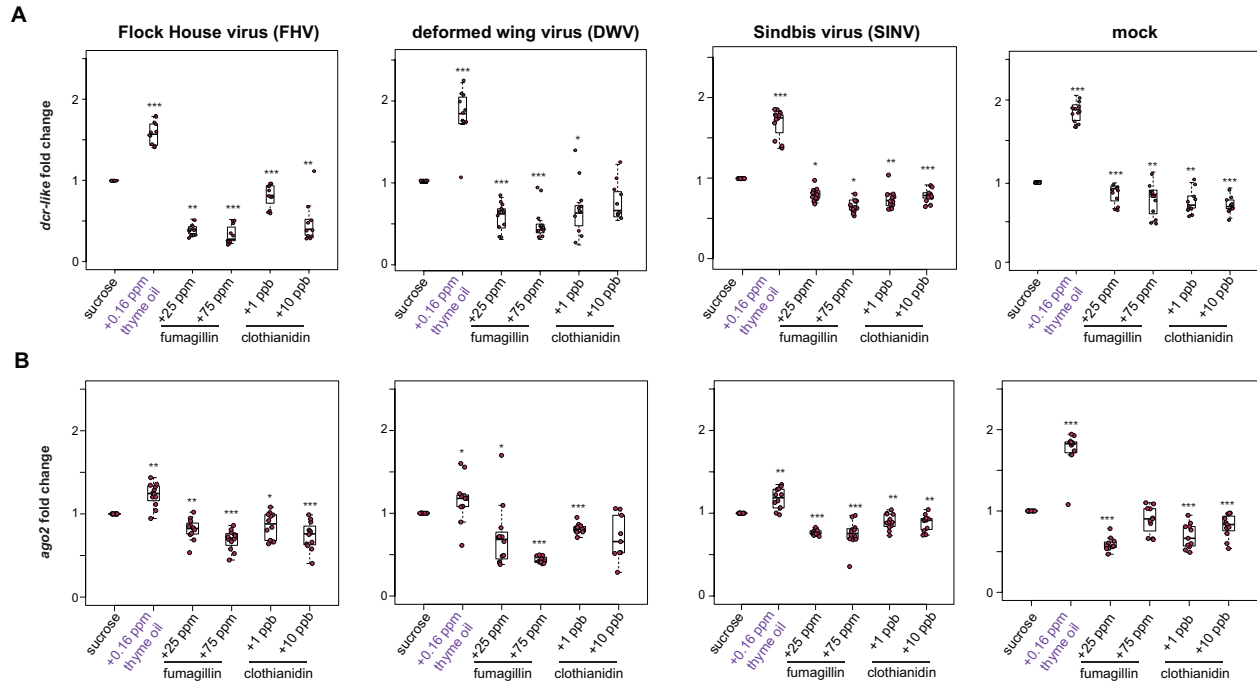


Figure 3. Expression of key RNAi genes was higher in honey bees fed thyme oil augmented sucrose syrup and lower in bees fed sucrose syrup containing fumagillin or clothianidin.

Expression of *dicer-like* and *argonaute-2* in honey bees that were either mock- or virus-infected (i.e., FHV, DWV, SINV) and fed sucrose syrup only or syrup containing either thyme oil (0.16 ppm), fumagillin (25 ppm or 75 ppm), or clothianidin (1 ppb or 10 ppb) was assessed by qPCR. The $\Delta\Delta C_t$ method with normalization to *rpl8* in mock or virus-infected bees fed sucrose syrup diet was utilized to determine the relative gene expression. **(A)** *Dicer-like* (*dcr-like*) expression was higher in virus-infected (i.e., FHV, DWV, SINV) and mock-infected bees fed thyme oil containing sucrose syrup, and lower in bees fed sucrose syrup containing fumagillin (25 ppm or 75 ppm) or clothianidin (1 ppb or 10 ppb) relative to bees fed sucrose syrup. **(B)** *Argonaute-2* (*ago2*) expression was higher in virus-infected (i.e., FHV, DWV, SINV) or mock-infected bees fed thyme oil augmented sucrose syrup and reduced in bees fed diets containing fumagillin (25 ppm or 75 ppm) or clothianidin (1 ppb or 10 ppb), except in mock-infected bees fed 75 ppm fumagillin, *ago2* expression was similar to expression levels in bees fed sucrose only. Data were analyzed by a pairwise Wilcoxon Rank Sums with a Benjamini–Hochberg correction for multiple comparisons. Asterisks indicate a significant change in gene expression compared to sucrose only control; significance levels: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$. Figure 3 shows representative biological replicate for each gene (i.e., rep2 for *dcr-like* and rep3 for *ago2*). The data for all three biological replicates are presented in Figure S4 and S5. Raw data are included in Supplemental Tables S7 - S10.

Comparably, SINV-infected bees fed thyme oil augmented sucrose syrup had higher levels of *dcr-like* expression than SINV-infected bees fed sucrose syrup only (i.e., 1.68 fold, $p < 0.001$) (Figure 3A). Whereas the combination of virus infection and chemical exposure resulted in reduced *dcr-like* expression (i.e., in SINV-infected bees fed 25 ppm fumagillin (0.81 fold, $p = 0.014$), 75 ppm fumagillin (0.66 fold, $p = 0.018$), 1 ppb clothianidin (0.74 fold, $p < 0.01$), and 10 ppb clothianidin containing sucrose syrup (0.79 fold, $p < 0.001$) compared to SINV-infected bees fed sucrose syrup only) (Figure 3A). Likewise, SINV-infected bees fed thyme oil containing sucrose syrup had 1.17 fold higher *ago2* expression ($p = 0.002$), whereas expression was slightly lower in bees fed sucrose syrup containing 25 ppm fumagillin (0.77 fold, $p < 0.001$), 75 ppm fumagillin (0.75 fold, $p < 0.001$), 1 ppb clothianidin (0.9 fold, $p < 0.01$), and 10 ppb clothianidin (0.89 fold, $p = 0.002$) (Figure 3B). The majority of these results were consistent in two additional biological replicates of this experiment (Supplemental Figure S4 and S5, $p < 0.05$) except *dcr-like* expression in SINV-infected bees fed 10 ppb clothianidin in rep3, and *ago2* expression in SINV-infected bees fed 25 ppm fumagillin in rep2 were similar to expression levels in virus-infected bees fed only sucrose syrup ($p > 0.05$). Together these results demonstrate that the expression of *dcr-like* and *ago2* were greater in virus-infected honey bees fed thyme oil (0.16 ppm) containing sucrose syrup, whereas expression of these genes was modestly reduced in virus-infected bees fed pesticide containing sucrose syrup.

To disentangle the gene expression results obtained from honey bees simultaneously impacted by two stressors (i.e., virus-infection and oral exposure to chemicals), gene expression was measured in non-virus infected honey bees (i.e., mock-infected). These analyses determined that the expression of both *dcr-like* and *ago2* were higher in thyme oil fed bees by 1.86 fold and 1.75 fold

higher respectively ($p < 0.001$) (Figure 3). Whereas the expression of *dcr-like* and *ago2* in bees fed fumagillin or clothianidin was reduced, albeit to a lesser degree compared to the increase observed with thyme oil (Figure 3). Specifically, mock-infected bees fed fumagillin in sucrose at 25 ppm and 75 ppm concentrations had 0.77 fold ($p < 0.001$) and 0.77 fold (lower *dcr-like* expression levels $p < 0.001$), and 0.59 fold ($p < 0.001$), and no appreciable change in *ago2* expression, respectively. Likewise, mock-infected bees fed clothianidin in sucrose syrup at 1 ppb and 10 ppb concentrations had 0.75 fold ($p < 0.01$) and 0.72 fold ($p < 0.001$) *dcr-like* expression levels, and 0.69 fold ($p < 0.001$) and 0.81 fold ($p < 0.001$) *ago2* expression levels, respectively.

2.2.2 Expression of heat shock protein encoding genes is higher in thyme oil, fumagillin, and clothianidin fed honey bees

The heat shock response may be induced by a variety of stressors; therefore, we examined the expression of a subset of heat shock response genes (i.e., *pl2*, *hsp90*, and *hsc70-4*) in the context of virus infection and/or chemical exposure. In general, the expression of heat shock protein encoding genes was higher in virus and mock-infected bees fed sucrose syrup containing thyme oil (0.16 ppm), fumagillin (25 ppm or 75 ppm) or clothianidin (1 ppb or 10 ppb), indicating that the expression of these genes is also induced in response to chemical stressors (Figure 4 and Supplemental Figure S6).

In this study, *pl2* exhibited the highest induction in response to virus-infection (i.e., FHV, DWV, and SINV) and chemical stress (Figure 4A). In FHV-infected bees, *pl2* expression in bees fed thyme oil augmented sucrose syrup was similar to bees fed non-augmented sucrose syrup, but *pl2* expression was greater in bees that ingested sucrose syrup containing 25 ppm fumagillin (1.42

fold, $p < 0.001$), 75 ppm fumagillin (2.26 fold, $p < 0.001$), 1 ppb clothianidin (2.69 fold, $p < 0.001$), and 10 ppb clothianidin (1.55 fold, $p < 0.01$) compared to FHV-infected bees fed only sucrose syrup. Similarly, DWV-infected bees exhibited higher *pl2* expression when fed sucrose syrup containing 0.16 ppm thyme oil (3.32 fold, $p < 0.001$), 25 ppm fumagillin (2.7 fold, $p < 0.001$), 75 ppm fumagillin (2.65 fold, $p < 0.001$), 1 ppb clothianidin (1.69 fold, $p < 0.001$), and 10 ppb clothianidin (3.74 fold, $p < 0.001$) relative to DWV-infected bees fed sucrose syrup only (Figure 4A). In SINV-infected bees, *pl2* expression was higher in bees fed sucrose syrup containing thyme oil (2.3 fold, $p < 0.01$), 25 ppm fumagillin (3.63 fold, $p < 0.01$), 75 ppm fumagillin (4.57 fold, $p < 0.01$), 1 ppb clothianidin (3.97 fold, $p = 0.023$), and 10 ppb clothianidin (3.58 fold, $p < 0.01$) compared to bees fed sucrose syrup only (Figure 4A). The expression level of *pl2* was higher in mock-infected bees fed sucrose syrup containing 25 ppm fumagillin (1.27 fold, $p < 0.001$), 75 ppm fumagillin (1.35 fold, $p < 0.01$), and 1 ppb clothianidin (2.04 fold, $p < 0.001$) relative to sucrose only fed bees (Figure 4A). Whereas *pl2* expression in mock-infected bees fed sucrose syrup containing thyme oil or 10 ppb clothianidin was similar to levels in mock-infected bees fed only sucrose syrup ($p > 0.05$). In two additional biological replicates, *pl2* expression was greater in virus or mock-infected bees fed sucrose syrup containing thyme oil (0.16 ppm), fumagillin (25 ppm or 75 ppm) or clothianidin (1 ppb or 10 ppb), except in a few treatment groups that exhibited *pl2* levels similar to those observed in honey bees fed sucrose syrup alone (i.e., in rep2, DWV-infected bees fed 25 ppm fumagillin; in rep3, FHV-infected bees fed 0.16 ppm thyme oil augmented sucrose syrup and DWV-infected bees fed 75 ppm fumagillin containing sucrose syrup (Supplemental Figure S6)).

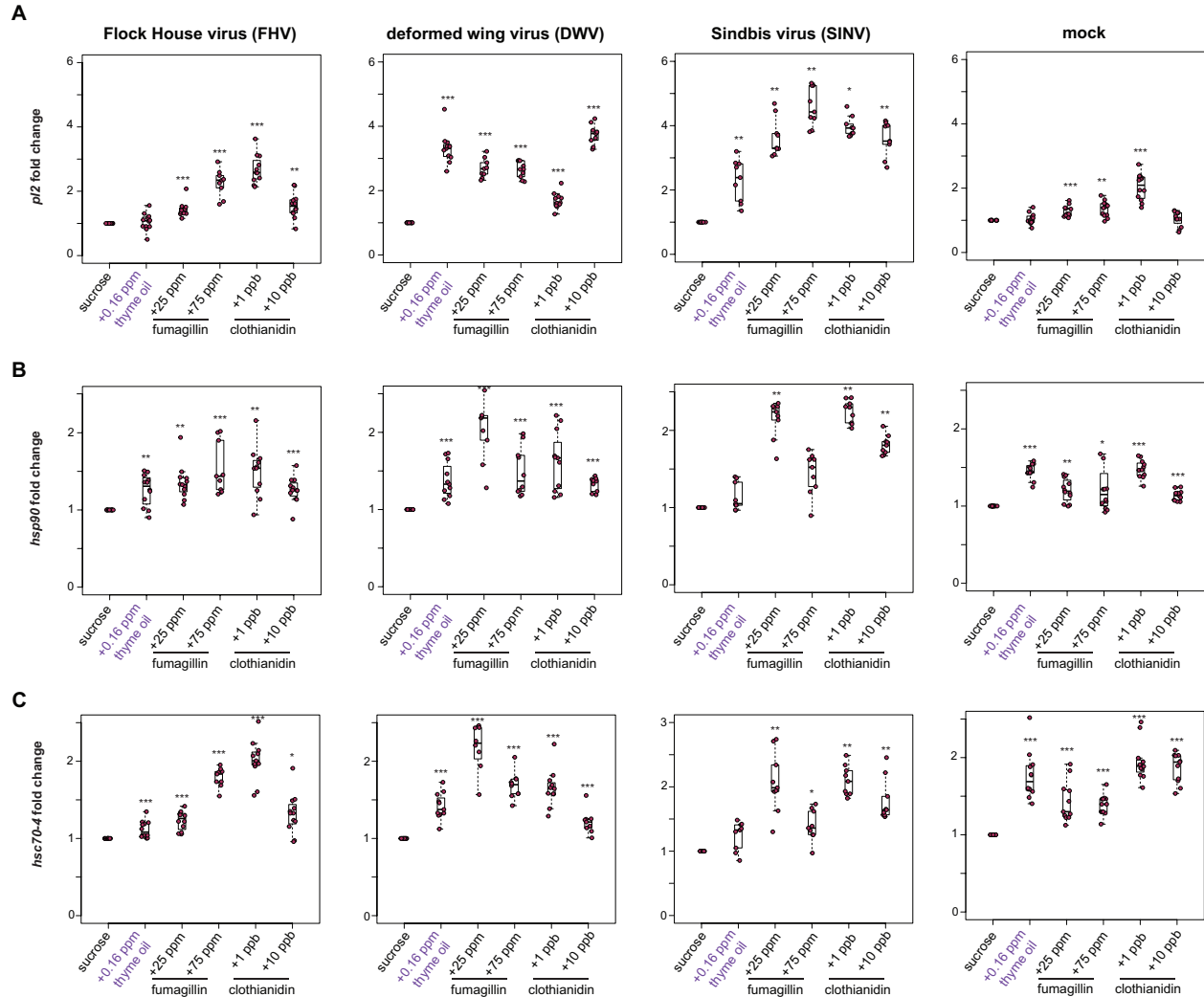


Figure 4. Expression of heat shock protein encoding genes was higher in honey bees fed sucrose syrup containing thyme oil, fumagillin, or clothianidin.

The relative expression of three genes encoding heat shock proteins (*p12*, *hsp90*, *hsc70-4*) was assessed using qPCR in mock or virus-infected bees fed sucrose syrup only or sucrose syrup containing additives (i.e., thyme oil, fumagillin, or clothianidin). The $\Delta\Delta C_t$ method with normalization to *rpl8* in mock or virus-infected bees fed sucrose syrup diet was utilized to determine the relative gene expression. **(A)** *Protein lethal(2)essential for life-like (p12)* expression in virus-infected bees (i.e., FHV, DWV, SINV) fed sucrose syrup containing thyme oil, fumagillin (25 ppm or 75 ppm) or clothianidin (1 ppb or 10 ppb) was higher compared to expression in bees fed sucrose only. In mock-infected bees, *p12* expression in bees fed sucrose syrup containing stimulant (0.16 ppm thyme oil) or 10 ppb clothianidin was similar to expression levels in bees fed sucrose only, whereas *p12* expression was higher in bees fed sucrose syrup containing fumagillin (25 ppm or 75 ppm) or 1 ppb clothianidin. **(B)** *Heat shock protein 90 (hsp90)* expression was higher in majority of the treatment groups, including virus- or mock-

infected infected bees fed augmented sucrose syrups compared to bees fed non-augmented sucrose syrup except in SINV-infected bees fed thyme oil or 75 ppm fumagillin, which had similar *hsp90* expression levels to bees fed sucrose only. (C) *Heat shock 70-kDa protein cognate 4 (hsc70-4)* expression was higher in the majority of treatment groups including mock and virus-infected bees fed sucrose syrup containing either thyme oil (0.16 ppm), fumagillin (25 ppm or 75 ppm) or clothianidin (1 ppb or 10 ppb), except in SINV-infected bees fed sucrose syrup containing thyme oil, which had *hsc70-4* expression levels similar to the controls. Data were analyzed by a pairwise Wilcoxon Rank Sums with a Benjamini–Hochberg correction for multiple comparisons. Asterisks indicate a significant change in gene expression compared to sucrose only control; significance levels: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$. Figure 4 shows representative biological replicate for *pl2* expression (i.e., rep1). The data for all three biological replicates for *pl2* are presented in Figure S6 and raw data are included in Supplemental Tables S11 - S16.

The expression of *heat shock protein 90 (hsp90)*, which encodes a protein chaperone that is involved in RISC assembly and siRNA loading, was greater in several treatment groups (169, 170) (Figure 4). Specifically, *hsp90* expression was higher in FHV-infected bees fed sucrose syrup containing thyme oil (1.26 fold, $p = 0.037$), 25 ppm fumagillin (1.36 fold, $p < 0.001$), 75 ppm fumagillin (1.54 fold, $p < 0.001$), 1 ppb clothianidin (1.5 fold, $p = 0.004$), and 10 ppb clothianidin (1.26 fold, $p = 0.003$) compared to expression levels in bees fed only sucrose syrup (Figure 4B). Likewise, the expression of *hsp90* in DWV-infected bees was higher in bees fed sucrose syrup containing thyme oil (1.38 fold, $p < 0.001$), 25 ppm fumagillin (2.02 fold, $p < 0.001$), 75 ppm fumagillin (1.49 fold, $p < 0.001$), 1 ppb clothianidin (1.63 fold, $p < 0.001$), and 10 ppb clothianidin (1.33 fold, $p < 0.001$), relative to sucrose syrup fed bees (Figure 4B). While in SINV-infected bees fed sucrose syrup augmented with 0.16 ppm thyme oil, *hsp90* expression was similar to bees fed only sucrose syrup. The expression of *hsp90* was higher in SINV-infected bees fed sucrose syrup containing 25 ppm fumagillin (2.15 fold, $p = 0.001$), 75 ppm fumagillin (1.44 fold, $p = 0.017$), 1 ppb clothianidin (2.24 fold, $p = 0.001$), and 10 ppb clothianidin (1.81 fold, $p = 0.001$), relative to

sucrose syrup fed bees (Figure 4B). Expression of *hsp90* was also greater in mock-infected bees fed sucrose syrup containing thyme oil (1.45 fold, $p < 0.001$), 25 ppm fumagillin (1.2 fold, $p = 0.001$), 75 ppm fumagillin (1.31 fold, $p = 0.017$), 1 ppb clothianidin (1.48 fold, $p < 0.001$), and 10 ppb clothianidin (1.14 fold, $p < 0.001$), compared to sucrose syrup fed bees (Figure 4B).

Heat shock protein 70 kDa cognate 4 (hsc70-4) is a constitutively expressed molecular chaperone and functions with the cooperation of co-chaperones. It is involved in RISC assembly and maintenance of cellular protein homeostasis, including protein folding, assembly, disassembly, and degradation (171-173). Expression of *hsc70-4* was higher in FHV-infected bees fed sucrose syrup containing thyme oil (1.12 fold, $p < 0.001$), 25 ppm fumagillin (1.23 fold, $p < 0.001$), 75 ppm fumagillin (1.79 fold, $p < 0.001$), 1 ppb clothianidin (2.01 fold, $p < 0.001$), and 10 ppb clothianidin (1.32 fold, $p = 0.016$) relative to sucrose syrup fed bees (Figure 4C). DWV-infected bees fed sucrose syrup containing either stimulant (thyme oil) or stressors (fumagillin or clothianidin) exhibited greater *hsc70-4* expression than bees fed sucrose syrup only i.e., thyme oil- 1.42 fold; 25 ppm fumagillin- 2 fold; 75 ppm fumagillin- 1.69 fold; 1 ppb clothianidin- 1.65 fold; and 10 ppb clothianidin-1.21 fold ($p < 0.001$) (Figure 4C). While SINV-infected bees fed thyme oil augmented sucrose syrup had similar *hsc70-4* expression relative to SINV-infected bees fed only sucrose syrup, SINV-infected bees fed chemical stressors exhibited greater *hsc70-4* expression i.e., 25 ppm fumagillin (2.07 fold, $p = 0.001$), 75 ppm fumagillin (1.4 fold, $p = 0.024$), 1 ppb clothianidin (2.1 fold, $p = 0.001$), and 10 ppb clothianidin (1.79 fold, $p = 0.001$) (Figure 4C). *Hsc70-4* expression was greater in mock-infected honey bees fed either augmented diets, compared to those fed only sucrose syrup i.e., thyme oil- 1.76 fold; 25 ppm fumagillin- 1.5 fold; 75 ppm fumagillin- 1.39 fold; 1 ppb clothianidin- 1.94 fold; and 10 ppb clothianidin- 1.87 fold

relative to sucrose syrup fed bees ($p < 0.001$) (Figure 4C). Since analyses of *hsp90* and *hsc70-4* expression in one biological replicate was similar to *pl2* expression data indicating that in general honey bee heat shock protein encoding genes exhibited higher expression in response to all chemicals, including thyme oil, fumagillin, and clothianidin, additional biological replicates were not analyzed for *hsp90* and *hsc70-4* expression.

2.2.3 Greater expression of antimicrobial peptide encoding genes in thyme oil fed bees, reduced in fumagillin and clothianidin fed bees

The honey bee genome encodes a suite of antimicrobial proteins (AMPs) that are expressed in response to microbial pathogens. Although the role of these genes in the context of virus infections in insects has not been well-characterized, increased expression is commonly used as a hallmark of active immune signaling pathways including the Toll, Imd, and Jak/STAT pathways [reviewed in (37, 40, 41)]. Therefore, we examined the expression of two honey bee AMP encoding genes *abaecin* and *hymenoptaecin* as an indicator of immune pathway status in virus-infected bees fed chemically augmented sucrose syrup. *Abaecin* is an effector molecule expressed as a result of an immune challenge that triggers the induction of Toll and Imd pathways (174-176). *Abaecin* expression in FHV-infected honey bees fed sucrose syrup supplemented with thyme oil was 1.28 fold higher relative to those fed only sucrose syrup ($p = 0.001$) (Figure 5A). In contrast, *abaecin* expression in FHV-infected bees fed either fumagillin or clothianidin was slightly lower than expression in FHV-infected bees fed plain sucrose syrup (i.e., 25 ppm fumagillin, 0.61 fold, $p = 0.001$; 75 ppm fumagillin, 0.59 fold, $p < 0.001$; 1 ppb clothianidin, 0.75 fold, $p = 0.002$; and 10 ppb clothianidin, 0.63 fold, $p < 0.001$) (Figure 5A). DWV-infected bees fed thyme oil augmented sucrose syrup had 1.58 fold greater *abaecin* expression than sucrose syrup fed bees ($p = 0.029$).

Whereas chemically stressed DWV-infected bees exhibited reduced *abaecin* expression than DWV-infected bees fed sucrose syrup alone (i.e., 25 ppm fumagillin, 0.88 fold, $p < 0.001$; 75 ppm fumagillin, 0.76 fold, $p = 0.003$; 1 ppb clothianidin, 0.3 fold, $p < 0.001$; and 10 ppb clothianidin, 0.4 fold, $p < 0.001$) (Figure 5A). Likewise, SINV-infected honey bees fed thyme oil augmented sucrose syrup had 1.4 fold higher *abaecin* expression compared to sucrose syrup fed bees ($p = 0.01$). Whereas SINV-infected bees fed chemical stressors exhibited reduced *abaecin* expression (i.e., 25 ppm fumagillin, 0.87 fold, $p = 0.01$; 75 ppm fumagillin, 0.45 fold, $p = 0.001$; 1 ppb clothianidin, 0.85 fold, $p = 0.01$; and 10 ppb clothianidin, 0.41 fold, $p = 0.001$) (Figure 5A). The same trend was also observed in mock-infected bees, which exhibited 1.33 fold higher *abaecin* expression in bees fed thyme oil augmented sucrose syrup compared to levels in bees fed non-augmented sucrose syrup ($p < 0.001$). Whereas *abaecin* expression was reduced in mock-infected bees fed sucrose syrup containing 25 ppm fumagillin (0.36 fold, $p < 0.001$), 75 ppm fumagillin (0.47 fold, $p < 0.001$), 1 ppb clothianidin (0.92 fold, $p < 0.001$), and 10 ppb clothianidin (0.47 fold, $p < 0.001$) (Figure 5A). In general, similar trends in *abaecin* expression was observed in one additional biological replicate except *abaecin* expression in FHV-infected bees fed sucrose syrup containing 75 ppm fumagillin or DWV-infected bees fed 1 ppb clothianidin was similar to expression levels in bees fed sucrose syrup (Supplemental Figure S7, $p > 0.05$).

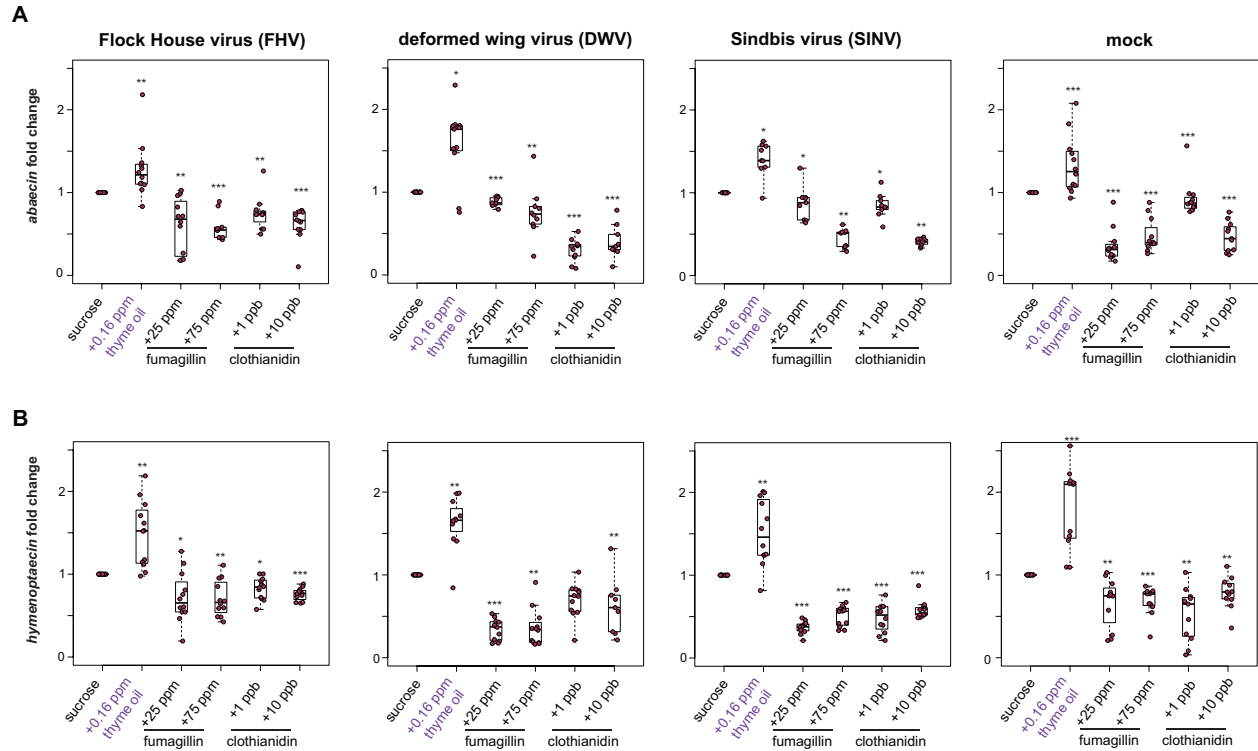


Figure 5. Expression of antimicrobial peptides encoding genes was greater in bees fed thyme oil augmented sucrose syrup and lower in bees fed fumagillin or clothianidin containing sucrose syrup.

The expression of two antimicrobial peptides (*abaecin* and *hymenoptaecin*) was assessed in mock and virus-infected bees fed sucrose syrup containing additives. **(A)** In virus- and mock-infected bees (i.e., FHV, DWV, SINV), *abaecin* expression was higher in bees fed sucrose syrup containing thyme oil and lower in bees fed sucrose syrup containing fumagillin (25 ppm or 75 ppm) or clothianidin (1 ppb or 10 ppb) compared to bees fed sucrose only. **(B)** In virus-infected bees (i.e., FHV, DWV, SINV), *hymenoptaecin* expression was higher in bees fed sucrose syrup containing thyme oil and lower in bees fed sucrose syrup containing fumagillin (25 ppm or 75 ppm) or clothianidin (1 ppb or 10 ppb). *Hymenoptaecin* expression was higher in mock-infected bees fed sucrose syrup augmented with 0.16 ppm thyme oil, but lower in mock-infected bees fed either fumagillin (i.e., 25 ppm or 75 ppm) or clothianidin (1 ppb or 10 ppb) containing sucrose syrup. Data were analyzed by a pairwise Wilcoxon Rank Sums with a Benjamini–Hochberg correction for multiple comparisons. Asterisks indicate a significant change in gene expression compared to sucrose only control; significance levels: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$. Figure 5 shows representative biological replicate for the expression of each gene (i.e., rep1 for *abaecin* and rep3 for *hymenoptaecin*). The data for one additional biological replicate is presented in supplemental Figures S7 and S8. Raw data are included in Supplemental Tables S17 – S20.

Hymenoptaecin is an antimicrobial peptide encoding gene that is induced via the nuclear factor kappa B (NF- κ B) signaling in Imd pathway (175). In the previous study by Palmer-Young et al., expression of *hymenoptaecin* in laboratory contained bees fed 0.16 ppm thymol for 7 days exhibited 12.9 to 61-fold greater expression than control bees (104). In our study, the overall trend in *hymenoptaecin* expression in bees that were either mock- or virus-infected and fed sucrose syrup containing thyme oil was higher, whereas in bees fed fumagillin, or clothianidin were lower relative to bees fed sucrose syrup (Figure 5B). FHV-infected bees fed thyme oil containing sucrose syrup had 1.48 fold higher expression of *hymenoptaecin* compared to bees fed sucrose syrup ($p = 0.002$). FHV-infection coupled with fumagillin or clothianidin containing sucrose syrup resulted in lower levels of *hymenoptaecin* expression compared to levels in bees fed plain sucrose syrup (i.e., 25 ppm fumagillin, 0.72 fold, $p = 0.019$; 75 ppm fumagillin, 0.7 fold, $p = 0.002$; 1 ppb clothianidin, 0.83 fold, $p = 0.027$; and 10 ppb clothianidin, 0.76 fold, $p < 0.001$) (Figure 5B). Likewise, DWV-infected honey bees fed thyme oil augmented sucrose syrup had 1.62 fold higher *hymenoptaecin* expression relative to bees fed sucrose syrup only ($p = 0.002$). However, *hymenoptaecin* expression was lower in DWV-infected bees fed sucrose syrup containing 25 ppm fumagillin (0.34 fold, $p < 0.001$), 75 ppm fumagillin (0.36 fold, $p < 0.001$), 1 ppb clothianidin (0.69 fold, $p = 0.001$), and 10 ppb clothianidin (0.62 fold, $p = 0.006$) than levels in bees fed only sucrose syrup (Figure 5B). SINV-infected bees fed sucrose syrup supplemented with 0.16 ppm thyme oil exhibited 1.51 fold greater expression of *hymenoptaecin*, relative to bees fed sucrose syrup only ($p = 0.001$). Bees infected with SINV and fed fumagillin or clothianidin containing sucrose syrup had lower levels of *hymenoptaecin* expression compared to bees fed sucrose syrup alone (i.e., 25 ppm fumagillin, 0.37 fold; 75 ppm fumagillin, 0.51 fold; 1 ppb clothianidin, 0.48

fold; and 10 ppb clothianidin, 0.58 fold; all with $p < 0.001$) (Figure 5B). Similar trends in *hymenoptaecin* expression were observed in mock-infected bees fed thyme oil, which exhibited 1.8 fold greater expression relative to bees fed sucrose syrup ($p < 0.001$), whereas mock-infected bees exposed to chemical stressors had reduced *hymenoptaecin* expression levels (i.e., in 25 ppm fumagillin, 0.66 fold, $p = 0.004$; 75 ppm fumagillin, 0.68 fold, $p < 0.001$; 1 ppb clothianidin, 0.52 fold, $p = 0.004$; and 10 ppb clothianidin, 0.78 fold, $p = 0.004$) (Figure 5B). Similar trends in *hymenoptaecin* expression were observed in one additional biological replicate of this experiment except in rep1, *hymenoptaecin* expression in bees fed thyme oil and either infected with FHV or SINV exhibited similar levels of expression to bees fed only sucrose syrup (Supplemental Figure S8).

2.2.4 Greater *vitellogenin* expression in thyme oil fed bees, reduced in fumagillin and clothianidin fed bees

In honey bees, *vitellogenin* (*vg*) has numerous functions including roles in immunity and stress resistance [reviewed in (77)]. Overall, the trends in *vg* expression were similar to the trends observed for other honey bee immune genes including *dcr-like*, *ago2*, *abaecin*, and *hymenoptaecin*. Virus infection in conjugation with thyme oil augmented diets resulted in higher levels of *vg* expression compared to virus-infected bees fed sucrose only (i.e., FHV- 1.47 fold, $p = 0.023$; DWV- 1.42 fold, $p < 0.001$; SINV- 1.23 fold, $p = 0.01$). The expression of *vg* was highest for 0.16 ppm thyme oil fed, mock-infected bees, which exhibited 2.71 fold greater expression than bees fed sucrose only ($p = 0.0001$). Whereas virus-infected bees fed either fumagillin or clothianidin containing diets had lower expression of *vg*.

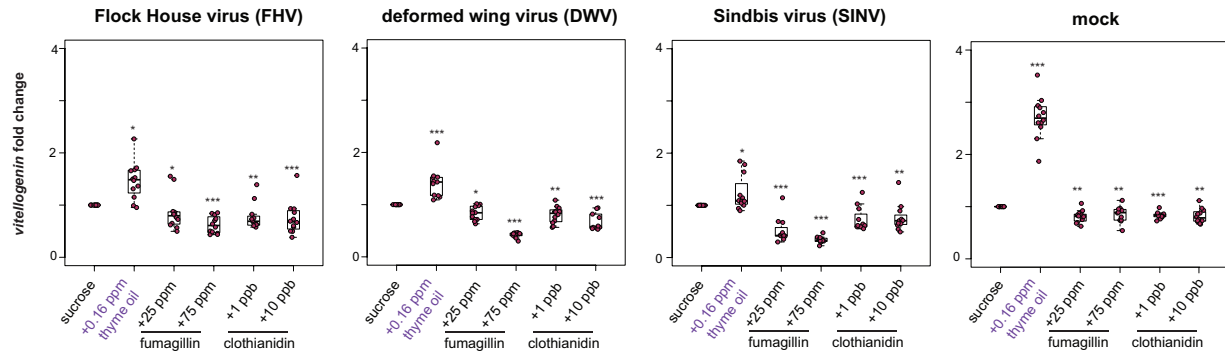


Figure 6. *Vitellogenin* expression was higher in bees fed sucrose syrup containing thyme oil and lower in bees fed sucrose syrup containing fumagillin or clothianidin.

Vitellogenin expression was assessed using qPCR and the relative gene expression was analyzed using the $\Delta\Delta C_t$ method with normalization to *rpl8* and relative to expression in mock or virus-infected bees fed sucrose syrup only. In virus-infected (i.e., FHV, DWV, SINV) and mock-infected honey bees *vitellogenin* expression was greater in bees fed sucrose syrup containing thyme oil and reduced in bees fed sucrose syrup containing fumagillin (25 ppm or 75 ppm) or clothianidin (1 ppb or 10 ppb) relative to sucrose only fed bees. Asterisks indicate a significant change in gene expression compared to sucrose only fed bees; significance levels: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$. Figure 6 shows representative biological replicate for *vitellogenin* expression. The data for one additional biological replicate is presented in supplemental Figure S9. Raw data are included in Supplemental Tables S21 and S22.

Specifically, in FHV-infected bees fed chemical containing sucrose syrup resulted in reduced *vg* expression (i.e., 25 ppm fumagillin, 0.86 fold, $p = 0.023$; 75 ppm fumagillin, 0.62 fold, $p = 0.002$; 1 ppb clothianidin, 0.77 fold, $p < 0.001$; and 10 ppb clothianidin, 0.74 fold, $p = 0.002$) compared to bees fed sucrose syrup (Figure 6). Analogously, dual stressed bees resulted in reduced *vg* expression in DWV-infected bees fed sucrose syrup containing 25 ppm fumagillin (0.84 fold, $p = 0.013$), 75 ppm fumagillin (0.42 fold, $p < 0.001$), 1 ppb clothianidin (0.8 fold, $p = 0.001$), and 10 ppb clothianidin (0.68 fold, $p < 0.001$) compared to bees fed sucrose syrup (Figure 6). *Vitellogenin* expression was reduced in SINV-infected bees fed with sucrose syrup containing 25 ppm fumagillin (0.51 fold, $p < 0.001$), 75 ppm fumagillin (0.34 fold, $p < 0.001$), 1 ppb clothianidin (0.73 fold, $p < 0.0001$), and 10 ppb clothianidin (0.77 fold, $p = 0.002$) compared to bees fed sucrose

syrup (Figure 6). In general, similar trends in *vg* expression were observed in mock-infected bees exposed to a single stressor, i.e., in bees fed sucrose syrup containing 25 ppm fumagillin (0.81 fold, $p = 0.001$), 75 ppm fumagillin (0.85 fold, $p = 0.001$), 1 ppb clothianidin (0.83 fold, $p < 0.001$), and 10 ppb clothianidin (0.82 fold, $p = 0.001$) compared to bees fed sucrose syrup (Figure 6). The expression of *vg* as analyzed in one additional biological replicate followed similar trends except in rep1, FHV-infected bees fed 10 ppb clothianidin and SINV-infected bees fed 25 ppm fumagillin, *vitellogenin* expression was constant relative to bees fed sucrose syrup (Supplemental Figure S9).

3 Discussion

Honey bee health is impacted by numerous factors including pathogens and chemical stressors. These stressors impact honey bee health at the colony, individual bee, and cellular levels. Honey bees have evolved various strategies to mitigate these threats (97, 177-179). However, the impact of multiple, simultaneous biotic and abiotic stressors on honey bee health and longevity is currently not well understood, and difficult to assess at the colony level due to the large number of confounding variables (e.g., weather, colony management, pathogen exposure, co-infections). Therefore, we performed laboratory-based experiments to examine the impact of virus infection in the context of putative chemical stimulant (i.e., thyme oil) and stressors (i.e., fumagillin and clothianidin), while limiting the effects of additional compounding variables. The results described herein demonstrate that honey bees fed sucrose syrup supplemented with 0.16 ppm thyme oil harbored 25-87% less virus than bees fed only sucrose syrup. This trend was consistent for a panel of viruses including FHV, SINV-GFP, and DWV. Honey bees fed thyme oil exhibited greater expression of genes involved in antiviral defense including the RNAi pathway (i.e., *ago2* and *dcr-*

like) and antimicrobial peptide genes regulated by Toll and Imd pathways (i.e., *abaecin* and *hymenoptaecin*), as well as a gene involved in immune competence and a marker of the overall bee health, vitellogenin. Together these results indicate that thyme oil stimulates honey bee immune responses and mitigates virus infections.

This is in line with previous studies that have documented modulation of honey bee gene expression in response to phytochemicals. Specifically, in the context of natural DWV infections, Palmer-Young *et al.* demonstrated greater *hymenoptaecin* expression (12.9 to 61-fold) in honey bees fed 0.16 ppm thymol relative to controls (104). Likewise, Mao *et al.* demonstrated that honey bees fed *p*-coumaric acid, a common pollen phytochemical, had greater expression of two other antimicrobial peptide encoding genes (i.e., 1.2 fold *defensin* and 1.9 fold *abaecin*), as well as genes involved in detoxification (107). Our results, coupled with previous studies are promising and may indicate that thyme oil augmented diets, either naturally acquired or fed could be used to enhance individual bee immunocompetence and, in turn, mitigate virus-associated colony deaths. However, further investigation is required to determine the range of thymol concentrations that positively impact honey bee health, since exposure to higher doses of thymol (i.e., 16 ppm) were lethal in previous studies (104). Additional studies are also required to further investigate the precise mechanisms of action, while results to date suggest thymol stimulates the expression of key honey bee immune genes. The signal transduction cascades, sensors, transcription factors, and effector proteins involved have yet to be fully elucidated.

The impact of agrochemicals, including neonicotinoid insecticides on honey bee health and colony longevity is an important area of research that includes a large body of literature including numerous studies that have examined field relevant exposure, colony longevity, and the impacts

on individual bees in laboratory-based experiments (139, 180-182). While acute toxicity is easy to measure in laboratory-based studies, sublethal impacts are more difficult to assess. Furthermore, the impact of sublethal doses of chemicals, including clothianidin, on the immune response and in turn the outcome of virus infection is even more difficult to discern. Previous studies demonstrated a negative impact of clothianidin (10 ppb) on the activation of Toll (NF- κ B/ Dorsal) pathway in honey bee larvae (154, 183). Clothianidin exposure enhances the transcription of the NF- κ B inhibitor and suppresses the immune response. This eventually results in higher DWV replication (154). Similar to previous results, we determined that honey bees fed sucrose containing clothianidin (1 ppb) had reduced expression of key immune genes (i.e., *ago2*, *dcr-like*, *abaecin*, *hymenoptaecin*, and *vitellogenin*), which resulted in more severe virus infections, using virus abundance as a proxy for infection severity for a panel of viruses. Together our results coupled with those from previous studies, suggest that sublethal doses of clothianidin may increase the overall virus burden in a colony. However, honey bees fed sucrose syrup containing 10 ppb clothianidin had virus levels that were similar to, or lower than, levels in bees fed only sucrose syrup. Although additional studies are needed, we hypothesize that bees fed 10 ppb clothianidin may have been less suitable hosts for viruses, which are obligate intracellular pathogens and, thus, rely on healthy host cells for replication. An alternative hypothesis, which is not well supported by the data described herein is that reduced virus abundance in 10 ppb clothianidin fed bees is due to hormesis (i.e., a dose-dependent beneficial biological response to a chemical stressor) (184, 185). Insecticide-induced hormesis has been reported to have ‘positive’ effects on survival, reproduction, and immunity in bees and other insects and is involved in various life stages and different insecticide active ingredients (184-187). The possibility of virus reduction due to

hormetic effects was mentioned in another study that found DWV-infected honey bees fed 0.12 - 0.35 ng clothianidin per bee ($\sim 1.2 - 3.5$ ppb) had lower DWV levels than bees fed 0.05 ng clothianidin per bee (~ 0.5 ppb) (143). However, in our study, the beneficial virus limiting response in bees fed 10 ppb clothianidin did not correspond with greater immune gene expression, therefore the hormetic effect hypothesis is not well-supported.

Beekeepers also use in-hive chemical treatments as part of integrated pest management (IPM) strategies to maintain healthy honey bee colonies. These beekeeper-applied treatments include miticides and the antifungal compound fumagillin, which is used to mitigate *Nosema spp.* infections. Previous studies indicated that fumagillin treatments may negatively impact bee health, therefore a limit of two treatments per colony is included as a label advisory. To further investigate the impacts of fumagillin treatment on the honey bee immune system and the outcome of virus infection, honey bees were fed sucrose containing the recommended dose of 25 ppm, or a greater dose of 75 ppm. We determined that virus abundance was higher in bees fed sucrose syrup containing 25 ppm or 75 ppm fumagillin, relative to bees fed sucrose syrup only. The greater virus abundance is likely due to immune suppression, as the expression of select honey bee immune genes (i.e., *ago2*, *dcr-like*, *abaecin*, *hymenoptaecin*, and *vitellogenin*) were reduced in fumagillin fed bees. Although future studies are warranted before adjusting recommendations for honey bee colony management, our results suggest that beekeepers should consider trade-off of reducing *Nosema* infections, but increasing virus abundance. This consideration may be more important at the end of the beekeeping season in temperate climates (i.e., late autumn), since DWV infections are usually great at that time of year (15, 27, 28). While the focus of our experiments was to evaluate the impact of dual stressors on individual honey bee health, we determined that honey

bees exposed to a single stressor, i.e., fumagillin (25 ppm or 75 ppm) or clothianidin (1 ppb) had reduced levels of key immune genes (i.e., *ago2*, *dcr-like*, *abaecin*, *hymenoptaecin*, and *vitellogenin*) compared to bees fed sucrose syrup alone. The reduced expression of *vitellogenin* in fumagillin or clothianidin (1 ppb) fed bees indicates poor health status of the host while the reduced expression of key immune genes suggests hindered immune responses.

Collectively, the results described herein indicate that 0.16 ppm thyme oil acts as an immunostimulant in honey bees and results in reduced levels of virus infection. Additional studies that further examine the kinetics of immune stimulation and the impact of multiple treatments on virus infections over a longer time frame are required to help move these studies beyond the laboratory and towards colony level investigations that may benefit beekeeping operations. It will also be important to examine the impact of simultaneous exposure to putative immune system stimulants including thyme oil, in the context of varying doses of chemical stressors (fungicide and/or insecticide) on the outcome of honey bee virus infections. In addition, chemical adjuvants can negatively impact the outcome of virus infections in the context of other chemical stressors, and therefore their impact also requires further investigation (188). Studies that more clearly mimic the real-world multifactorial and variable biotic and abiotic stressors encountered by honey bees, and other beneficial insect pollinators, will be instrumental to the development of strategies aimed at mitigating their losses (11, 79, 133, 189-192).

4 Materials and Methods

4.1 Honey bees

Honey bee colonies were originally established as packages containing naturally mated *Apis mellifera carnica* queens and maintained in Langstroth hives in an apiary located at Montana State

University's Horticulture Farm in Bozeman, MT, USA. Frames of capped brood with emerging bees were obtained from established colonies one day prior to each experiment and kept at 32°C and in a laboratory incubator overnight. Age-matched (~24 h post-emergence) adult female, worker bees were collected and housed in modified deli-containers and randomly assigned into treatment groups. Honey bees in each treatment group were maintained in laboratory incubators at 32°C and ~ 20% relative humidity and fed 50% sucrose solution *ad libitum* with or without additives including thyme oil, fumagillin, and clothianidin as described below. Experiments described herein were carried out using honey bees collected from three separate colonies obtained during May-August 2020, designated as rep1, rep2, and rep3.

4.2 Virus preparation

4.2.1 Flock House virus (FHV)

Flock House virus (FHV) was propagated in *Drosophila melanogaster* Schneider 2 (S2) cells derived from *D. melanogaster* embryos. S2 cells were grown as monolayers in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and 1% penicillin-streptomycin as per manufacturer's instructions. To generate FHV particles, 4×10^7 S2 cells/ml were seeded in a sterile T75 flask (Thermo Scientific). An aliquot of FHV was kindly provided by Dr. Anette Schneemann (The Scripps Research Institute, La Jolla, California). S2 cells were infected with FHV at a multiplicity of infection of 1 pfu/cell and incubated at 28°C for 48 h as previously described (193). After incubation, the cells were lysed by addition of 10% (vol/vol) Nonidet P-40 and incubated on ice for 10 min with periodic swirling. Cell debris was pelleted in a Sorvall LYNX 4000 Centrifuge (Thermo Fisher) at 13,800x g for 10

min at 4°C and the clarified supernatant was transferred to a fresh tube. Virus was pelleted through a 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 (Beckman Coulter). The pellets were resuspended in 0.5 mL 50 mM HEPES (pH 7) and centrifuged at 10,000 rpm for 10 min to remove any insoluble material. The clarified supernatant was layered on continuous sucrose gradients (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 halfway down the tube. The sedimented virus was further pelleted in 10 mM Tris HCl buffer (pH 7.5) at 40,000 rpm for 2.5 h at 11°C for injections in honey bees. RNA was isolated from 100 µL virus preparations (as described below) and FHV abundance was quantified by RT-PCR and copy number was based on a standard curve. The dose utilized for laboratory-based honey bee infection studies was 3.5×10^8 FHV RNA copies per bee. FHV RNA 2 plasmid standards were used as templates, with concentrations ranging from 10^3 to 10^9 copies per reaction to create a linear standard curve, with a limit of detection of 10^3 copies of FHV cDNA using primers FHV-FW and FHV-REV (Supplemental Table S1). The linear equation for the plasmid standard for FHV was $Ct = -3.240x + 40.138$ ($R^2 = 0.980$, efficiency = 103%) where 'x' is the log (FHV RNA copies).

4.2.2 Deformed wing virus (DWV)

DWV was propagated in white eyed pupae collected from a frame of capped brood. Specifically, honey bee pupae were injected with 3.41×10^7 DWV RNA copies in 2 µL between the 2nd and 3rd integuments of the abdomen using microcapillary glass needle and a Harbo syringe (Honey bee Insemination Service). Post-injection, pupae were held at 30°C in a humid incubator for the course of infection. Infection progression was tracked by the eye color to ensure the pupae were alive. Live pupae were harvested at 10 days post infection and place in a 2 mL safe-lock Eppendorf tube

with 1 mL PBS (pH 7.4) and homogenized using a Tissue Lyser II (Qiagen) at 30 Hz for 2 min. The homogenate was centrifuged at 14,000x g for 15 min at 4°C and the clarified supernatant was transferred to a fresh tube. DWV RNA copies were quantified using qPCR to be 3.41×10^7 DWV RNA copies/ μ L. The inoculum was tested for copurifying/contaminating viruses (ABPV, BQCV, CBPV, IAPV, KBV, LSVs, and SBV) via PCR. No other viruses except DWV were detected via PCR. (Supplemental Figure S11). The dose utilized for laboratory-based honey bee infection studies was 3.5×10^8 DWV RNA copies per bee. DWV plasmid standards were used as templates, with concentrations ranging from 10^3 to 10^9 copies per reaction to create a linear standard curve, with a limit of detection of 10^3 copies of DWV cDNA using primers DWV-F 1170 and DWV-R 1364 (Supplemental Table S1). The linear equation for the plasmid standard for DWV was $Ct = -3.469x + 40.803$ ($R^2 = 0.999$, efficiency = 94.5%) where 'x' is the log (DWV RNA copies).

4.2.3 Sindbis-green fluorescent protein tagged (SINV-GFP)

We utilized a recombinant model virus, Sindbis virus expressing green fluorescent protein (42, 93, 194). We and others have used SINV to investigate honey bee, fruit fly, and mosquito antiviral defense mechanisms (42, 43, 61, 93). SINV was propagated in Baby Hamster Kidney fibroblast (BHK-21) cells. BHK-21 cells were grown as a monolayer in Eagle's Minimum Essential Medium with 10% fetal bovine serum. A 90% confluent flask was infected with a multiplicity of infection of 0.001 as previously described (94). Infected cells were incubated at 37°C with 5% carbon dioxide for 24 h. Cells were collected, and the cell debris was removed by centrifugation at 1000x g for 10 min. The clarified supernatant was quantified using a standard plaque assay (93). Each bee was injected with 3,750 plaque forming units (PFUs) SINV. 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, with a limit of detection of 10^3 copies of SINV-GFP cDNA using primers qSindbisFW4495 and qSindbisREV4635 (Supplemental Table S1). The linear equation for the plasmid standard for SINV-GFP was $Ct = -3.335x + 40.137$ ($R^2 = 0.993$, efficiency = 99.5%) where 'x' is the log (SINV-GFP RNA copies).

4.3 Honey bee virus infection

Age-matched (~24 h post-emergence) adult female, worker bees were cold anesthetized at 4°C for 10 min prior to intrathoracic injection of virus or buffer (mock-infection). Intra-thoracic injections were performed using a Harbo syringe (Honey Bee Insemination Service) and microcapillary glass needles, 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). Honey bees were infected with 3.5×10^8 FHV RNA copies/bee in 10mM Tris HCl buffer pH 7.5, 3.5×10^8 DWV RNA copies/bee in 10mM Tris HCl buffer pH 7.5 or 3,750 PFUs SINV-GFP. Mock-infected bees were injected with 2 μ L buffer (10mM Tris HCl, pH 7.5).

4.4 Honey bee diet preparation

Post-injection, honey bees were housed in modified deli containers for the duration of the study. Bees in the control group were fed 50% sucrose syrup only, whereas bees in treatment groups were fed sucrose syrup containing one of the following additives: 0.16 ppm thyme oil (Body wonders), Fumagilin-B®, fumagillin dicyclohexyl ammonium (Medivet Pharmaceuticals Ltd.) at manufacturer's recommended dose of 25 ppm or a higher dose of 75 ppm, or clothianidin at the field relevant sublethal concentration of 1 ppb or near lethal dose of 10 ppb (130, 195, 196). Thyme

oil contains 10% - 64% thymol (~37% average) depending on the plant species, geographical sources, and harvest season, which may affect the volatile composition of the plant (113, 116-118). We estimate that 0.16 ppm thyme oil used in this experiment may contain approximately 0.06 ppm (60 ppb) thymol (37% thymol in 0.16 ppm thyme oil corresponds to ~ 60 ppb). For clothianidin treatments we utilized the commercially available Poncho® 600, which contains 48% of the active ingredient clothianidin. A working stock of 1000 ppb clothianidin was prepared by 1:10 serial dilutions in 50% sucrose solution, which was further diluted in 50% sucrose to prepare 1 ppb (i.e., 10 μ L of 1000 ppb Poncho® 600) and 10 ppb (i.e., 100 μ L of 1000 ppb Poncho® 600) clothianidin solutions. Honey bees were fed sucrose solution either alone or with additives using cage feeders that were made by putting two holes on each side of a 1.5 mL centrifuge tube; sucrose solution was checked daily and refilled as needed throughout the study.

4.5 RNA isolation

Individual honey bee sample was dissected into head, thorax, and abdomen. The abdomen was utilized for further analysis since it is distal to the injection site and thus virus infection naturally spread into that tissue, and it contains the immune cell generating fat body. Honey bee abdomens were transferred into 2 mL Eppendorf safe-lock tubes and homogenized in 300 μ L deionized water with a sterile steel ball (5 mm) using a Tissue Lyser II (Qiagen) at 30 Hz for 2 min. An equal volume (300 μ L) of TRIzol reagent (Invitrogen) was added to homogenate and then samples were vortexed for 15 s followed by a 5 min incubation at room temperature. Next, 100 μ L chloroform was added and each sample was shaken by hand for 15 s and incubated at room temperature for 2 min. Samples were then centrifuged at 12,000x g at 4°C for 15 min and the aqueous phase was

transferred to a fresh 1.5 mL centrifuge tube. An equal volume of isopropanol was added to the aqueous phase, vortexed, and incubated at room temperature for 10 min to precipitate the nucleic acid. The nucleic acid was pelleted by centrifugation at 12000x g at 4°C for 10 min. Supernatant was carefully removed and the pellets were washed with 75% ethanol by centrifugation at 7500x g at 4°C for 5 min. The resulting supernatant was discarded, and the pellets were air dried for 10 min at room temperature, then dissolved in 50 µL deionized water. RNA concentrations were assessed on Nanodrop 2000 spectrophotometer (Thermo Fisher). All RNA samples were stored in -80°C until further analysis.

4.6 Reverse transcription/cDNA synthesis

Reverse transcription reactions were carried out to synthesize complimentary DNA (cDNA) by incubating 2,000 ng of RNA with M-MLV reverse transcriptase (Promega) and 500 ng random hexamer primers (IDT) for 1 h at 37°C according to manufacturer's instructions. The resulting cDNA was diluted 1:2 with sterile distilled water.

4.7 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used to test mock-infected bees in all experimental replicates for pre-existing infections with honey bee infecting viruses, since bees were obtained from honey bee colonies that may have varying levels of naturally prevalent bee infecting viruses (i.e., BQCV, CBPV, ABPV, DWV, IAPV, KBV, SBV, LSVs 1–4). To reduce the number of individual PCR tests, individual bee cDNA samples (n= 11-12) were pooled, resulting in three pooled samples per treatment. Each pool was tested for pre-existing virus infections using pathogen-specific primers

(Supplemental Table S1 and Supplemental Figure S10). PCR was performed according to standard methods (197). In brief, 2 μ L cDNA template was combined with 10 pmol of each forward and reverse primer, and amplified with Denville 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, 57°C for 30 s, 72°C for 30s, 35 cycles, followed by final elongation at 72°C for 4 min.

4.8 Quantitative PCR (qPCR)

Quantitative PCR was used to quantify relative abundances of viruses and honey immune gene transcripts using the primers listed in Supplemental Table S1. All qPCR reactions were performed in triplicate with 2 μ L of cDNA template. Each 20 μ L reaction contained 1 $\times$ ChoiceTaq Mastermix (Thomas Scientific), 0.4 μ M each forward and reverse primer, 1 $\times$ SYBR Green (Life Technologies), and 3 mM MgCl₂. A CFX Connect Real Time instrument (BioRad) was used to carry out reactions with the following thermo-profile: pre-incubation at 95°C for 1 min followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 15 s; final extension at 72 °C for 1 min, followed by melt curve analysis. Reactions without template were carried out as negative controls. The qPCR specificity was verified through melt point analysis and gel electrophoresis of select samples. The sequence amplicons were previously verified by chain-termination sequencing. As described above in section 4.2 for virus stock quantification, virus abundance in individual bee samples was determined relative to a standard curve that was generated by the use of virus amplicon specific plasmids, with concentrations ranging from 10³ to 10⁹ copies per reaction, as templates to create linear standard curves. The linear equations for each virus amplicon are included in section 4.2 above. The qPCR and mortality data for individual samples is included in

Supplemental Tables S3 and S5. The virus fold change for dead and live virus-infected honey bees fed chemical treatments was plotted for each treatment group and no significant difference in fold change was recorded in dead vs. live bees. Thus, in our analyses, data from dead and live bees was combined and presented.

To assess the relative expression of honey bee genes including immune genes (i.e., *argonaute-2*, *dicer-like*, *abaecin*, *hymenoptaecin*, *heat shock protein 90*, *heat shock protein 70 cognate 4*, *protein lethal (2) essential for life-like*), and *vitellogenin*, relative to the expression of a reference gene (i.e., *ribosomal protein L8*) was carried out using the gene-specific primer sets in Supplemental Table S1 and the $\Delta\Delta C_t$ method (198). Specifically, the relative expression of host genes was determined by a ranked $\Delta\Delta C_t$ method in which the ΔC_t was calculated by subtracting the *rpl8* C_t value from the C_t of the gene of interest. Then, the within-group ΔC_t values were ranked from highest to lowest, and the relevant corresponding control ΔC_t value was subtracted from the treatment group ΔC_t to obtain the $\Delta\Delta C_t$. The fold-change in cDNA abundance was calculated by the equation $2^{-\Delta\Delta C_t}$. Efficiency of each primer set was evaluated using cDNA dilution series and calculated by plotting $\log_{10}$ of the concentration versus the crossing point threshold ($C(t)$) values and using the primer efficiency equation $(10^{(1/\text{Slope})-1}) \times 100$. The efficiency equations of the primers utilized for honey bee immune genes are- *ago-2* $C_p = -3.36x + 30.28$, $R^2 = 0.99$; *dcr-like* $C_p = -3.56x + 31.22$, $R^2 = 0.99$; *abaecin* $C_p = -3.37x + 29.8$, $R^2 = 0.99$; *hymenoptaecin* $C_p = -3.44x + 30.50$, $R^2 = 0.99$; *hsp90* $C_p = -3.43x + 32.98$, $R^2 = 0.99$; *hsc70-4* $C_p = -3.36x + 31.52$, $R^2 = 0.99$; *pl2* $C_p = -3.16x + 30.12$, $R^2 = 0.99$; *vitellogenin* $C_p = -3.04x + 29.44$, $R^2 = 0.99$.

4.9 Statistical analysis

All data were analyzed using R v4.0.2 in RStudio V1.3.1073. Pairwise comparisons for virus abundance and gene expression were evaluated using the *pairwise.wilcox.test* function in the base R stats package to perform a Wilcoxon Rank Sums test with Benjamini-Hochberg correlation for multiple comparisons (199).

Conflict of Interest

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.

Author Contributions

Conceptualization, F.P., K.F.D., and M.L.F.; methodology, F.P., K.F.D., and M.L.F.; formal analysis F.P.; investigation, F.P. and M.L.F.; resources, M.L.F.; data curation, F.P. and M.L.F.; writing—original draft preparation, F.P. and M.L.F.; writing—review and editing, F.P. and M.L.F.; visualization, 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.

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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.

Supplementary Material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.747848/full#supplementary-material>

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REFERENCES CITED

1. Gallai N, Salles J-M, Settele J, Vaissière BE. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics* (2009) 68(3):810-21. doi: <https://doi.org/10.1016/j.ecolecon.2008.06.014>.
2. Eilers EJ, Kremen C, Smith Greenleaf S, Garber AK, Klein A-M. Contribution of Pollinator-Mediated Crops to Nutrients in the Human Food Supply. *PLOS ONE* (2011) 6(6):e21363. doi: 10.1371/journal.pone.0021363.
3. Klein A-M, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, et al. Importance of pollinators in changing landscapes for world crops. *Proceedings of the royal society B: biological sciences* (2007) 274(1608):303-13.
4. Calderone NW. Insect Pollinated Crops, Insect Pollinators and US Agriculture: Trend Analysis of Aggregate Data for the Period 1992–2009. *PLOS ONE* (2012) 7(5):e37235. doi: 10.1371/journal.pone.0037235.
5. Kulhanek K, Steinhauer N, Rennich K, Caron DM, Sagili RR, Pettis JS, et al. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. *Journal of Apicultural Research* (2017) 56(4):328-40. doi: 10.1080/00218839.2017.1344496.
6. Spleen AM, Lengerich EJ, Rennich K, Caron D, Rose R, Pettis JS, et al. A national survey of managed honey bee 2011–12 winter colony losses in the United States: results from the Bee Informed Partnership. *Journal of Apicultural Research* (2013) 52(2):44-53. doi: 10.3896/IBRA.1.52.2.07.
7. Steinhauer NA, Rennich K, Wilson ME, Caron DM, Lengerich EJ, Pettis JS, et al. A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. *Journal of Apicultural Research* (2014) 53(1):1-18. doi: 10.3896/IBRA.1.53.1.01.
8. Traynor KS, Rennich K, Forsgren E, Rose R, Pettis J, Kunkel G, et al. Multiyear survey targeting disease incidence in US honey bees. *Apidologie* (2016) 47(3):325-47. doi: 10.1007/s13592-016-0431-0.
9. vanEngelsdorp D, Caron D, Hayes J, Underwood R, Henson M, Rennich K, et al. A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. *Journal of Apicultural Research* (2012) 51(1):115-24. doi: 10.3896/IBRA.1.51.1.14.
10. Seitz N, Traynor KS, Steinhauer N, Rennich K, Wilson ME, Ellis JD, et al. A national survey of managed honey bee 2014–2015 annual colony losses in the USA. *Journal of Apicultural Research* (2015) 54(4):292-304. doi: 10.1080/00218839.2016.1153294.

11. McMenamin AJ, Brutscher LM, Glennly W, Flenniken ML. Abiotic and biotic factors affecting the replication and pathogenicity of bee viruses. *Current Opinion in Insect Science* (2016) 16:14-21. doi: <https://doi.org/10.1016/j.cois.2016.04.009>.
12. Brutscher LM, McMenamin AJ, Flenniken ML. The Buzz about Honey Bee Viruses. *PLOS Pathogens* (2016) 12(8):e1005757. doi: [10.1371/journal.ppat.1005757](https://doi.org/10.1371/journal.ppat.1005757).
13. Cornman RS, Tarpy DR, Chen Y, Jeffreys L, Lopez D, Pettis JS, et al. Pathogen Webs in Collapsing Honey Bee Colonies. *PLOS ONE* (2012) 7(8):e43562. doi: [10.1371/journal.pone.0043562](https://doi.org/10.1371/journal.pone.0043562).
14. vanEngelsdorp D, Meixner MD. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *Journal of Invertebrate Pathology* (2010) 103:S80-S95. doi: <https://doi.org/10.1016/j.jip.2009.06.011>.
15. Genersch E, von der Ohe W, Kaatz H, Schroeder A, Otten C, B  chler R, et al. The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies*. *Apidologie* (2010) 41(3):332-52.
16. Tentcheva D, Gauthier L, Zappulla N, Dainat B, Cousserans F, Colin ME, et al. Prevalence and Seasonal Variations of Six Bee Viruses in *Apis mellifera* L. and *Varroa destructor* Mite Populations in France. *Applied and Environmental Microbiology* (2004) 70(12):7185-91. doi: [doi:10.1128/AEM.70.12.7185-7191.2004](https://doi.org/10.1128/AEM.70.12.7185-7191.2004).
17. 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(1):417-32. doi: [10.1146/annurev-ento-010715-023731](https://doi.org/10.1146/annurev-ento-010715-023731).
18. Boecking O, Genersch E. Varroosis – the Ongoing Crisis in Bee Keeping. *Journal f  r Verbraucherschutz und Lebensmittelsicherheit* (2008) 3(2):221-8. doi: [10.1007/s00003-008-0331-y](https://doi.org/10.1007/s00003-008-0331-y).
19. de D'Aubeterre JP, Myrold DD, Royce LA, Rossignol PA. A scientific note of an application of isotope ratio mass spectrometry to feeding by the mite, *Varroa jacobsoni* Oudemans, on the honeybee, *Apis mellifera* L. *Apidologie* (1999) 30(4):351-2.
20. Annoscia D, Brown SP, Di Prisco G, De Paoli E, Del Fabbro S, Frizzera D, et al. Haemolymph removal by *Varroa* mite destabilizes the dynamical interaction between immune effectors and virus in bees, as predicted by Volterra's model. *Proceedings of the Royal Society B: Biological Sciences* (2019) 286(1901):20190331. doi: [doi:10.1098/rspb.2019.0331](https://doi.org/10.1098/rspb.2019.0331).
21. Ramsey SD, Ochoa R, Bauchan G, Gulbranson C, Mowery JD, Cohen A, et al. *Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph. *Proceedings of the National Academy of Sciences* (2019) 116(5):1792. doi: [10.1073/pnas.1818371116](https://doi.org/10.1073/pnas.1818371116).

22. Grozinger CM, Flenniken ML. Bee Viruses: Ecology, Pathogenicity, and Impacts. *Annual Review of Entomology* (2019) 64(1):205-26. doi: 10.1146/annurev-ento-011118-111942. PubMed PMID: 30629896.
23. Beaufort A, Piot N, Doublet V, Antunez K, Campbell E, Chantawannakul P, et al. Diversity and Global Distribution of Viruses of the Western Honey Bee, *Apis mellifera*. *Insects* (2020) 11(4):239. PubMed PMID: doi:10.3390/insects11040239.
24. McMenamin AJ, Flenniken ML. Recently identified bee viruses and their impact on bee pollinators. *Current Opinion in Insect Science* (2018) 26:120-9. doi: <https://doi.org/10.1016/j.cois.2018.02.009>.
25. de Miranda JR, Genersch E. Deformed wing virus. *Journal of Invertebrate Pathology* (2010) 103:S48-S61. doi: <https://doi.org/10.1016/j.jip.2009.06.012>.
26. Highfield AC, Nagar AE, Mackinder LCM, Noël LM-LJ, Hall MJ, Martin SJ, et al. Deformed Wing Virus Implicated in Overwintering Honeybee Colony Losses. *Applied and Environmental Microbiology* (2009) 75(22):7212-20. doi: doi:10.1128/AEM.02227-09.
27. Faurot-Daniels C, Glenny W, Daughenbaugh KF, McMenamin AJ, Burkle LA, Flenniken ML. 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(9):e0237544. doi: 10.1371/journal.pone.0237544.
28. Glenny W, Cavigli I, Daughenbaugh KF, Radford R, Kegley SE, Flenniken ML. Honey bee (*Apis mellifera*) colony health and pathogen composition in migratory beekeeping operations involved in California almond pollination. *PLoS One* (2017) 12(8):e0182814. Epub 2017/08/18. doi: 10.1371/journal.pone.0182814. PubMed PMID: 28817641; PubMed Central PMCID: PMC5560708.
29. 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(18):10241-52. doi: <https://doi.org/10.1002/ece3.5544>.
30. Antúnez K, Anido M, Branchiccela B, Harriet J, Campa J, Invernizzi C, et al. Seasonal Variation of Honeybee Pathogens and its Association with Pollen Diversity in Uruguay. *Microbial Ecology* (2015) 70(2):522-33. doi: 10.1007/s00248-015-0594-7.
31. Nazzi F, Brown SP, Annoscia D, Del Piccolo F, Di Prisco G, Varricchio P, et al. Synergistic Parasite-Pathogen Interactions Mediated by Host Immunity Can Drive the Collapse of Honeybee Colonies. *PLOS Pathogens* (2012) 8(6):e1002735. doi: 10.1371/journal.ppat.1002735.
32. Di Prisco G, Annoscia D, Margiotta M, Ferrara R, Varricchio P, Zanni V, et al. 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(12):3203-8.

33. Wilfert L, Long G, Leggett H, Schmid-Hempel P, Butlin R, Martin S, et al. Deformed wing virus is a recent global epidemic in honeybees driven by Varroa mites. *Science* (2016) 351(6273):594-7.
34. Ryabov EV, Wood GR, Fannon JM, Moore JD, Bull JC, Chandler D, et al. 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(6):e1004230. doi: 10.1371/journal.ppat.1004230.
35. Annoscia D, Del Piccolo F, Covre F, Nazzi F. Mite infestation during development alters the in-hive behaviour of adult honeybees. *Apidologie* (2015) 46(3):306-14.
36. Benaets K, Van Geystelen A, Cardoen D, De Smet L, de Graaf DC, Schoofs L, et al. Covert deformed wing virus infections have long-term deleterious effects on honeybee foraging and survival. *Proceedings of the Royal Society B: Biological Sciences* (2017) 284(1848):20162149. doi: doi:10.1098/rspb.2016.2149.
37. McMenamin AJ, Daughenbaugh KF, Parekh F, Pizzorno MC, Flenniken ML. Honey Bee and Bumble Bee Antiviral Defense. *Viruses* (2018) 10(8):395. PubMed PMID: doi:10.3390/v10080395.
38. Lourenço AP, Florecki MM, Simões ZLP, Evans JD. Silencing of *Apis mellifera* dorsal genes reveals their role in expression of the antimicrobial peptide defensin-1. *Insect molecular biology* (2018) 27(5):577-89.
39. Danihlík J, Aronstein K, Petřivalský M. Antimicrobial peptides: a key component of honey bee innate immunity. *Journal of Apicultural Research* (2015) 54(2):123-36. doi: 10.1080/00218839.2015.1109919.
40. Brutscher LM, Daughenbaugh KF, Flenniken ML. Antiviral defense mechanisms in honey bees. *Current Opinion in Insect Science* (2015) 10:71-82. doi: <https://doi.org/10.1016/j.cois.2015.04.016>.
41. Evans J, Aronstein K, Chen YP, Hetru C, Imler JL, Jiang H, et al. Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect molecular biology* (2006) 15(5):645-56.
42. Flenniken ML, Andino R. Non-specific dsRNA-mediated antiviral response in the honey bee. *Plos one* (2013) 8(10):e77263.
43. Brutscher LM, Daughenbaugh KF, Flenniken ML. Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. *Scientific reports* (2017) 7(1):1-15.

44. Chen YP, Pettis JS, Corona M, Chen WP, Li CJ, Spivak M, et al. Israeli Acute Paralysis Virus: Epidemiology, Pathogenesis and Implications for Honey Bee Health. *PLOS Pathogens* (2014) 10(7):e1004261. doi: 10.1371/journal.ppat.1004261.
45. Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E, et al. IAPV, a bee-affecting virus associated with colony collapse disorder can be silenced by dsRNA ingestion. *Insect molecular biology* (2009) 18(1):55-60.
46. 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):3172-85.
47. Niu J, Smagghe G, De Coninck DI, 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) 70:127-37. Epub 2015/12/30. doi: 10.1016/j.ibmb.2015.12.006. PubMed PMID: 26711439.
48. Hammond SM. Dicing and slicing: The core machinery of the RNA interference pathway. *FEBS Letters* (2005) 579(26):5822-9. doi: <https://doi.org/10.1016/j.febslet.2005.08.079>.
49. Ding S-W. RNA-based antiviral immunity. *Nature Reviews Immunology* (2010) 10(9):632-44. doi: 10.1038/nri2824.
50. Bronkhorst AW, van Rij RP. 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>.
51. Kingsolver MB, Hardy RW. Making connections in insect innate immunity. *Proceedings of the National Academy of Sciences* (2012) 109(46):18639-40. doi: 10.1073/pnas.1216736109.
52. Van Rij RP, Saleh M-C, Berry B, Foo C, Houk A, Antoniewski C, et al. The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in *Drosophila melanogaster*. *Genes & development* (2006) 20(21):2985-95.
53. Zamboni RA, Vakharia VN, Wu LP. RNAi is an antiviral immune response against a dsRNA virus in *Drosophila melanogaster*. *Cellular microbiology* (2006) 8(5):880-9.
54. Parker JS, Barford D. Argonaute: a scaffold for the function of short regulatory RNAs. *Trends in Biochemical Sciences* (2006) 31(11):622-30. doi: <https://doi.org/10.1016/j.tibs.2006.09.010>.
55. Wang X-H, Aliyari R, Li W-X, Li H-W, Kim K, Carthew R, et al. RNA Interference Directs Innate Immunity Against Viruses in Adult *Drosophila*. *Science* (2006) 312(5772):452-4. doi: 10.1126/science.1125694.

56. Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, Imler J-L. Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. *Nature Immunology* (2006) 7(6):590-7. doi: 10.1038/ni1335.
57. Galbraith DA, Yang X, Niño EL, Yi S, Grozinger C. Parallel Epigenomic and Transcriptomic Responses to Viral Infection in Honey Bees (*Apis mellifera*). *PLOS Pathogens* (2015) 11(3):e1004713. doi: 10.1371/journal.ppat.1004713.
58. 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) 454-455:176-83. doi: <https://doi.org/10.1016/j.virol.2014.02.012>.
59. Desai S, Eu YJ, Whyard S, Currie R. 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(4):446-55.
60. Merkling SH, Overheul GJ, van Mierlo JT, Arends D, Gilissen C, van Rij RP. The heat shock response restricts virus infection in *Drosophila*. *Scientific Reports* (2015) 5(1):12758. doi: 10.1038/srep12758.
61. McMenamin AJ, Daughenbaugh KF, Flenniken ML. The heat shock response in the Western honey bee (*Apis Mellifera*) is antiviral. *Viruses* (2020) 12(2):245.
62. McKinsty M, Chung C, Truong H, Johnston BA, Snow JW. The heat shock response and humoral immune response are mutually antagonistic in honey bees. *Scientific Reports* (2017) 7(1):8850. doi: 10.1038/s41598-017-09159-4.
63. Kampmueller KM, Miller DJ. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in *Drosophila* cells. *J Virol* (2005) 79(11):6827-37. Epub 2005/05/14. doi: 10.1128/jvi.79.11.6827-6837.2005. PubMed PMID: 15890922; PubMed Central PMCID: PMC1112161.
64. Glotzer JB, Saltik M, Chiocca S, Michou AI, Moseley P, Cotten M. Activation of heat-shock response by an adenovirus is essential for virus replication. *Nature* (2000) 407(6801):207-11. Epub 2000/09/23. doi: 10.1038/35025102. PubMed PMID: 11001061.
65. 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>.
66. Ryabov EV, Fannon JM, Moore JD, Wood GR, Evans DJ. 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.

67. Li Z, Zhang S, Zhang J, Liu M, Liu Z. Vitellogenin is a cidal factor capable of killing bacteria via interaction with lipopolysaccharide and lipoteichoic acid. *Molecular Immunology* (2009) 46(16):3232-9. doi: <https://doi.org/10.1016/j.molimm.2009.08.006>.
68. Li Z, Zhang S, Liu Q. Vitellogenin Functions as a Multivalent Pattern Recognition Receptor with an Opsonic Activity. *PLOS ONE* (2008) 3(4):e1940. doi: [10.1371/journal.pone.0001940](https://doi.org/10.1371/journal.pone.0001940).
69. Salmela H, Amdam GV, Freitak D. Transfer of Immunity from Mother to Offspring Is Mediated via Egg-Yolk Protein Vitellogenin. *PLOS Pathogens* (2015) 11(7):e1005015. doi: [10.1371/journal.ppat.1005015](https://doi.org/10.1371/journal.ppat.1005015).
70. Amdam GV, Omholt SW. The Regulatory Anatomy of Honeybee Lifespan. *Journal of Theoretical Biology* (2002) 216(2):209-28. doi: <https://doi.org/10.1006/jtbi.2002.2545>.
71. Amdam GV, Fennern E, Havukainen H. Vitellogenin in Honey Bee Behavior and Lifespan. In: Galizia CG, Eisenhardt D, Giurfa M, editors. *Honeybee Neurobiology and Behavior: A Tribute to Randolph Menzel*. Dordrecht: Springer Netherlands (2012). p. 17-29.
72. Corona M, Velarde RA, Remolina S, Moran-Lauter A, Wang Y, Hughes KA, et al. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proceedings of the National Academy of Sciences* (2007) 104(17):7128-33. doi: [10.1073/pnas.0701909104](https://doi.org/10.1073/pnas.0701909104).
73. Seehuus S-C, Norberg K, Gimsa U, Krekling T, Amdam GV. Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America* (2006) 103(4):962-7. doi: [10.1073/pnas.0502681103](https://doi.org/10.1073/pnas.0502681103).
74. Marco Antonio DS, Guidugli-Lazzarini KR, do Nascimento AM, Simões ZLP, Hartfelder K. RNAi-mediated silencing of vitellogenin gene function turns honeybee (*Apis mellifera*) workers into extremely precocious foragers. *Naturwissenschaften* (2008) 95(10):953-61. doi: [10.1007/s00114-008-0413-9](https://doi.org/10.1007/s00114-008-0413-9).
75. Münch D, Amdam G, Wolschin F. Ageing in a eusocial insect: molecular and physiological characteristics of life span plasticity in the honey bee. *Functional ecology* (2008) 22(3):407-21.
76. Pluta P, Sokół R. Changes in the expression of antimicrobial peptide genes in honey bees (*Apis mellifera*) under the influence of various pathogens. *Ann Parasitol* (2020) 66(4):457-65. Epub 2020/01/01. doi: [10.17420/ap6604.286](https://doi.org/10.17420/ap6604.286). PubMed PMID: 33646735.
77. Page RE, Rueppell O, Amdam GV. Genetics of Reproduction and Regulation of Honeybee (*Apis mellifera* L.) Social Behavior. *Annual Review of Genetics* (2012) 46(1):97-119. doi: [10.1146/annurev-genet-110711-155610](https://doi.org/10.1146/annurev-genet-110711-155610).

78. Dainat B, Evans JD, Chen YP, Gauthier L, Neumann P. Predictive Markers of Honey Bee Colony Collapse. *PLOS ONE* (2012) 7(2):e32151. doi: 10.1371/journal.pone.0032151.
79. Smart M, Pettis J, Rice N, Browning Z, Spivak M. Linking Measures of Colony and Individual Honey Bee Health to Survival among Apiaries Exposed to Varying Agricultural Land Use. *PLOS ONE* (2016) 11(3):e0152685. doi: 10.1371/journal.pone.0152685.
80. Zanni V, Galbraith DA, Annoscia D, Grozinger CM, Nazzi F. Transcriptional signatures of parasitization and markers of colony decline in Varroa-infested honey bees (*Apis mellifera*). *Insect Biochemistry and Molecular Biology* (2017) 87:1-13. doi: <https://doi.org/10.1016/j.ibmb.2017.06.002>.
81. Carrillo-Tripp J, Dolezal AG, Goblirsch MJ, Miller WA, Toth AL, Bonning BC. In vivo and in vitro infection dynamics of honey bee viruses. *Scientific Reports* (2016) 6(1):22265. doi: 10.1038/srep22265.
82. Lamp B, Url A, Seitz K, Eichhorn J, Riedel C, Sinn LJ, et al. Construction and Rescue of a Molecular Clone of Deformed Wing Virus (DWV). *PLOS ONE* (2016) 11(11):e0164639. doi: 10.1371/journal.pone.0164639.
83. Ryabov EV, Christmon K, Heerman MC, Posada-Florez F, Harrison RL, Chen Y, et al. Development of a Honey Bee RNA Virus Vector Based on the Genome of a Deformed Wing Virus. *Viruses* (2020) 12(4):374. doi: 10.3390/v12040374. PubMed PMID: 32231059.
84. Seitz K, Buczolic K, Dikunová A, Plevka P, Power K, Rümenapf T, et al. A molecular clone of Chronic Bee Paralysis Virus (CBPV) causes mortality in honey bee pupae (*Apis mellifera*). *Scientific Reports* (2019) 9(1):16274. doi: 10.1038/s41598-019-52822-1.
85. O'Neal ST, Swale DR, Anderson TD. ATP-sensitive inwardly rectifying potassium channel regulation of viral infections in honey bees. *Scientific reports* (2017) 7(1):1-9.
86. Dasgupta R, Free HM, Zietlow SL, Paskewitz SM, Aksoy S, Shi L, et al. Replication of Flock House Virus in Three Genera of Medically Important Insects. *Journal of Medical Entomology* (2007) 44(1):102-10. doi: 10.1093/jmedent/41.5.102.
87. Dasgupta R, Cheng L-L, Bartholomay LC, Christensen BM. Flock house virus replicates and expresses green fluorescent protein in mosquitoes. *Journal of General Virology* (2003) 84(7):1789-97. doi: <https://doi.org/10.1099/vir.0.18938-0>.
88. Dearing SC, Scotti PD, Wigley PJ, Dhana SD. A small RNA virus isolated from the grass grub, *Costelytra zealandica* (Coleoptera: Scarabaeidae). *New Zealand Journal of Zoology* (1980) 7(2):267-9. doi: 10.1080/03014223.1980.10423785.
89. Dasgupta R, Selling B, Rueckert R, editors. Flock house virus: a simple model for studying persistent infection in cultured *Drosophila* cells. 1994; Vienna: Springer Vienna.

90. Taning CNT, Christiaens O, Li X, Swevers L, Casteels H, Maes M, et al. Engineered Flock House Virus for Targeted Gene Suppression Through RNAi in Fruit Flies (*Drosophila melanogaster*) in Vitro and in Vivo. *Frontiers in Physiology* (2018) 9(805). doi: 10.3389/fphys.2018.00805.
91. Scotti PD, Dearing S, Mossop DW. Flock house virus: A Nodavirus isolated from *Costelytra zealandica* (White) (Coleoptera: Scarabaeida). *Archives of Virology* (1983) 75(3):181-9. doi: 10.1007/BF01315272.
92. Bonning BC, Saleh M-C. The Interplay Between Viruses and RNAi Pathways in Insects. *Annual Review of Entomology* (2021) 66(1):61-79. doi: 10.1146/annurev-ento-033020-090410. PubMed PMID: 33417818.
93. Saleh M-C, Tassetto M, Van Rij RP, Goic B, Gausson V, Berry B, et al. Antiviral immunity in *Drosophila* requires systemic RNA interference spread. *Nature* (2009) 458(7236):346-50.
94. Nayak A, Berry B, Tassetto M, Kunitomi M, Acevedo A, Deng C, et al. Cricket paralysis virus antagonizes Argonaute 2 to modulate antiviral defense in *Drosophila*. *Nature Structural & Molecular Biology* (2010) 17(5):547-54. doi: 10.1038/nsmb.1810.
95. Kemp C, Imler J-L. Antiviral immunity in *drosophila*. *Current opinion in immunology* (2009) 21(1):3-9.
96. Blair CD. Mosquito RNAi is the major innate immune pathway controlling arbovirus infection and transmission. *Future microbiology* (2011) 6(3):265-77.
97. Alaux C, Ducloz F, Crauser D, Le Conte Y. Diet effects on honeybee immunocompetence. *Biology letters* (2010) 6(4):562-5.
98. Dolezal AG, Carrillo-Tripp J, Judd TM, Allen Miller W, Bonning BC, Toth AL. Interacting stressors matter: diet quality and virus infection in honeybee health. *Royal Society open science* (2019) 6(2):181803.
99. Negri P, Villalobos E, Szawarski N, Damiani N, Gende L, Garrido M, et al. Towards Precision Nutrition: A Novel Concept Linking Phytochemicals, Immune Response and Honey Bee Health. *Insects* (2019) 10(11):401. PubMed PMID: doi:10.3390/insects10110401.
100. DeGrandi-Hoffman G, Chen Y. Nutrition, immunity and viral infections in honey bees. *Current Opinion in Insect Science* (2015) 10:170-6. doi: <https://doi.org/10.1016/j.cois.2015.05.007>.
101. Brodschneider R, Crailsheim K. Nutrition and health in honey bees*. *Apidologie* (2010) 41(3):278-94.

102. Drescher N, Klein A-M, Schmitt T, Leonhardt SD. A clue on bee glue: New insight into the sources and factors driving resin intake in honeybees (*Apis mellifera*). PLOS ONE (2019) 14(2):e0210594. doi: 10.1371/journal.pone.0210594.
103. Tauber JP, Collins WR, Schwarz RS, Chen Y, Grubbs K, Huang Q, et al. Natural Product Medicines for Honey Bees: Perspective and Protocols. Insects (2019) 10(10):356. doi: 10.3390/insects10100356. PubMed PMID: 31635365.
104. Palmer-Young EC, Tozkar CÖ, Schwarz RS, Chen Y, Irwin RE, Adler LS, et al. Nectar and Pollen Phytochemicals Stimulate Honey Bee (Hymenoptera: Apidae) Immunity to Viral Infection. Journal of Economic Entomology (2017) 110(5):1959-72. doi: 10.1093/jee/tox193.
105. Strachecka A, Krauze M, Olszewski K, Borsuk G, Paleolog J, Merska M, et al. Unexpectedly strong effect of caffeine on the vitality of western honeybees (*Apis mellifera*). Biochemistry (Moscow) (2014) 79(11):1192-201. doi: 10.1134/S0006297914110066.
106. Negri P, Ramirez L, Quintana S, Szawarski N, Maggi MD, Eguaras MJ, et al. Immune-related gene expression of *Apis mellifera* larvae in response to cold stress and Absciscic Acid (ABA) dietary supplementation. Journal of Apicultural Research (2020) 59(4):669-76. doi: 10.1080/00218839.2019.1708653.
107. Mao W, Schuler MA, Berenbaum MR. Honey constituents up-regulate detoxification and immunity genes in the western honey bee *Apis mellifera*. Proceedings of the National Academy of Sciences (2013) 110(22):8842-6. doi: 10.1073/pnas.1303884110.
108. Wiese N, Fischer J, Heidler J, Lewkowski O, Degenhardt J, Erler S. The terpenes of leaves, pollen, and nectar of thyme (*Thymus vulgaris*) inhibit growth of bee disease-associated microbes. Scientific Reports (2018) 8(1):14634. doi: 10.1038/s41598-018-32849-6.
109. Palmer-Young EC, Sadd BM, Stevenson PC, Irwin RE, Adler LS. Bumble bee parasite strains vary in resistance to phytochemicals. Scientific Reports (2016) 6(1):37087. doi: 10.1038/srep37087.
110. Rennich K, Pettis J, VanEngelsdorp D, Bozarth R, Eversole H, Roccacaccia K, et al. 2011-2012 National Honey Bee Pests and Diseases Survey Report. USDA Animal Plant and Health Inspection (2012).
111. Nozal MJ, Bernal JL, Jiménez JJ, González MJ, Higes M. Extraction of thymol, eucalyptol, menthol, and camphor residues from honey and beeswax: Determination by gas chromatography with flame ionization detection. Journal of Chromatography A (2002) 954(1):207-15. doi: [https://doi.org/10.1016/S0021-9673\(02\)00153-X](https://doi.org/10.1016/S0021-9673(02)00153-X).
112. Šegvić Klarić M, Kosalec I, Mastelić J, Piecková E, Pepeljnak S. Antifungal activity of thyme (*Thymus vulgaris* L.) essential oil and thymol against moulds from damp dwellings. Letters in applied microbiology (2007) 44(1):36-42.

113. McGimpsey JA, Douglas MH, Van Klink JW, Beauregard DA, Perry NB. Seasonal variation in essential oil yield and composition from naturalized *Thymus vulgaris* L. in New Zealand. *Flavour and Fragrance Journal* (1994) 9(6):347-52.
114. Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology* (2004) 94(3):223-53. doi: <https://doi.org/10.1016/j.ijfoodmicro.2004.03.022>.
115. Lens-Lisbonne C, Cremieux A, Maillard C, Balansard G. Methodes d'évaluation de l'activité antibactérienne des huiles essentielles: application aux essences de thym et de cannelle. *J Pharm Belg* (1987) 42(5):297-302.
116. Arras G, Grella GE. Wild thyme, *Thymus capitatus*, essential oil seasonal changes and antimycotic activity. *Journal of Horticultural Science* (1992) 67(2):197-202. doi: 10.1080/00221589.1992.11516237.
117. Cosentino S, Tuberoso CIG, Pisano B, Satta M, Mascia V, Arzedi E, et al. In-vitro antimicrobial activity and chemical composition of Sardinian thymus essential oils. *Letters in applied microbiology* (1999) 29(2):130-5.
118. Juliano C, Mattana A, Usai M. Composition and in vitro Antimicrobial Activity of the Essential Oil of *Thymus herba-barona* Loisel Growing Wild in Sardinia. *Journal of Essential Oil Research* (2000) 12(4):516-22. doi: 10.1080/10412905.2000.9699578.
119. Gregorc A, Planinc I. The control of *Varroa destructor* in honey bee colonies using the thymol-based acaricide-Apiguard. *American Bee Journal* (2005) 145(8):672-5.
120. Floris I, Satta A, Cabras P, Garau VL, Angioni A. Comparison Between Two Thymol Formulations in the Control of *Varroa destructor*: Effectiveness, Persistence, and Residues. *Journal of Economic Entomology* (2004) 97(2):187-91. doi: 10.1093/jee/97.2.187.
121. Chen Y, Evans JD, Smith IB, Pettis JS. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. *Journal of Invertebrate Pathology* (2008) 97(2):186-8. doi: <https://doi.org/10.1016/j.jip.2007.07.010>.
122. Klee J, Besana AM, Genersch E, Gisder S, Nanetti A, Tam DQ, et al. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. *Journal of Invertebrate Pathology* (2007) 96(1):1-10. doi: <https://doi.org/10.1016/j.jip.2007.02.014>.
123. Richardson LL, Adler LS, Leonard AS, Andicoechea J, Regan KH, Anthony WE, et al. Secondary metabolites in floral nectar reduce parasite infections in bumblebees. *Proceedings of the Royal Society B: Biological Sciences* (2015) 282(1803):20142471.

124. Costa C, Lodesani M, Maistrello L. Effect of thymol and resveratrol administered with candy or syrup on the development of *Nosema ceranae* and on the longevity of honeybees (*Apis mellifera* L.) in laboratory conditions*. *Apidologie* (2010) 41(2):141-50.
125. Krupke CH, Hunt GJ, Eitzer BD, Andino G, Given K. Multiple Routes of Pesticide Exposure for Honey Bees Living Near Agricultural Fields. *PLOS ONE* (2012) 7(1):e29268. doi: 10.1371/journal.pone.0029268.
126. Krupke CH, Holland JD, Long EY, Eitzer BD. Planting of neonicotinoid-treated maize poses risks for honey bees and other non-target organisms over a wide area without consistent crop yield benefit. *Journal of Applied Ecology* (2017) 54(5):1449-58. doi: <https://doi.org/10.1111/1365-2664.12924>.
127. Jeschke P, Nauen R. Neonicotinoids—from zero to hero in insecticide chemistry. *Pest Management Science: formerly Pesticide Science* (2008) 64(11):1084-98.
128. Cabirol A, Haase A. The Neurophysiological Bases of the Impact of Neonicotinoid Pesticides on the Behaviour of Honeybees. *Insects* (2019) 10(10):344. PubMed PMID: doi:10.3390/insects10100344.
129. Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends in Pharmacological Sciences* (2001) 22(11):573-80. doi: [https://doi.org/10.1016/S0165-6147\(00\)01820-4](https://doi.org/10.1016/S0165-6147(00)01820-4).
130. Laurino D, Manino A, Patetta A, Porporato M. Toxicity of neonicotinoid insecticides on different honey bee genotypes. (2013).
131. Iwasa T, Motoyama N, Ambrose JT, Roe RM. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Protection* (2004) 23(5):371-8. doi: <https://doi.org/10.1016/j.cropro.2003.08.018>.
132. Decourtye A, Devillers J. Ecotoxicity of Neonicotinoid Insecticides to Bees. In: Thany SH, editor. *Insect Nicotinic Acetylcholine Receptors*. New York, NY: Springer New York (2010). p. 85-95.
133. Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, vanEngelsdorp D, et al. High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. *PLOS ONE* (2010) 5(3):e9754. doi: 10.1371/journal.pone.0009754.
134. Wu JY, Anelli CM, Sheppard WS. Sub-Lethal Effects of Pesticide Residues in Brood Comb on Worker Honey Bee (*Apis mellifera*) Development and Longevity. *PLOS ONE* (2011) 6(2):e14720. doi: 10.1371/journal.pone.0014720.
135. Rundlöf M, Andersson GKS, Bommarco R, Fries I, Hederström V, Herbertsson L, et al. Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature* (2015) 521(7550):77-80. doi: 10.1038/nature14420.

136. Sanchez-Bayo F, Goka K. Pesticide Residues and Bees – A Risk Assessment. PLOS ONE (2014) 9(4):e94482. doi: 10.1371/journal.pone.0094482.
137. Bonmatin JM, Giorio C, Girolami V, Goulson D, Kreutzweiser DP, Krupke C, et al. Environmental fate and exposure; neonicotinoids and fipronil. Environmental Science and Pollution Research (2015) 22(1):35-67. doi: 10.1007/s11356-014-3332-7.
138. Botías C, David A, Horwood J, Abdul-Sada A, Nicholls E, Hill E, et al. Neonicotinoid Residues in Wildflowers, a Potential Route of Chronic Exposure for Bees. Environmental Science & Technology (2015) 49(21):12731-40. doi: 10.1021/acs.est.5b03459.
139. Johnson RM, Ellis MD, Mullin CA, Frazier M. Pesticides and honey bee toxicity – USA*. Apidologie (2010) 41(3):312-31.
140. Cutler CG, Scott-Dupree CD. Exposure to Clothianidin Seed-Treated Canola Has No Long-Term Impact on Honey Bees. Journal of Economic Entomology (2007) 100(3):765-72. doi: 10.1093/jee/100.3.765.
141. Morfin N, Goodwin PH, Correa-Benitez A, Guzman-Novoa E. Sublethal exposure to clothianidin during the larval stage causes long-term impairment of hygienic and foraging behaviours of honey bees. Apidologie (2019) 50(5):595-605. doi: 10.1007/s13592-019-00672-1.
142. Morfin N, Goodwin PH, Guzman-Novoa E. Interaction of field realistic doses of clothianidin and Varroa destructor parasitism on adult honey bee (*Apis mellifera* L.) health and neural gene expression, and antagonistic effects on differentially expressed genes. PLOS ONE (2020) 15(2):e0229030. doi: 10.1371/journal.pone.0229030.
143. Morfin N, Goodwin PH, Hunt GJ, Guzman-Novoa E. Effects of sublethal doses of clothianidin and/or *V. destructor* on honey bee (*Apis mellifera*) self-grooming behavior and associated gene expression. Scientific Reports (2019) 9(1):5196. doi: 10.1038/s41598-019-41365-0.
144. Abdelkader FB, Kairo G, Bonnet M, Barbouche N, Belzunces LP, Brunet JL. Effects of clothianidin on antioxidant enzyme activities and malondialdehyde level in honey bee drone semen. Journal of Apicultural Research (2019) 58(5):740-5. doi: 10.1080/00218839.2019.1655182.
145. Matsumoto T. Reduction in homing flights in the honey bee *Apis mellifera* after a sublethal dose of neonicotinoid insecticides. Bulletin of Insectology (2013) 66(1):1-9.
146. Tison L, Duer A, Púčiková V, Greggers U, Menzel R. Detrimental effects of clothianidin on foraging and dance communication in honey bees. PLOS ONE (2020) 15(10):e0241134. doi: 10.1371/journal.pone.0241134.
147. Samson-Robert O, Labrie G, Chagnon M, Fournier V. Planting of neonicotinoid-coated corn raises honey bee mortality and sets back colony development. PeerJ (2017) 5:e3670.

148. Tsvetkov N, Samson-Robert O, Sood K, Patel HS, Malena DA, Gajiwala PH, et al. Chronic exposure to neonicotinoids reduces honey bee health near corn crops. *Science* (2017) 356(6345):1395-7. doi: 10.1126/science.aam7470.
149. Zhu YC, Adamczyk J, Rinderer T, Yao J, Danka R, Luttrell R, et al. Spray Toxicity and Risk Potential of 42 Commonly Used Formulations of Row Crop Pesticides to Adult Honey Bees (Hymenoptera: Apidae). *Journal of Economic Entomology* (2015) 108(6):2640-7. doi: 10.1093/jee/fov269.
150. Brandt A, Gorenflo A, Siede R, Meixner M, Büchler R. The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (*Apis mellifera* L.). *Journal of Insect Physiology* (2016) 86:40-7. doi: <https://doi.org/10.1016/j.jinsphys.2016.01.001>.
151. Sánchez-Bayo F, Goulson D, Pennacchio F, Nazzi F, Goka K, Desneux N. Are bee diseases linked to pesticides? - A brief review. *Environ Int* (2016) 89-90:7-11. Epub 2016/01/31. doi: 10.1016/j.envint.2016.01.009. PubMed PMID: 26826357.
152. 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). Epub 2018/03/31. doi: 10.3390/v10040159. PubMed PMID: 29601473; PubMed Central PMCID: PMC5923453.
153. DeGrandi-Hoffman G, Chen Y, Simonds R. The Effects of Pesticides on Queen Rearing and Virus Titers in Honey Bees (*Apis mellifera* L.). *Insects* (2013) 4(1):71-89. PubMed PMID: doi:10.3390/insects4010071.
154. Di Prisco G, Cavaliere V, Annoscia D, Varricchio P, Caprio E, Nazzi F, et al. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences* (2013) 110(46):18466-71. doi: 10.1073/pnas.1314923110.
155. Alburaki M, Boutin S, Mercier P-L, Loublier Y, Chagnon M, Derome N. Neonicotinoid-Coated Zea mays Seeds Indirectly Affect Honeybee Performance and Pathogen Susceptibility in Field Trials. *PLOS ONE* (2015) 10(5):e0125790. doi: 10.1371/journal.pone.0125790.
156. Doublet V, Labarussias M, de Miranda JR, Moritz RF, Paxton RJ. Bees under stress: sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle. *Environmental microbiology* (2015) 17(4):969-83.
157. Coulon M, Schurr F, Martel A-C, Cougoule N, Bégaud A, Mangoni P, et al. Influence of chronic exposure to thiamethoxam and chronic bee paralysis virus on winter honey bees. *PLOS ONE* (2019) 14(8):e0220703. doi: 10.1371/journal.pone.0220703.
158. Bailey L. The Treatment of Nosema Disease with Fumagillin. *Bee World* (1953) 34(7):136-7. doi: 10.1080/0005772X.1953.11094806.

159. Higes M, Nozal MJ, Alvaro A, Barrios L, Meana A, Martín-Hernández R, et al. The stability and effectiveness of fumagillin in controlling *Nosema ceranae* (Microsporidia) infection in honey bees (*Apis mellifera*) under laboratory and field conditions. *Apidologie* (2011) 42(3):364-77. doi: 10.1007/s13592-011-0003-2.
160. Didier ES. Effects of albendazole, fumagillin, and TNP-470 on microsporidial replication in vitro. *Antimicrobial Agents and Chemotherapy* (1997) 41(7):1541-6. doi: 10.1128/AAC.41.7.1541.
161. Pajuelo AG, Torres C, Bermejo FJO. Colony losses: a double blind trial on the influence of supplementary protein nutrition and preventative treatment with fumagillin against *Nosema ceranae*. *Journal of Apicultural Research* (2008) 47(1):84-6.
162. Williams GR, Shutler D, Little CM, Burgher-MacLellan KL, Rogers REL. The microsporidian *Nosema ceranae*, the antibiotic Fumagilin-B®, and western honey bee (*Apis mellifera*) colony strength. *Apidologie* (2011) 42(1):15-22. doi: 10.1051/apido/2010030.
163. Williams GR, Sampson MA, Shutler D, Rogers REL. Does fumagillin control the recently detected invasive parasite *Nosema ceranae* in western honey bees (*Apis mellifera*)? *Journal of Invertebrate Pathology* (2008) 99(3):342-4. doi: <https://doi.org/10.1016/j.jip.2008.04.005>.
164. Huang W-F, Solter LF, Yau PM, Imai BS. *Nosema ceranae* Escapes Fumagillin Control in Honey Bees. *PLOS Pathogens* (2013) 9(3):e1003185. doi: 10.1371/journal.ppat.1003185.
165. Lopez MI, Pettis JS, Smith IB, Chu P-S. Multiclass Determination and Confirmation of Antibiotic Residues in Honey Using LC-MS/MS. *Journal of Agricultural and Food Chemistry* (2008) 56(5):1553-9. doi: 10.1021/jf073236w.
166. Webster TC. Fumagillin Affects *Nosema apis* and Honey Bees (Hymenoptera: Apidae). *Journal of Economic Entomology* (1994) 87(3):601-4. doi: 10.1093/jee/87.3.601.
167. Rada V, Máchová M, Huk J, Marounek M, Dušková D. Microflora in the honeybee digestive tract: counts, characteristics and sensitivity to veterinary drugs. *Apidologie* (1997) 28(6):357-65.
168. van den Heever JP, Thompson TS, Otto SJG, Curtis JM, Ibrahim A, Pernal SF. The effect of dicyclohexylamine and fumagillin on *Nosema ceranae*-infected honey bee (*Apis mellifera*) mortality in cage trial assays. *Apidologie* (2016) 47(5):663-70. doi: 10.1007/s13592-015-0411-9.
169. Miyoshi T, Takeuchi A, Siomi H, Siomi MC. A direct role for Hsp90 in pre-RISC formation in *Drosophila*. *Nat Struct Mol Biol* (2010) 17(8):1024-6. Epub 2010/07/20. doi: 10.1038/nsmb.1875. PubMed PMID: 20639883.
170. Zhao R, Houry WA. Hsp90: a chaperone for protein folding and gene regulation. *Biochemistry and Cell Biology* (2005) 83(6):703-10. doi: 10.1139/o05-158 %M 16333321.

171. Aquino DA, Peng D, Lopez C, Farooq M. The Constitutive Heat Shock Protein-70 Is Required for Optimal Expression of Myelin Basic Protein During Differentiation of Oligodendrocytes. *Neurochemical Research* (1998) 23(3):413-20. doi: 10.1023/A:1022473904335.
172. Liu T, Daniels CK, Cao S. Comprehensive review on the HSC70 functions, interactions with related molecules and involvement in clinical diseases and therapeutic potential. *Pharmacology & Therapeutics* (2012) 136(3):354-74. doi: <https://doi.org/10.1016/j.pharmthera.2012.08.014>.
173. Iwasaki S, Kobayashi M, Yoda M, Sakaguchi Y, Katsuma S, Suzuki T, et al. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol Cell* (2010) 39(2):292-9. Epub 2010/07/08. doi: 10.1016/j.molcel.2010.05.015. PubMed PMID: 20605501.
174. Lourenço AP, Guidugli-Lazzarini KR, Freitas FCP, Bitondi MMG, Simões ZLP. Bacterial infection activates the immune system response and dysregulates microRNA expression in honey bees. *Insect Biochemistry and Molecular Biology* (2013) 43(5):474-82. doi: <https://doi.org/10.1016/j.ibmb.2013.03.001>.
175. Schlüns H, Crozier R. Relish regulates expression of antimicrobial peptide genes in the honeybee, *Apis mellifera*, shown by RNA interference. *Insect molecular biology* (2007) 16(6):753-9.
176. Gättschenberger H, Azzami K, Tautz J, Beier H. Antibacterial Immune Competence of Honey Bees (*Apis mellifera*) Is Adapted to Different Life Stages and Environmental Risks. *PLOS ONE* (2013) 8(6):e66415. doi: 10.1371/journal.pone.0066415.
177. Spivak M, Reuter GS. Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. *Apidologie* (2001) 32(6):555-65.
178. Di Pasquale G, Salignon M, Le Conte Y, Belzunces LP, Decourtye A, Kretzschmar A, et al. Influence of Pollen Nutrition on Honey Bee Health: Do Pollen Quality and Diversity Matter? *PLOS ONE* (2013) 8(8):e72016. doi: 10.1371/journal.pone.0072016.
179. Pritchard DJ. Grooming by honey bees as a component of varroa resistant behavior. *Journal of Apicultural Research* (2016) 55(1):38-48. doi: 10.1080/00218839.2016.1196016.
180. Sandrock C, Tanadini M, Tanadini LG, Fauser-Misslin A, Potts SG, Neumann P. Impact of Chronic Neonicotinoid Exposure on Honeybee Colony Performance and Queen Supersedure. *PLOS ONE* (2014) 9(8):e103592. doi: 10.1371/journal.pone.0103592.
181. Wade A, Lin C-H, Kurkul C, Regan ER, Johnson RM. Combined toxicity of insecticides and fungicides applied to California almond orchards to honey bee larvae and adults. *Insects* (2019) 10(1):20.

182. Shi J, Liao C, Wang Z, Zeng Z, Wu X. Effects of sublethal acetamiprid doses on the lifespan and memory-related characteristics of honey bee (*Apis mellifera*) workers. *Apidologie* (2019) 50(4):553-63. doi: 10.1007/s13592-019-00669-w.
183. Annoscia D, Di Prisco G, Becchimanzi A, Caprio E, Frizzera D, Linguadoca A, et al. Neonicotinoid Clothianidin reduces honey bee immune response and contributes to Varroa mite proliferation. *Nature Communications* (2020) 11(1):5887. doi: 10.1038/s41467-020-19715-8.
184. Cutler GC. Insects, Insecticides and Hormesis: Evidence and Considerations for Study. *Dose-Response* (2013) 11(2):dose-response.12-008.Cutler. doi: 10.2203/dose-response.12-008.Cutler. PubMed PMID: 23930099.
185. Guedes RNC, Cutler GC. Insecticide-induced hormesis and arthropod pest management. *Pest Management Science* (2014) 70(5):690-7.
186. Cutler GC, Rix RR. Can poisons stimulate bees? Appreciating the potential of hormesis in bee–pesticide research. *Pest management science* (2015) 71(10):1368-70.
187. Cohen E. Pesticide-mediated homeostatic modulation in arthropods. *Pesticide Biochemistry and Physiology* (2006) 85(1):21-7. doi: <https://doi.org/10.1016/j.pestbp.2005.09.002>.
188. Fine JD, Cox-Foster DL, Mullin CA. An Inert Pesticide Adjuvant Synergizes Viral Pathogenicity and Mortality in Honey Bee Larvae. *Scientific Reports* (2017) 7(1):40499. doi: 10.1038/srep40499.
189. O’Neal ST, Anderson TD, Wu-Smart JY. Interactions between pesticides and pathogen susceptibility in honey bees. *Current Opinion in Insect Science* (2018) 26:57-62. doi: <https://doi.org/10.1016/j.cois.2018.01.006>.
190. Vaudo AD, Tooker JF, Grozinger CM, Patch HM. Bee nutrition and floral resource restoration. *Current Opinion in Insect Science* (2015) 10:133-41. doi: <https://doi.org/10.1016/j.cois.2015.05.008>.
191. Goulson D, Nicholls E, Botías C, Rotheray EL. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* (2015) 347(6229):1255957. doi: 10.1126/science.1255957.
192. Collison E, Hird H, Cresswell J, Tyler C. Interactive effects of pesticide exposure and pathogen infection on bee health - a critical analysis. *Biol Rev Camb Philos Soc* (2016) 91(4):1006-19. Epub 2015/07/08. doi: 10.1111/brv.12206. PubMed PMID: 26150129.
193. Marshall D, Schneemann A. Specific Packaging of Nodaviral RNA2 Requires the N-Terminus of the Capsid Protein. *Virology* (2001) 285(1):165-75. doi: <https://doi.org/10.1006/viro.2001.0951>.

194. Rice CM, Levis R, Strauss JH, Huang HV. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. *Journal of virology* (1987) 61(12):3809-19. doi: 10.1128/JVI.61.12.3809-3819.1987. PubMed PMID: 3479621.
195. Lin CH, Sponsler DB, Richardson RT, Watters HD, Glinski DA, Henderson WM, et al. Honey bees and neonicotinoid-treated corn seed: contamination, exposure, and effects. *Environmental toxicology and chemistry* (2021) 40(4):1212-21.
196. Blacqui re T, Smagghe G, van Gestel CAM, Mommaerts V. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology* (2012) 21(4):973-92. doi: 10.1007/s10646-012-0863-x.
197. de Miranda JR, Bailey L, Ball BV, Blanchard P, Budge GE, Chejanovsky N, et al. Standard methods for virus research in *Apis mellifera*. *Journal of Apicultural Research* (2013) 52(4):1-56. doi: 10.3896/IBRA.1.52.4.22.
198. Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol* (2002) 30(6):503-12. Epub 2002/06/14. doi: 10.1016/s0301-472x(02)00806-8. PubMed PMID: 12063017.
199. Bauer DF. Constructing Confidence Sets Using Rank Statistics. *Journal of the American Statistical Association* (1972) 67(339):687-90. doi: 10.1080/01621459.1972.10481279.

CHAPTER FOUR

INVESTIGATING VIRUS-HOST INTERACTIONS IN
CULTURED PRIMARY HONEY BEE CELLS

Contributions of authors and Co-authors

Manuscript in Chapter Four

Co- First Author: Alexander J. McMenamin

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

Co-First 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

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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- κ B (Nuclear Factor κ B) 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 patriline. 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 hemolentin, 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 μ L 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 $1\times$ penicillin/streptomycin (ThermoFisher, cat# 15070063), 10 at a time [33,175]. Sterile 18-gauge 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 ($\sim 1 \times 10^5$ 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-gauge 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 μ L).

Hemolymph was diluted with 100 μ L WH2 media; then, hemocytes were pelleted by centrifuging at $1000\times g$ for 10 min. Supernatant was removed, and hemocytes were suspended in 100 μ L WH2 media. Then, 800 μ L 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, Nuair). Surface sterilization was carried out in a sterile polystyrene petri dish (100 mm $\times$ 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 ($\sim 24 \times 10^6$ cells) were pooled. Cell suspensions with approximately 10^6 cells per 300 μ L 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 μ L) 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 $10,000 \times g$ for 10 min at 4 °C. To characterize this virus preparation, RNA was isolated from 100 μ L 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/ μ L. For DWV infection studies, approximately 10^6 pupal cells in 300 μ L (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 μ L 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 μ L) was collected into a microcentrifuge tube. Then, 300 μ L 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 μ L of TRIzol was added to each sample, which was vortexed and incubated at room temperature for 5 min. Then, 160 μ L of chloroform was added, and samples were shaken by hand (15 s) and incubated at room temperature (2 min). Samples were centrifuged at $12,000\times g$ for 15 min, and the aqueous phase was transferred to a clean microcentrifuge tube. An equal volume of isopropanol (~ 550 μ L) and 20 μ g 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 $14,000\times g$. 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 μ L nuclease-free water; then, 3 μ L 3M sodium acetate (pH 5.5) and 120 μ L ethanol were added, and RNA was precipitated overnight at -20°C . Next, samples were centrifuged at $14,000\times g$ 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 μ L nuclease-free water. RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher).

To extract RNA from larval carcasses, 300 μ L of deionized H₂O 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 μ L TRIzol[®] reagent (Invitrogen) was added to the lysate and vortexed and incubated at room temperature for 5 min; 200 μ L 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 $\times$ g. The aqueous phase was transferred to a clean microcentrifuge tube (approximately 800 μ L), 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 $\times$ g. The supernatant was decanted, and the pellet was washed by adding 500 μ L 75% ethanol and centrifuging for 5 min (7500 $\times$ g) at 4 °C. The supernatant was decanted, and pellets were air-dried at room temperature. Pellets were suspended in 100 μ L of dH₂O.

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 μ L cDNA template in 12.5 μ L aqueous buffer containing 10 pmol of each forward and reverse primer (Table S1) was amplified with 0.5 μ L (5 U/ μ L) 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 μ L of cDNA template and a no-template negative control was run in triplicate for each qPCR reaction. Each 20 μ L reaction contained 1 $\times$ Denville ChoiceTaq

Mastermix, 0.4 μ M each forward and reverse primer, 1 $\times$ 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 $C_t = -3.240x + 40.138$ ($R^2 = 0.980$, efficiency = 103%), determined by a previously described method [181]. Similarly, the linear equation for DWV was $C_t = -3.5627x + 39.091$ ($R^2 = 0.9973$, efficiency = 90.5%). The linear equation for the SBV standard curve was $C_t = -3.39x + 37.42$ ($R^2 = 0.994$, efficiency = 97.2%). The relative expression of host genes was determined by a ranked $\Delta\Delta C_t$ method in which the ΔC_t was calculated by subtracting the *rpl8* C_t value from the C_t of the gene of interest. Then, the within-group ΔC_t values were ranked from highest to lowest, and the relevant corresponding control ΔC_t value was subtracted from the treatment group ΔC_t to obtain the $\Delta\Delta C_t$. The fold-change in cDNA abundance was calculated by the equation $2^{-\Delta\Delta C_t}$. 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 13x over the initial levels by 24 hpi ($p < 0.001$), and it rose to 69.2x by 48 hpi ($p < 0.001$), to 147.9x by 72 hpi ($p < 0.0001$), and peaked at 354.8x 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 24x increase from 0 hpi to 24 hpi ($p = 0.0001$), and by 48 hpi SBV had increased 182x ($p < 0.0001$) and reached peak infection. There was a decrease in SBV relative to 48 hpi at 72 hpi (i.e., 49x greater virus levels than at 0 hpi, $p < 0.0001$), with a subsequent rise at 96 hpi (i.e., 132x 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.

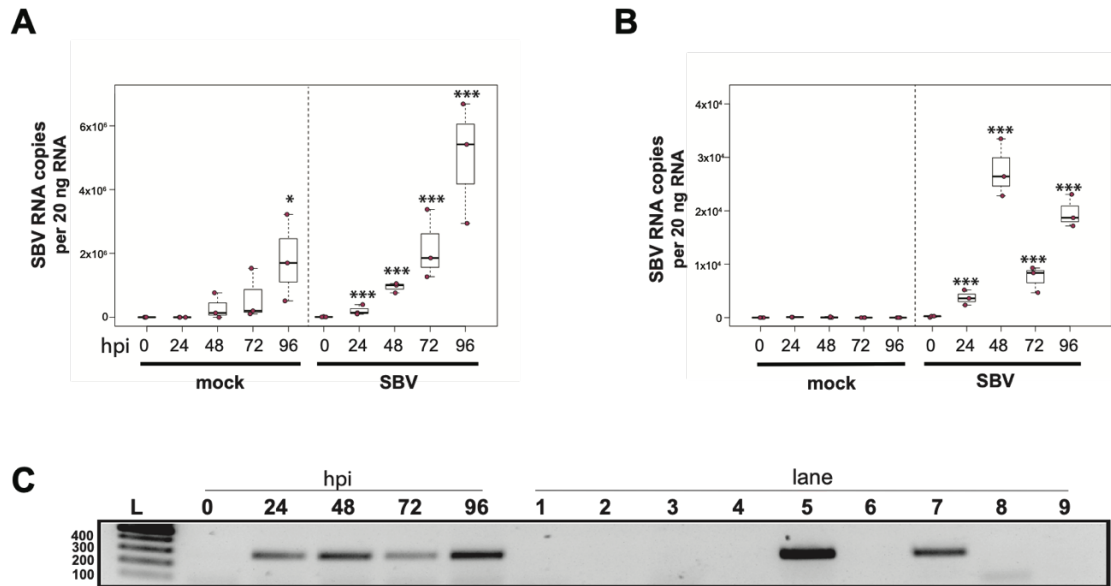


Figure 1. Sacbrood virus (SBV) naturally infects hemocytes and replicates in primary honey bee hemocyte cell cultures. SBV abundance was assessed in mock- or SBV-treated larval hemocytes by qPCR over a time course (i.e., 0 hpi, 24 hpi, 48 hpi, 72 hpi, and 96 hpi). (A) Cells isolated from asymptomatic 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 *Ame1102*, 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

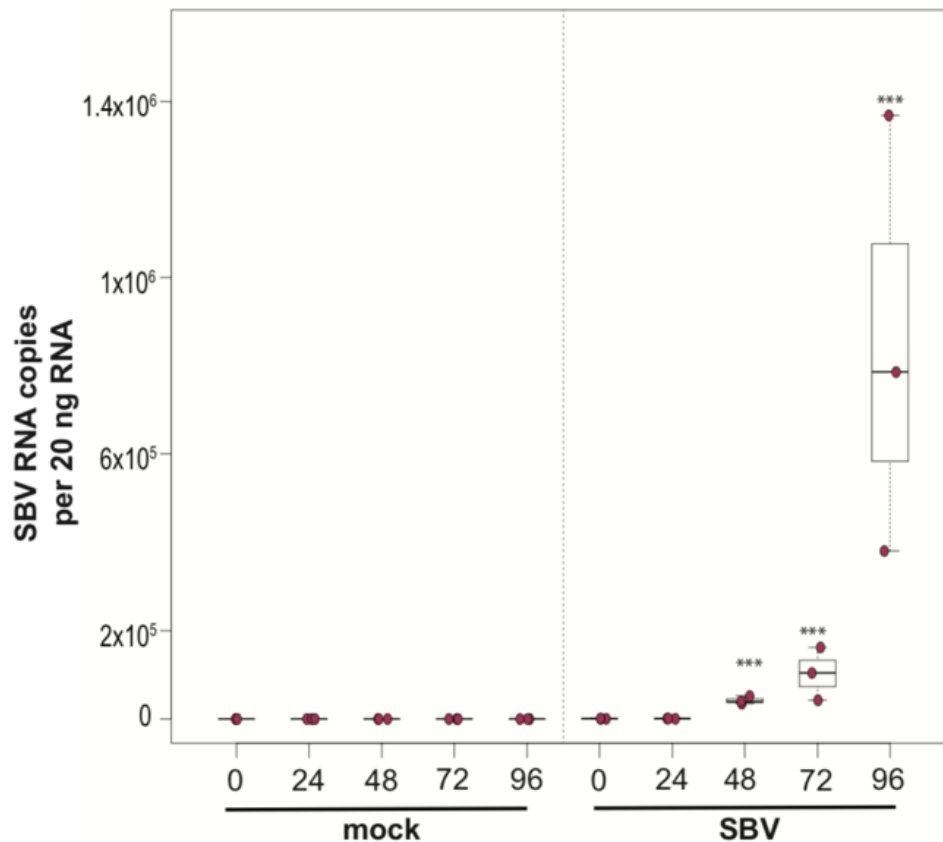
aimed at elucidating their role in antiviral defense, including further investigation of the genes and processes highlighted by transcriptional level studies.

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.

A



B

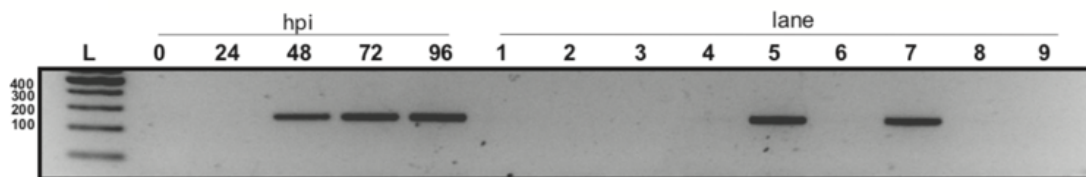


Figure 2. Sacbrood virus (SBV) replicates in primary honey bee pupal cell cultures. (A) SBV abundance was assessed in mock- or SBV-treated pupal cells by qPCR over a time course (i.e., 0 hpi, 24 hpi, 48 hpi, 72 hpi, and 96 hpi). A representative replicate of SBV-infection in

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.

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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 1×10^6 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 $2.7\times$ greater at 48 hpi ($p = 0.002$). DWV abundance increased $3.5\times$ and $4\times$ 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 $3.2\times$ higher at 72 hpi ($p = 0.001$) and $6.6\times$ 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.

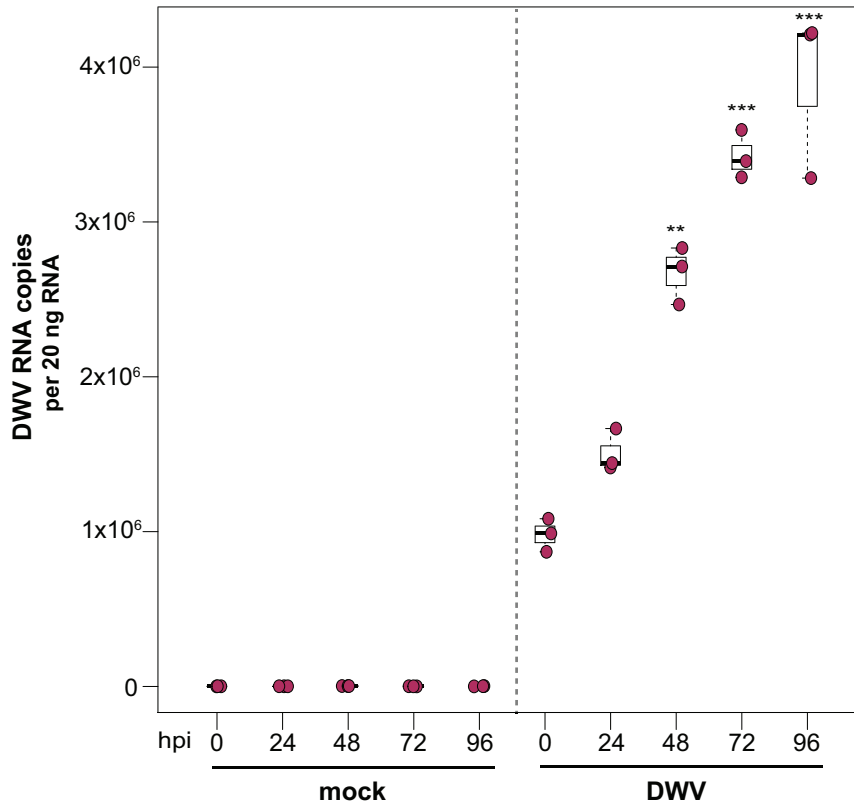
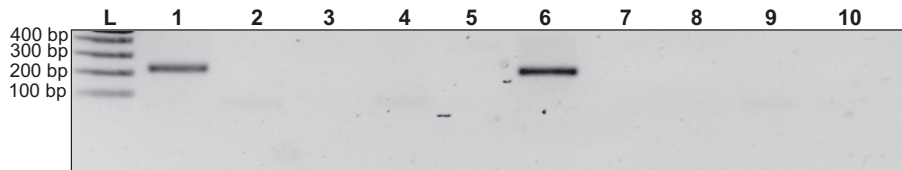
A**B**

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 $2.7\times$ ($p < 0.001$) and $3.5\times$ ($p < 0.001$) increase in mean DWV abundance at 48 hpi and 72 hpi, respectively, relative to 0 hpi. At 96 hpi, mean DWV abundance was $4\times$ 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 $\times$ by 24 hpi ($p = 0.026$) and 10.7 $\times$ 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 $\times$ ($p < 0.001$) 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 $\times$ higher at 48 hpi ($p < 0.01$) relative to 0 hpi. At 72 hpi, FHV levels peaked, with 39.8 $\times$ 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 $\times$ higher than 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.

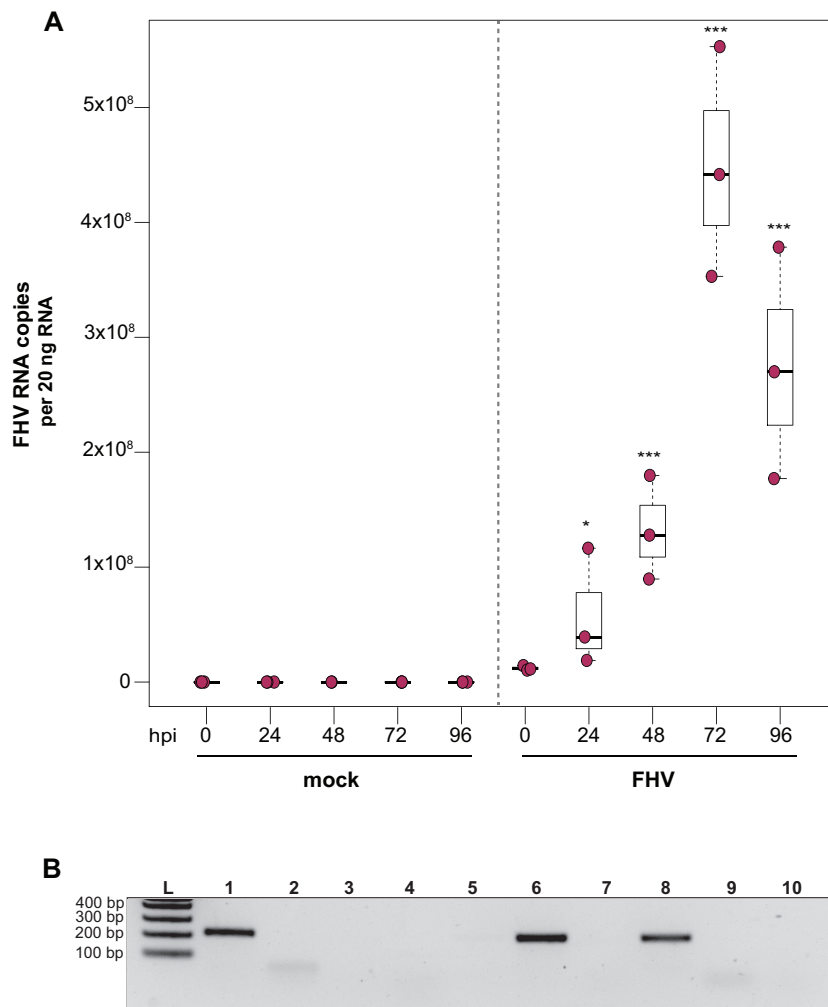


Figure 4. Flock House virus (FHV) replicates in primary honey bee hemocytes. FHV abundance in mock- or FHV-infected (1×10^6 FHV RNA copies) hemocytes was assessed over time (i.e., 0 hpi, 24 hpi, 48 hpi, 72 hpi, and 96 hpi). (A) FHV abundance was higher at 24 hpi ($3.7\times$, $p = 0.026$), 48 hpi ($10.7\times$, $p < 0.001$), and 72 hpi ($37\times$, $p < 0.001$), 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- Δ 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 $1.7\times$ at 24 hpi ($p = 0.024$) and by $3.4\times$ at 48 hpi, relative to initial levels at 0 hpi ($p < 0.001$). The peak of infection was reached at 72 hpi, with $5\times$ 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 $4\times$ higher than at 0 hpi ($p < 0.001$) (Figure 5A). Similarly, in the second biological replicate, FHV abundance was $2.6\times$ higher at 24 hpi ($p = 0.013$) and $7.5\times$ higher at 48 hpi ($p < 0.001$), relative to levels at 0 hpi. FHV abundance increased $20\times$ ($p < 0.001$) 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 $15.8\times$ 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].

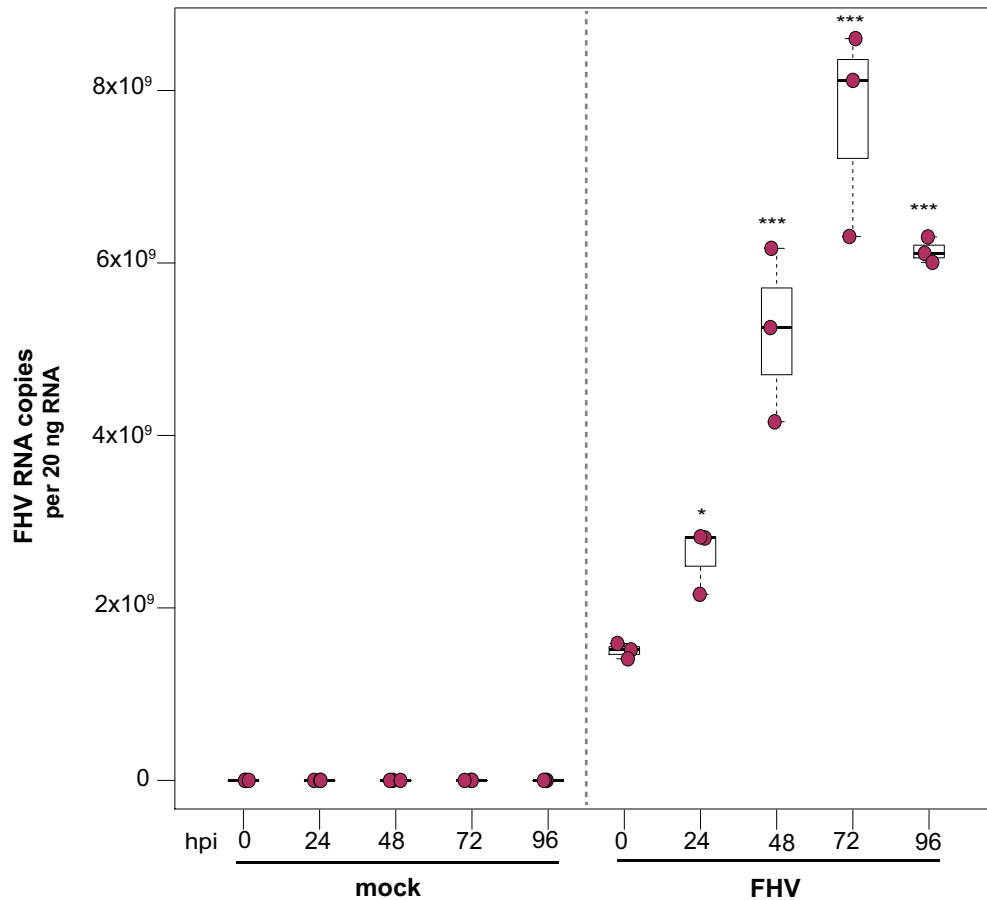
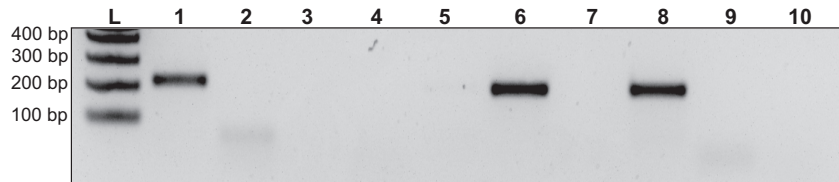
A**B**

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 ($1.7\times$, $p = 0.024$) and 48 hpi ($3.4\times$ $p < 0.001$), with a peak in FHV infection at 72 hpi ($5\times$, $p < 0.001$), relative to 0 hpi. FHV abundance did not increase from 72 hpi to 96 hpi but remained $4\times$ 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 μ L 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 $\times$ greater ($p = 0.003$) and at 72 hpi 28 $\times$ greater than levels at 0 hpi ($p < 0.001$). In the second replicate, FHV-infected pupal cells (i.e., 10 μ L 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 $\times$ 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).

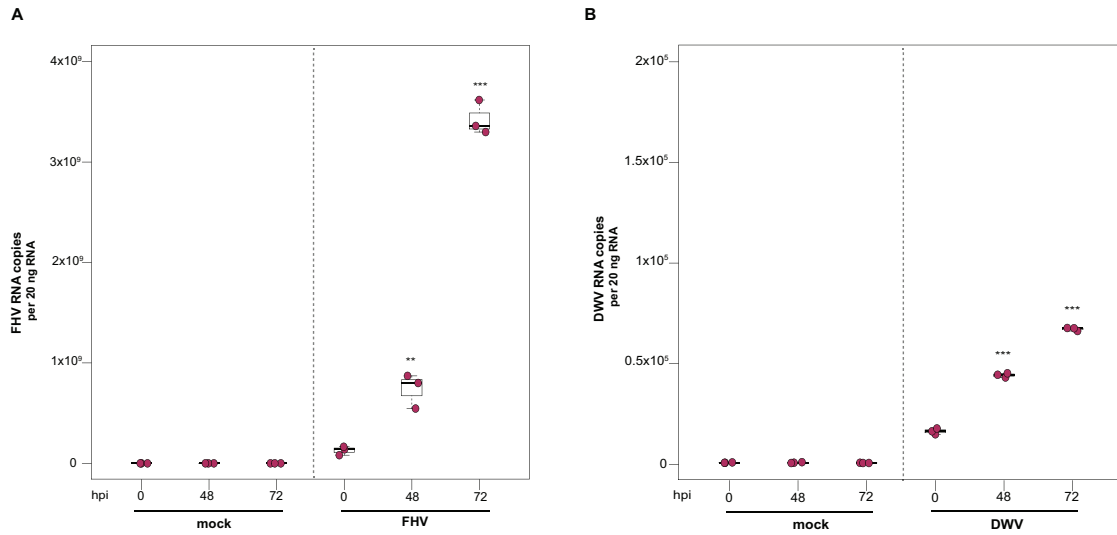


Figure 6. DWV and FHV infection transferred from infected to naïve honey bee pupal cells. To confirm that infectious DWV and FHV virions were produced in infected primary honey bee pupal cells, mock- or virus-infected honey bee pupal cell cultures (10 μ L) at 72 hpi were transferred to naïve pupal cells. 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 $\times$ at 48 hpi ($p = 0.003$), with a subsequent 28 $\times$ increase at 72 hpi relative to 0 hpi ($p < 0.001$). **(B)** DWV abundance was 2.7 $\times$ ($p < 0.001$) higher at 48 hpi and 4.1 $\times$ 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 μ L) 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 $\times$ relative to 0 hpi ($p < 0.001$). DWV levels continued to increase over time, with 4.1 $\times$ increase at 72 hpi ($p < 0.001$) (Figure 6B). In replicate two, naïve pupal cells were treated with 10 μ L DWV-infected

pupal cells (i.e., 9×10^4 DWV RNA copies). DWV abundance increased by $2.3\times$ at 48 hpi ($p < 0.01$) and was $10.4\times$ 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).

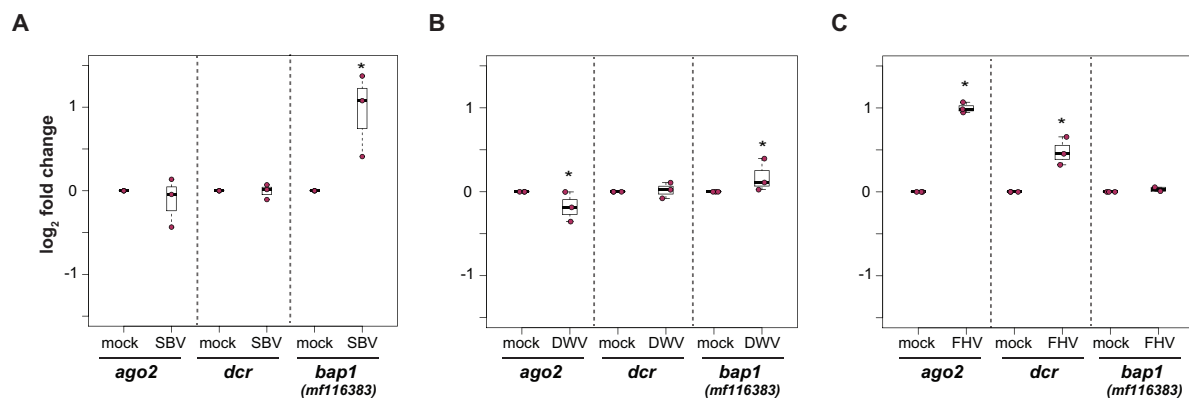


Figure 7. Virus infections of primary honey bee pupal cells differentially impact the expression of select immune genes. The relative expression of three honey bee immune genes, *argonaute-2* (*ago2*), *dicer-like* (*dcr-like*), and *bee antiviral protein-1* (*bap1*)/*mf116383*, were assessed by qPCR at 72 hpi. The $\Delta\Delta C_t$ 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.

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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.; Glenney, 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.; Gulbranson, 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 from *Costelytra 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. Zamboni, 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-Medzihradsky, K.; Darula, Z.; Andó, I.; Kurucz, É. Hemolymph 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 merguensis*. *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 FIVE

HONEY BEE ANTIVIRAL RESPONSE TO FLOCK HOUSE VIRUS INFECTION

Contributions of authors and Co-authors

Manuscript in Chapter Five

First Author: Fenali Parekh

Contributions: 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 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

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Fenali Parekh, Katie F. Daughenbaugh, Michelle L. Flenniken

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Honey bee antiviral response to Flock House virus infection

Fenali Parekh^{1,2,3}, Katie F. Daughenbaugh^{2,3}, Michelle L. Flenniken^{1,2,3,*}

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

*corresponding author: michelle.flenniken@montana.edu

Introduction

Honey bees (*Apis mellifera*) play a vital role in global agriculture as pollinators of fruit, nut, and vegetable crops (1-3). The value of pollination services in the United States is \$14.6 billion annually (1, 4). Unfortunately, the US beekeeping operations have suffered high colony losses, averaging ~38% annual losses from 2008 to 2018 (5-9). Various biotic and abiotic factors contribute to these losses, including habitat loss, poor nutrition, exposure to agrochemicals, the *Varroa destructor* mite, and pathogens (reviewed in (10-12)). Honey bee pathogens, including bacteria, microsporidia, fungi, and viruses contribute to honey bee colony deaths (13-17). Viruses, especially positive single-stranded RNA (+ssRNA) viruses comprise the largest and most diverse group of honey bee pathogens. These include acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), sacbrood virus (SBV), and the Lake Sinai virus (LSV) group (reviewed in (10, 12, 18, 19)). Virus transmission occurs vertically from infected queen or drone to offspring, or horizontally from nest mates via trophallaxis (mouth-to-mouth food exchange) and shared floral resources (19-22). In addition, varroa mites transmit viruses as they

feed on honey bees (23-25). Viral infections in honey bees may cause symptoms including deformity, paralysis and/or death, or remain asymptomatic (10, 19, 26). Virus-infected asymptomatic bees may harbor high viral loads ($\sim 10^8 - 10^9$ viral RNA copies per individual bee). These asymptomatic infections are likely energetically taxing and negatively affect bee physiology and overall health status (27, 28).

Honey bees have evolved antiviral defense mechanisms to limit viral infections at the cellular, individual bee, and colony levels. Antiviral defense mechanisms at the cellular level include NF- κ B mediated Toll and Imd (Immune Deficiency), 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 (29, 30)). RNAi is a post-transcriptional gene silencing mechanism that is triggered by the presence of cytosolically located long double-stranded RNA (dsRNA), a virus-associated molecular pattern (VAMP) (31-33). The dsRNAs are detected and cleaved by Dicer into 21-22 nucleotide long short interfering RNA (siRNA) and loaded into Argonaute-2, the catalytic component of the RNA-induced silencing complex (RISC) (32, 34, 35). One strand of the siRNA, the passenger strand is then released leaving the guide strand bound to Argonaute-2. RISC surveils the cell, binds and cleaves cognate RNAs, including virus or host genome transcripts, thereby reducing viral abundance (36). In addition, honey bees and bumble bees exhibit a non-sequence specific dsRNA antiviral response that limits virus infection (37, 38). However, the non-specific dsRNA-mediated antiviral response requires further characterization.

Investigations aimed at better understanding the honey bee antiviral immune response have utilized naturally infected bees which often harbor variable viral loads , or experimentally infected honey bees (39, 40). While it is important to investigate honey bee host virus interactions using naturally infecting honey bee viruses, these virus stocks are produced in honey bee pupae, which sometimes harbor confounding co-purifying viruses. In addition, the limited availability of infectious clones of honey bee viruses has made it challenging to further our knowledge on understanding the interactions between the honey bee immune system and infecting viruses (41-43). Model viruses including Sindbis virus tagged with green florescent protein (SINV-GFP) and Flock House virus (FHV) have been used to investigate insect antiviral responses in fruit flies, mosquitoes, and honey bees (37, 38, 44-48). The advantages of utilizing model viruses to investigate the host immune responses are that they are easy to propagate and purify, genetically and structurally well-studied, similar to naturally-infecting honey bees viruses, infect a range of insects to enable comparative immunology studies, and are easily trackable (48, 49). FHV is a non-enveloped, icosahedral virus, originally isolated from New Zealand grass grub (*Costelytra zealandica*) (50, 51). FHV has been utilized as a model virus system in a broad range of insect hosts, including mosquitoes, fruit flies, honey bees, and medically important insects including tsetse fly, thus enabling comparative immunology studies across insect genera (46, 47, 49). FHV contains a bipartite positive sense RNA genome and encodes a viral suppressor of RNAi, protein B2. FHV B2 suppresses the host antiviral RNAi response by binding long dsRNAs and siRNAs to prevent Dicer-mediated cleavage or inhibit loading into RISC (52, 53). Intriguingly, FHV protein B2 suppresses the RNAi mechanism in plants, insects, and worms, suggesting that B2 has evolved to evade the host immune system in a host-independent manner (53-55). Previous studies by

O'Neal et al. utilized FHV as an experimental model virus system to investigate the role of ATP-sensitive inwardly rectifying potassium (K_{ATP}) channels in honey bee antiviral immunity (46). Honey bees were infected with FHV via intrathoracic injections and exhibited increased virus abundance over time, suggesting that adult honey bees support FHV infection (46). Moreover, FHV has been previously utilized to better understand honey bee host-virus interactions at the cellular level using primary honey bee cells (56). Honey bee larval derived hemocytes and pupae derived mixed cell cultures support FHV replication and mount antiviral immune responses (56). The ability of FHV to infect honey bee derived primary cells provides a powerful tool to investigate cellular mechanisms honey bee antiviral defense network.

Herein, we carried out laboratory-based experiments to assess the transcriptional regulation of honey bee immune genes in response to FHV infection. In addition, we verified the role of key immune genes involved in FHV-infected honey bees. Honey bees injected with FHV supported FHV replication and virus abundance increased over time. The expression of a suite of immune genes was assessed to determine the specific genes and antiviral pathways involved in limiting FHV infection. FHV-infected honey bees exhibited differential expression of *dicer-like (dcr)*, *argonaute-2 (ago2)*, *bee antiviral protein-1 (bap1)*, *heat shock protein-90 (hsp90)*, *protein lethal (2) essential for life-like (pl2)* and *vitellogenin (vg)* compared to mock-infected bees. Furthermore, we utilized RNA interference to knockdown the expression of key genes in the RNAi pathway (*dcr* and *ago2*) to investigate the function of these genes in honey bee antiviral response to FHV infection. FHV levels were not affected by the introduction of non-sequence specific dsRNA, or sequence specific dsRNA i.e., *dcr* dsRNA or *ago2* dsRNA. Overall, the use of well-studied model

virus systems like FHV will help us further our understanding of the fundamentals of honey bee immunity, which may be otherwise challenging to discern using naturally infecting honey bee viruses.

Results and Discussion

Honey bees exhibit dose dependent mortality in response to Flock House virus infection

To examine FHV associated mortality in honey bees, age-matched adult bees (i.e., 24 hours post-emergence) were infected via intrathoracic injection with four different FHV doses (i.e., 3.5×10^3 , 3.5×10^6 , 3.5×10^7 , and 3.5×10^8 FHV RNA copies/bee) and survival was assessed at 0, 6, 24, 48, and 72 hours post infection (hpi). In general, honey bees exhibited FHV-dose dependent mortality, i.e., higher FHV dose resulted in more rapid mortality (Figure 1).

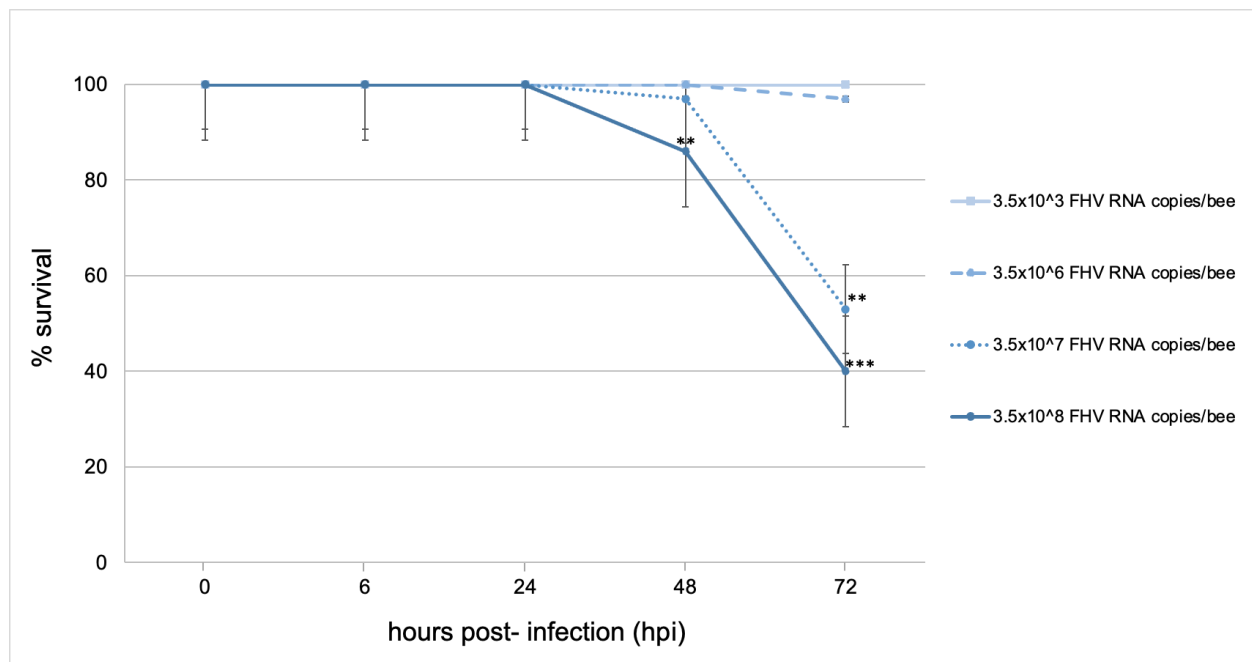


Figure 1 Honey bees exhibit dose dependent mortality in response to Flock House virus infection.

Honey bees at 24 hours post-emergence were infected via intrathoracic injection with four different FHV doses, i.e., 3.5×10^3 FHV RNA copies/bee, 3.5×10^6 FHV RNA copies/bee, 3.5×10^7 FHV RNA copies/bee, and 3.5×10^8 FHV RNA copies/bee and survival was assessed at 6, 24, 48, and 72 hpi. Bees infected with 3.5×10^8 FHV RNA copies /bee (solid line with diamonds) exhibited 14% and 60% ($p < 0.05$, Kaplan-Meier log-rank test) mortality at 72 hpi and 48 hpi respectively. In bees injected with 3.5×10^7 FHV RNA copies /bee (dotted line), 47% mortality was recorded. Bees infected with lower FHV doses i.e., 3.5×10^6 FHV RNA copies/bee (dashed line), 3.5×10^3 FHV RNA copies/bee (solid line with squares) did not exhibit appreciable mortality until 72 hpi. Data were analyzed using Kaplan-Meier log-rank test in three biological replicates ($n=10-12$ bees per treatment), significance levels: ** $p < 0.001$, *** $p < 0.0001$.

Bees injected with highest FHV dose, i.e., 3.5×10^8 FHV RNA copies/bee exhibited 14% mortality ($p < 0.001$, Kaplan-Meier log-rank test) within the first 48 hpi and 60% mortality at 72 hpi ($p < 0.0001$) (Figure 1). Bees infected with 3.5×10^7 FHV RNA copies/bee did not exhibit appreciable mortality in the first 48 hpi. At 72 hpi, bees infected with 3.5×10^7 FHV RNA copies/bee exhibited 47% mortality ($p < 0.001$). In honey bees injected with 3.5×10^6 FHV RNA copies /bee, no mortality was recorded until 48 hpi, with modest mortality (i.e., 3%) at 72 hpi. Honey bees infected with 3.5×10^3 FHV RNA copies/bee exhibited the least amount of mortality. These results indicate that FHV causes dose dependent mortality in honey bees, i.e., higher mortality with increasing FHV dose (Figure 1).

Virus abundance increases over time in Flock House virus- infected honey bees

To examine the kinetics of FHV infection, adult honey bees (i.e., 24 hrs post-emergence) were inoculated with FHV (3.5×10^8 RNA copies/bee) via intrathoracic injection and virus abundance was assessed over a time course (i.e., 6 , 48 , 72 , and 96 hpi). FHV abundance was quantified via RT-qPCR using RNA copy number, including both viral genomes and transcripts, as a proxy for overall virus abundance. In general, FHV abundance increased over time from 6 hpi to 72 hpi and remained constant from 72 hpi to 96 hpi (Figure 2). In replicate one, $\log_{10}$ FHV RNA levels increased 2.9 logs at 48 hpi and 3.9 logs at 72 hpi relative to 6 hpi ($p < 0.001$, Dunnett's test). By 96 hpi, FHV abundance was constant relative to 72 hpi, but still 2.6 logs higher relative to 6 hpi ($p < 0.001$) (Figure 2A,). In replicate two, FHV infection level increased by 3.8 logs at 48 hpi relative to 6 hpi ($p < 0.001$) (Supplemental Figure S1). Infection peak was achieved at 72 hpi, with 4.2 logs increase in FHV levels relative to 6 hpi ($p < 0.001$). FHV levels did not change from 72 hpi to 96 hpi, but remained 4 logs higher than 6 hpi ($p < 0.001$) (Supplemental Figure S1). In replicate three, FHV abundance in infected honey bees increased 2.1 logs by 48 hpi, and 1.8 logs by 72 hpi ($p < 0.001$), relative to 6 hpi. However, FHV abundance at 72 hpi was reduced compared to 48 hpi. FHV levels remained constant at 96 hpi relative to 72 hpi, but 1.7 logs higher relative to 6 hpi ($p < 0.05$) (Supplemental Figure S1). To confirm active replication of FHV in virus-infected honey bees, strand-specific RT-PCR was carried out to detect the presence of negative strand at 72 hpi since FHV abundance peaked at this time point. The FHV genome is positive-sense single-stranded RNA. Thus, a negative sense template is synthesized during virus replication. FHV-

specific negative strand was successfully detected in FHV-infected honey bees at 72 hpi (Figure 2B).

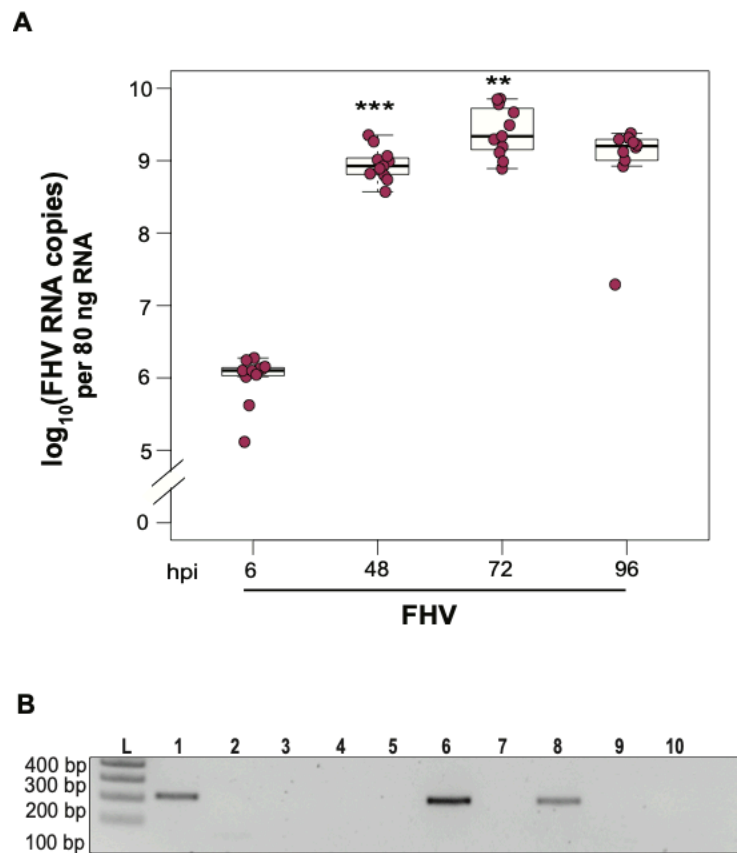


Figure 2 Virus abundance increases over time in Flock House virus-infected honey bees.

FHV abundance was assessed in FHV-infected honey bees by qPCR over a time course (i.e., 0 hpi, 48 hpi, 72 hpi, and 96 hpi). **(A)** In honey bees infected with 3.5×10^8 FHV RNA copies/bee via intrathoracic injection, $\log_{10}$ FHV RNA levels increased 2.9 logs at 48 hpi, and 3.9 logs at 72 hpi relative to 6 hpi ($p < 0.001$, Dunnett's test). FHV abundance was constant at 96 hpi relative to 72 hpi, but still 2.6 logs higher relative to 6 hpi ($p < 0.001$). Figure 2 includes results from one representative biological replicate ($n=10-12$ per treatment group). The data for additional replicates are presented in Supplemental Figure S1. All differences in means relative to 6 hpi were assessed by a Dunnett's test. Significance levels: * $p < 0.005$; *** $p < 0.0005$. **(B)** To confirm productive replication of FHV in honey bees, the presence of negative strand (replicative intermediate) was assessed using negative-strand specific PCR. Negative strand was detected in pooled FHV-infected honey bee sample ($n=5$) at 72 hpi (Lane 1). Additional control reactions were

performed using pooled RNA (Lanes labeled 2–10). Specifically, RNA isolated from FHV-infected bees (n=5) was pooled and reverse transcribed with the primers 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) 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-R. (6) Positive control: RT with random hexamers, PCR with FHV-F and FHV-R. (7) Negative control: RT with random hexamers, PCR with only FHV-R primer. (8) Self-priming: RT without a primer, PCR with FHV-F and FHV-R. (9) Negative control: No template, no RT, PCR with TAGS and FHV-R. (10) Negative control: No template, no RT, PCR with FHV-F. Primer sequences provided in Supplemental Table S1. When reverse transcription was performed without primer, FHV cDNA was detectable (lane 8), indicating that the secondary structure in the RNA genome potentially serves as a primer for cDNA synthesis.

Together these data verify previous findings that FHV can productively infect adult honey bees and thus may be utilized as a model virus to investigate honey bee host- virus interactions. An advantage of utilizing FHV to study the honey bee antiviral defense mechanisms is its ability to infect adult honey bees, as well as primary honey bee cells i.e., larval derived hemocytes and pupae derived mixed cell cultures (45, 46, 56). This allows for better understanding the honey bee immune mechanisms at the individual bee level and more precisely at the cellular level. The use of FHV enables a better understanding of the honey bee immune response upon infection with a virus that encodes a viral suppressor of RNAi (VSR), since RNAi is the key antiviral immune pathway in honey bees (57, 58). In addition, RNAi suppressor proteins play a critical role in establishment of virus infection. For instance, *Drosophila melanogaster* embryos injected with wild type RNA1 produced abundant amount of RNA1 and subgenomic RNA3, whereas embryos injected with B2 deletion mutant did not accumulate viral RNA (33). This indicates that protein B2 plays a critical role in the suppression of host RNAi which enables FHV to establish infection. Future work should focus on studying FHV accumulation and host immune response in honey

bees infected with wild type (wt) FHV and FHV- Δ B2 mutant to better understand the role of VSR's in honey-bee infecting viruses that may encode a suppressor.

Transcriptional response to Flock House virus infection in honey bees

We hypothesized that FHV replication would trigger the honey bee antiviral immune pathways and result in differential expression of antiviral genes. The expression of select immune genes was assessed in honey bees infected with 3.5×10^8 FHV RNA copies/bee at 72 hpi. This time point was selected since FHV abundance peaked at this time. The expression of six genes was assessed including key genes in the RNAi pathway (*dicer-like (dcr-like)*, *argonaute-2 (ago2)*), heat-shock protein encoding genes (*heat shock protein 90 (hsp90)*, *protein lethal (2) essential for life-like (pl2)*), *bee antiviral protein-1 (bap1)*, and *vitellogenin (vg)* relative to mock-infected bees. Transcription levels of these genes was analyzed by qPCR in three experimental replicates (Figure 3 and Supplemental Figure S2).

A key endoribonuclease in the RNAi pathway, Dicer-like, recognizes and cleaves long dsRNAs into 21-22 bp long short interfering RNAs (35). Transcription of *dcr-like* was higher in FHV-infected bees in three biological replicates at 72 hpi relative to mock-infected bees (Figure 3 and Supplemental Figure S2). Specifically, in the first biological replicate, *dcr-like* expression was 4.6 fold greater in FHV-infected bees relative to expression level in mock-infected bees ($p < 0.001$, Wilcoxon Rank Sums) (Figure 3). Likewise, *dcr-like* expression was 1.2 fold ($p < 0.001$) and 0.9 fold ($p < 0.001$) greater in FHV-infected bees in replicate two and three respectively, compared to

mock-infected bees (Figure 3 and Supplemental Figure S2). Previous studies have validated the role of *dcr-like* in response to virus infection in adult honey bees, larvae, and honey bee pupae-derived cells (37, 56, 59).

Argonaute-2 is the catalytic component of RISC and plays a crucial role in the RNAi pathway (60). In general, FHV infection triggered *ago2* expression in honey bees. In replicate one, *ago2* expression was 2.7 fold higher in FHV-infected bees compared to mock-infected bees ($p < 0.001$). In replicate two and three, *ago2* expression was consistently higher in FHV-infected bees with 2.5 fold and 0.85 fold higher expression respectively ($p < 0.001$), compared to mock-infected bees (Figure 3 and Supplemental Figure S2).

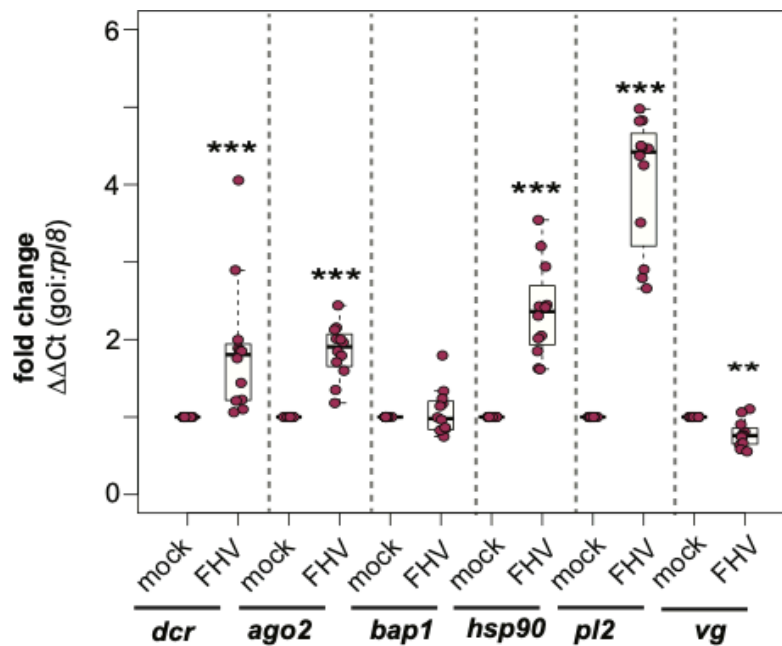


Figure 3 Flock House virus infection results in differential expression of select honey bee immune genes.

The relative expression of six honey bee genes including *dicer-like* (*dcr*), *argonaute-2* (*ago2*), *bee antiviral protein-1* (*bap1*), *heat shock protein 90* (*hsp90*), *protein lethal (2) essential for life-like*

(*pl2*) and *vitellogenin* (*vg*), were assessed in FHV-infected honey bees at 72 hpi via qPCR. The data were analyzed using $\Delta\Delta C_t$ method with normalization to *rpl8*, and then the fold change values were calculated relative to mock-infected honey bees. The expression of *dcr* was 1.85 fold higher, *ago-2* was 1.9 fold higher, *hsp90* was 1.4 fold higher, and *pl2* was 3 fold higher in FHV-infected bees relative to mock-infected bees ($p < 0.001$). The expression of *vg* was 0.2 fold lower ($p = 0.005$), whereas *bap1* expression remained constant in FHV-infected honey bees relative to mock-infected bees. The differences in relative immune gene expression in FHV-infected honey bees relative to mock-infected bees at 72 hpi was assessed using a one-sided Wilcoxon Rank Sums test. Significance levels: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

Bee antiviral protein-1 (*bap1*) is a rapidly evolving, taxonomically restricted antiviral gene in honey bees. A previous transcriptomic study showed induced expression of *bap1* in response to infection with Sindbis virus (SINV), a model virus (37, 61). In addition, SINV infected bees with reduced *bap1* expression exhibited higher SINV abundance, suggesting that *bap1* has an antiviral function in honey bees (37, 61). The expression of *bap1* in FHV-infected bees was not impacted in replicate one and three, but was 0.7 fold lower in the second replicate compared to mock-infected bees ($p < 0.001$) (Figure 3 and Supplemental Figure S2). This observation is intriguing since *bap1* expression is higher in honey bees infected with SINV. One potential explanation to the differences in *bap1* expression in FHV-infected bees compared to SINV-infected bees is that FHV encodes an RNAi suppressor protein while SINV does not (53, 62). The interactions between the RNAi suppressor protein and the host RNAi pathway perhaps results in unique gene expression profiles. If the presence of a RNAi suppressor protein impacts the expression of *bap1*, it is intriguing to speculate that *bap1* may play a role in the RNAi pathway. Future studies should focus on better understanding whether *bap1* works in tandem or independent of the RNAi pathway or whether the presence of an RNAi suppressor protein which binds long dsRNA and siRNA molecules, may impact *bap1* expression.

Heat shock protein 90 (hsp90) is a molecular chaperone that is involved in protein folding and protein stability (63). *Hsp90* acts as a chaperone for RISC assembly thereby playing a significant role in the RNAi-mediated immune response (64). The expression of heat shock proteins is induced in response to various stressors including virus infection (44). Transcription level of *hsp90* was 2 fold higher in FHV-infected bees compared to mock-infected bees in replicate one ($p < 0.001$) (Figure 3). In replicate two and three, *hsp90* expression was 1.7 fold and 1.4 fold higher respectively, compared to mock-infected bees ($p < 0.001$) (Figure 3 and Supplemental Figure S2). This is consistent with previous studies that demonstrate that the heat shock response is antiviral in honey bees (44, 65).

Protein lethal(2)essential for life-like (pl2) is a small heat shock protein that belongs to the Hsp20 family. In FHV-infected bees, *pl2* expression was highly induced. The expression of *pl2* was consistently higher in all three replicates: 19.7 fold higher in replicate one, 9.3 fold higher in replicate two, and 3 fold higher in replicate three ($p < 0.001$) (Figure 3 and Supplemental Figure S2). This is in line with previous studies that have documented higher *pl2* expression in virus-infected honey bees, specifically in bees infected with IAPV, SINV, and DWV (44, 59, 66).

Vitellogenin (vg) is a honey bee egg yolk protein mainly synthesized in the fat body of honey bee queens and sterile female workers (67). *Vg* serves multiple roles including its role in nutrition, immune function, oxidative stress, and ageing and thus is a common marker of honey bee health and lifespan (68-70) (reviewed in (71)). Previous work carried out at the colony level illustrates

that *vg* gene expression is negatively correlated with DWV abundance (72, 73). Transcriptomic analysis of SINV-infected bees at the individual bee level also demonstrated reduced *vg* levels relative to mock-infected bees (37). Thus, we hypothesized that FHV infection would result in reduced *vg* expression relative to mock-infected bees at 72 hpi. In replicate one, *vg* expression was slightly lower in FHV-infected bees relative to mock-infected bees (i.e., 0.3 fold, $p < 0.05$) (Figure 3 and Supplemental Figure S2). Likewise *vg* expression was slightly lower in FHV-infected bees compared to mock-infected bees in two additional biological replicates (i.e., rep2, 0.2-fold, $p < 0.001$; rep3 0.2 fold, $p = 0.005$). Since *vg* is utilized as a proxy for honey bee health status, lower levels of *vg* expression may indicate poor health status of the host.

Together these results indicate that the honey bee immune responses elicited against a model virus, FHV, resembles the immune response elicited on infection with commonly infecting honey bee viruses (39, 59). For instance, *dcr*, *ago-2*, and *pl2* expression is induced in honey bees experimentally infected with IAPV, expression of *pl2* is higher in naturally IAPV-infected bees and bees infected with DWV and SBV (39, 66, 74). Thus, FHV serves as a valuable model system to study the honey bee host-virus interactions. Moreover, the fact that honey bee immune response elicited at the individual level recapitulates the response elicited at the cellular level (i.e., honey bee hemocytes and pupal cells in culture), suggests that FHV can serve as an effective model to answer basic immunology questions important to better understand the honey bee antiviral immunity (56).

Reduced expression of key RNAi genes does not impact FHV abundance in honey bees

RNA viruses produce long dsRNA during replication, which triggers the RNAi-mediated antiviral immunity in honey bees (57). To verify the role of two key RNAi genes in response to FHV infection in honey bees, we utilized RNAi-mediated knockdown to reduce *dcr* and *ago2* expression in FHV-infected bees and quantified virus abundance at 72 hpi using RT-qPCR. Based on previous studies and the importance of RNAi in honey bee immunity, we hypothesized that lower expression of *dcr* or *ago2* will result in higher virus abundance (37). Honey bees were injected with FHV only, or co-injected with FHV containing non-sequence-specific dsRNA (ns-dsRNA) corresponding to Drosophila C virus which has no sequence specificity to the honey bee genome or to honey bee-infecting viruses. The ns-dsRNA has been shown to trigger an antiviral response in honey bees and was thus utilized as a control. Alternatively, to reduce expression of *dcr* or *ago2*, honey bees were co-injected with FHV containing *dcr* specific dsRNA (*dcr* dsRNA) or *ago2* specific dsRNA (*ago2* dsRNA). In replicate one, FHV-infected bees treated with *dcr* dsRNA exhibited 69% reduction in *dcr* expression ($p < 0.001$, Dunnett's test) whereas, bees co-injected with FHV and ns-dsRNA did not exhibit a change in *dcr* expression relative to FHV-infected bees (Figure 4A). This suggests that the reduction in *dcr* expression was reduced in a sequence dependent manner (i.e., RNAi-mediated). FHV levels were not impacted by reduced *dcr* expression in bees co-injected with FHV and *dcr* dsRNA relative to FHV infected bees (Figure 4A). In replicate two, FHV-infected bees co-injected with *dcr* dsRNA-treated had 18% reduction in *dcr* transcript levels compared to FHV-infected bees ($p = 0.04$). However, FHV abundance was similar in bees co-injected with FHV and *dcr*-dsRNA compared to FHV-infected bees (Supplemental Figure S3). To investigate the impact of *ago2* knockdown in FHV-infected bees,

honey bees were co-injected with FHV and *ago2*-dsRNA. To determine the knockdown efficiency, *ago2* transcript levels were quantified 72 hpi via RT-qPCR. The expression levels of *ago2* were 33% ($p < 0.001$) and 89% ($p < 0.001$) lower in bees co-injected with FHV and *ago2* dsRNA in replicate one and two respectively, relative to FHV- infected bees. FHV abundance was similar in bees with reduced expression of *ago2* compared to FHV-infected bees (Figure 4B, Supplemental Figure S4). Thus, FHV abundance in honey bees was not impacted by the administration of non-specific dsRNA or *dcr/ago2*-dsRNA.

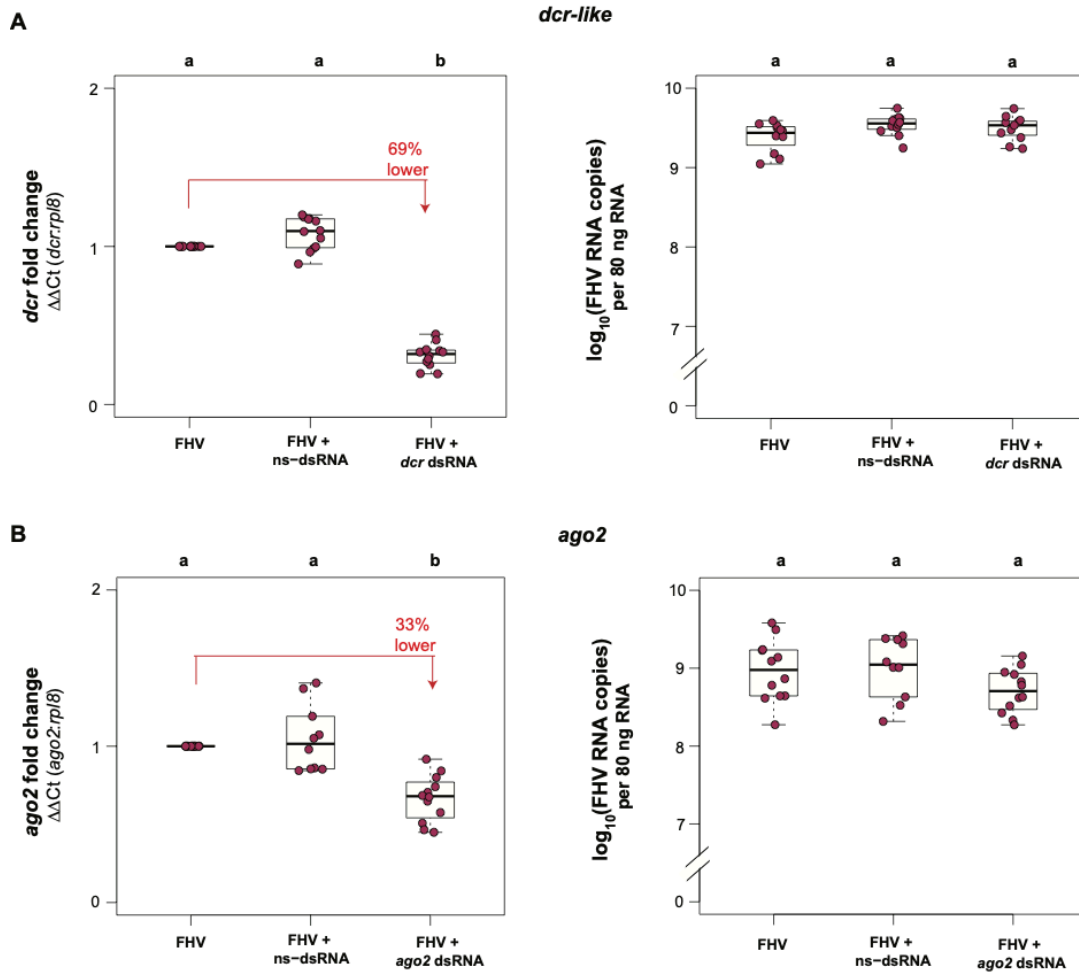


Figure 4 Reduced expression of key RNAi genes does not impact FHV abundance in honey bees.

The relative expression of *dicer* (*dcr*) and argonaute-2 (*ago2*) was assessed by qPCR, normalized to *rpl8* and fold changes were calculated relative to FHV-injected bees. FHV-abundance was measured by determining the FHV RNA copies at 72 hours post-infection (hpi). **(A)** Bees co-injected with FHV and *dcr* dsRNA had 69% reduction in *dcr* transcript levels compared to FHV-infected bees ($p < 0.001$, Dunnett's test). Co-injection with FHV and non-specific dsRNA (ns-dsRNA) resulted in no change in *dcr* expression, confirming successful *dcr* knockdown. FHV abundance was similar in bees co-injected with FHV and *dcr*-dsRNA compared to FHV-infected bees. **(B)** In honey bees co-injected with FHV and *ago2* dsRNA, *ago2* transcript levels were quantified at 72 hpi. The expression level of *ago2* was 33% lower ($p < 0.001$) in bees co-injected with FHV and *ago2* dsRNA relative to FHV-infected bees. FHV abundance was similar in bees co-injected with FHV and *ago2* dsRNA compared to bees infected with FHV only. Different letters above the treatment groups indicates a statistically significant difference.

RNAi is the primary antiviral immune pathway in insects which is activated by the presence of dsRNA molecules produced by RNA viruses during the viral replication cycle (57). In solitary insects such as fruit flies and mosquitos, virus-specific dsRNA triggers an RNAi-mediated immune response, whereas non-sequence-specific dsRNA does not lower virus levels. Previous research suggests that in honey bees and bumble bees, the introduction of virus-specific dsRNA or ns-dsRNA triggers an immune response, resulting in lower virus abundance (38, 75, 76). The sequence specific dsRNA-mediate immune response can be utilized to knockdown the expression of gene(s) of interest to investigate/verify their function in vivo. It has proved to be a powerful tool in answering fundamental questions about honey bee immune pathways (37). We employed the RNAi-mediated knockdown technique to reduce the expression of key RNAi genes, *dcr* and *ago2* to investigate their role in response to FHV infection in honey bees. Considering the importance of *dcr* and *ago2* in the RNAi pathway, we expected FHV-infected honey bees with reduced expression of *dcr* or *ago2* would have greater virus abundance compared to controls. However, we determined that FHV abundance in honey bees co-injected with FHV and *dcr* or *ago2*-dsRNA was similar to FHV-infected bees. A potential explanation for similar FHV levels in these experimental treatment groups is that the FHV-B2 may prevent Dicer- mediated cleavage or siRNA loading into RISC and reduce the impact of lowering their expression. To test this hypothesis, future work should include similar experiments in FHV-infected and FHV Δ B2- infected bees to determine if FHV abundance is lower in FHV Δ B2- infected bees co-injected with *dcr* or *ago2*-dsRNA.

Materials and Methods

Age-matched honey bees

Honey bee (*Apis mellifera*) colonies were established from ~1.5 kg of worker bees of *Apis mellifera carnica* from a commercial producer in Montana. Honey bees were kept in Langstroth hives located on Montana State University's Horticulture Farm in Bozeman, MT, USA. Frames of newly emerging honey bees for laboratory-based experiments were obtained one day prior to each experiment and maintained in an incubator at 32°C. Age-matched (~24 h post-emergence), female, adult honey bees were collected and housed in modified deli-containers and randomly assigned into treatment groups. Honey bees in each treatment group were fed bee candy (powdered sugar mixed with corn syrup until pliable) and water *ad libitum*. Experiments described herein were carried out using honey bees collected from three separate colonies, designated as rep1, rep2, and rep3.

Flock house virus preparation

FHV was propagated in *Drosophila melanogaster* Schneider 2 (S2) cells derived from *D. melanogaster* embryos. S2 cells were grown as monolayers in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and 1% penicillin-streptomycin as per manufacturer's instructions. To generate FHV particles, 4×10^7 S2 cells/ml were seeded in a sterile T75 flask (Thermo Scientific). An aliquot of FHV was kindly provided by Dr. Anette Schneemann (The Scripps Research Institute, La Jolla, California). S2 cells

were infected with FHV at a multiplicity of infection of 1 pfu/cell and incubated at 28°C for 48 h as previously described (77). After incubation, the cells were lysed by addition of 10% (vol/vol) Nonidet P-40 and incubated on ice for 10 min with periodic swirling. Cell debris was pelleted in a Sorvall LYNX 4000 Centrifuge (Thermo Fisher) at 13,800x g for 10 min at 4°C and the clarified supernatant was transferred to a fresh tube. Virus was pelleted through a 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 (Beckman Coulter). The pellets were resuspended in 0.5 mL 50 mM HEPES (pH 7) and centrifuged at 10,000 rpm for 10 min to remove any insoluble material. The clarified supernatant was layered on continuous sucrose gradients (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 halfway down the tube. The sedimented virus was further pelleted in 10 mM Tris HCl buffer (pH 7.5) at 40,000 rpm for 2.5 h at 11°C for injections in honey bees. RNA was isolated from 100 µL virus preparations (as described below) and FHV abundance was quantified by RT-PCR and copy number was based on a standard curve. FHV RNA 2 plasmid standards were used as templates, with concentrations ranging from 10^3 to 10^9 copies per reaction to create a linear standard curve, with a limit of detection of 10^3 copies of FHV cDNA using primers FHV-FW and FHV-REV (Supplemental Table S1). The linear equation for the plasmid standard for FHV was $Ct = -3.335x + 40.137$ ($R^2 = 0.993$, efficiency = 99.5%) where 'x' is the log (FHV RNA copies).

dsRNA preparation

Double stranded RNA was generated by *in vitro* transcription with T7 RNA polymerase (37, 78). 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 elongation at 72°C (5 min). PCR products were used as template for T7 polymerase transcription reactions (100 µL): NTPs (each 7.5 mM final), RNase OUT (40 units) (Invitrogen, Carlsbad, CA, USA), buffer (400 mM HEPES pH 7.5, 120 mM MgCl₂, 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, WI, 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 µL 10 mM Tris HCl (pH 7.5). The dsRNA 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.

Honey bee virus infection

Age-matched honey bees (~24 h post-emergence, n=10-12 bees per treatment) were cold anesthetized at 4°C for 10 min prior to intrathoracic injection of virus or buffer (mock-infection). Intra-thoracic injections were performed using a Harbo syringe (Honey Bee Insemination Service) and microcapillary glass needles, 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). Honey bees were injected intrathoracically with varying doses of

FHV or mock-infected with 2 μ L buffer (10mM Tris HCl, pH 7.5) and incubated at 28°C for the duration of the experiment.

RNA isolation

Individual honey bees were transferred into 2 mL Eppendorf safe-lock tubes and homogenized in 300 μ L deionized water with a sterile steel ball (5 mm) using a Tissue Lyser II (Qiagen) at 30 Hz for 2 min. An equal volume (300 μ L) of TRIzol reagent (Invitrogen) was added to homogenate and then samples were vortexed for 15 s. Samples were incubated for 5 min at room temperature, followed by addition of 100 μ L chloroform. Each sample was shaken by hand for 15 s and incubated at room temperature for 2 min. Samples were then centrifuged at 12,000x *g* at 4°C for 15 min and the aqueous phase was transferred to a fresh 1.5 mL centrifuge tube. An equal volume of isopropanol (~300 μ L) was added to the aqueous phase, vortexed, and incubated at room temperature for 10 min to precipitate the nucleic acid. The nucleic acid was pelleted by centrifugation at 12,000x *g* at 4°C for 10 min. Supernatant was carefully removed and the pellets were washed with 75% ethanol by centrifugation at 7,500x *g* at 4°C for 5 min. The resulting supernatant was discarded, and the pellets were air dried for 10 min at room temperature, then dissolved in 50 μ L deionized water. RNA concentrations were assessed on Nanodrop 2000 spectrophotometer (Thermo Fisher). All RNA samples were stored in -80°C until further analysis.

Reverse transcription/cDNA synthesis

Reverse transcription reactions were carried out to synthesize complimentary DNA (cDNA) by incubating 2,000 ng of RNA with M-MLV reverse transcriptase (Promega) and 500 ng random hexamer primers (IDT) for 1 h at 37°C according to manufacturer's instructions. The resulting cDNA was diluted 1:2 with sterile distilled water.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used to test mock-infected bees in all experimental replicates for pre-existing infections with naturally infecting honey bee viruses, since bees were obtained from honey bee colonies that may have varying levels of naturally prevalent bee infecting viruses (i.e., BQCV, CBPV, ABPV, DWV, IAPV, KBV, SBV, LSVs 1–4). To reduce the number of individual PCR tests, individual bee cDNA samples (n= 11-12) were pooled, resulting in three pooled samples per treatment. Each pool was tested for pre-existing virus infections using pathogen-specific primers. PCR was performed according to standard methods (79). In brief, 2 µL cDNA template was combined with 10 pmol of each forward and reverse primer, and amplified with Denville 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, 57°C for 30 s, 72°C for 30s, 35 cycles, followed by final elongation at 72°C for 4 min.

Negative strand specific PCR

FHV-infected honey bee samples were pooled (n=5) and analyzed for the presence of the negative-strand RNA of the viral genome (i.e., the replicative intermediate form) using strand-specific PCR (80-82). 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 FHV nor with the honey bee genome. Reactions were carried out by incubating 500 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 1 h 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, 2 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 (Supplemental 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 FHV-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. The 72 hpi time point was selected for the detection of negative strand since FHV abundance was highest at this time.

Quantitative PCR (qPCR)

Quantitative PCR was used to quantify relative virus abundance and honey immune gene transcript levels. All qPCR reactions were performed in triplicate with 2 μ L of cDNA template. Each 20 μ L reaction contained 1 $\times$ ChoiceTaq Mastermix (Thomas Scientific), 0.4 μ M each forward and reverse primer, 1 $\times$ SYBR Green (Life Technologies), and 3 mM MgCl₂. A CFX Connect Real Time instrument (BioRad) was used to carry out reactions with the following thermo-profile: pre-incubation at 95°C for 1 min followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 15 s; final extension at 72°C for 1 min, followed by melt curve analysis. Reactions without template were carried out as negative controls. qPCR specificity was verified through melt point analysis, and all products had previously been verified by sequencing. Plasmid standards containing 10³-10⁹ copies per reaction were used as qPCR templates to determine the primer efficiency and create linear standard curves used to determine viral RNA copies in each sample. The relative expression of host genes was determined by a ranked $\Delta\Delta$ Ct method in which the Δ Ct was calculated by subtracting the *rpl8* Ct value from the Ct of the gene of interest (goi) (83). Then, the within-group Δ Ct values were ranked from highest to lowest, and the relevant corresponding control Δ Ct value

was subtracted from the treatment group ΔCt to obtain the $\Delta\Delta\text{Ct}$. The fold-change in cDNA abundance was calculated by the equation $2^{-\Delta\Delta\text{Ct}}$.

Statistical analysis

All data were analyzed using R v4.0.2 in RStudio V1.3.1073. Pairwise comparisons for virus abundance and gene expression were evaluated using the `pairwise.wilcox.test` function in the base R stats package to perform a Wilcoxon Rank Sums test with Benjamini-Hochberg correlation for multiple comparisons (84).

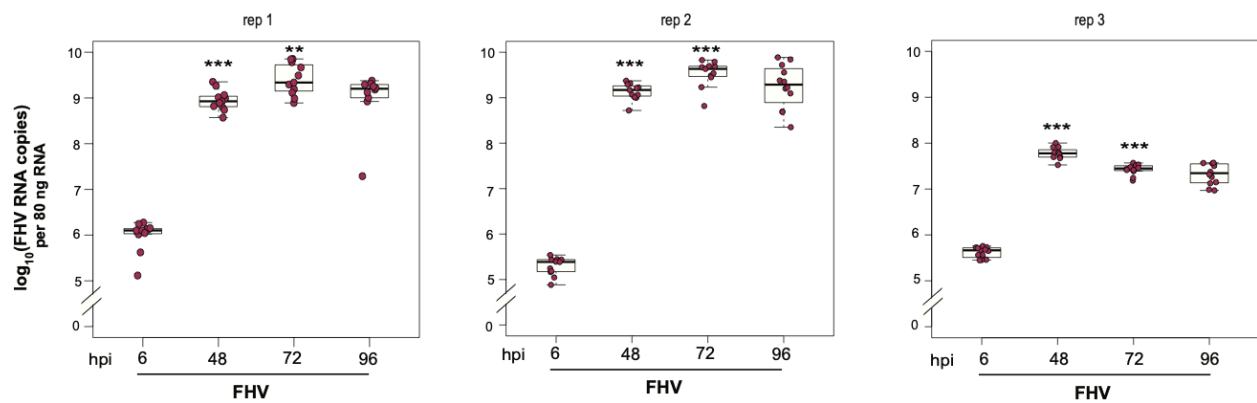
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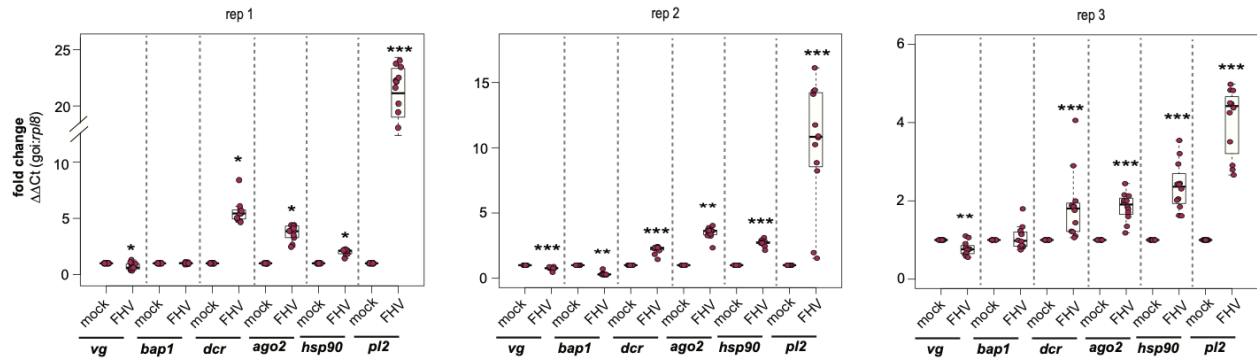
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Supplementary figures



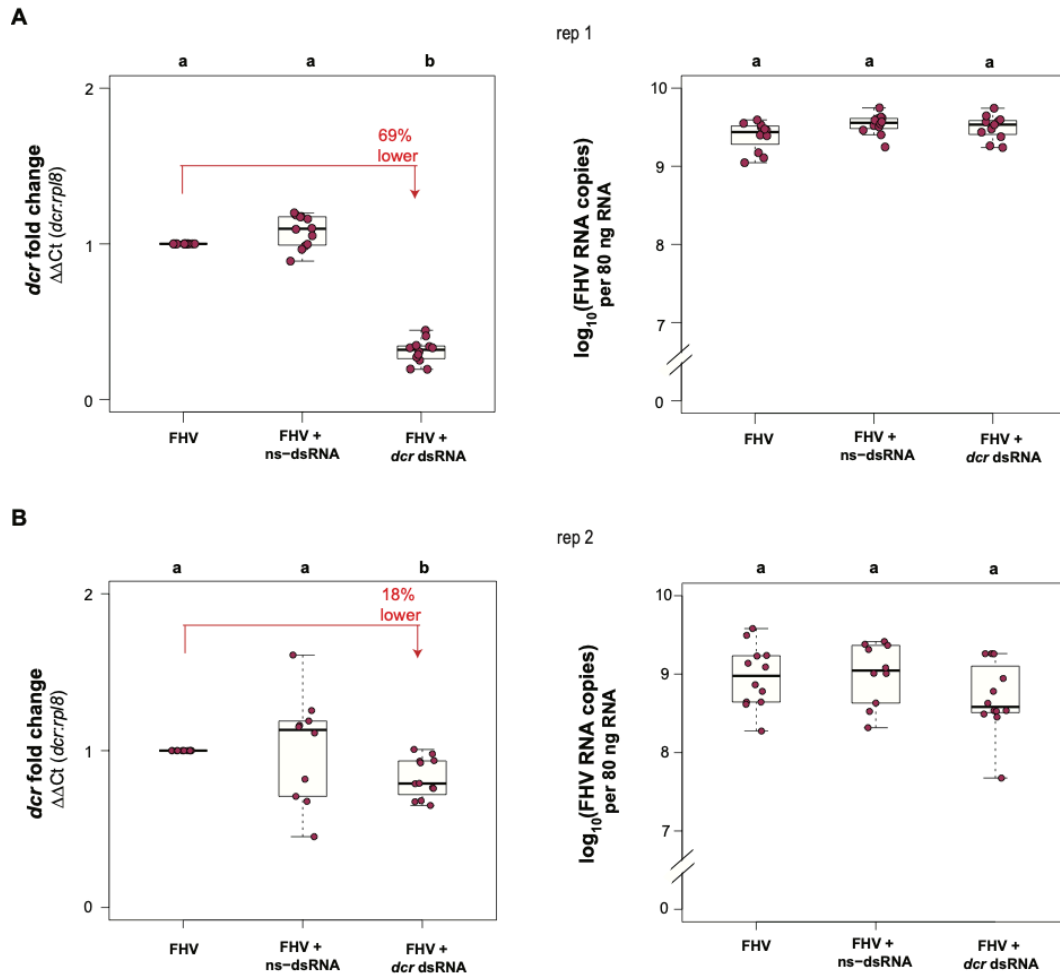
Supplemental Figure S1. Flock House virus (FHV) abundance increases over time in virus-infected honey bees.

FHV abundance in individual honey bees injected with 3.5×10^8 FHV RNA copies/bee was assessed over a time course (i.e., 6 hpi, 48 hpi, 72 hpi, 96 hpi) by qPCR (n= 9-12 per treatment group) in each of the three experimental replicates (rep1, rep2, rep3), one of which is included in Figure 2. In replicate one and two, log₁₀ FHV RNA levels were greater at 48 hpi and 72 hpi relative to 6 hpi. FHV levels did not change from 72 hpi to 96 hpi, but remained higher at 96 hpi relative to 6 hpi. In replicate three, log₁₀ FHV RNA levels were greater at 48 hpi relative to 6 hpi. FHV levels did not change from 48 hpi to 96 hpi, but remained higher at both these time points relative to 6 hpi. Statistical significance was assessed using Dunnett's test. Asterisks indicate a significant difference in virus abundance, significance levels: ** $p < 0.005$, *** $p < 0.0005$.



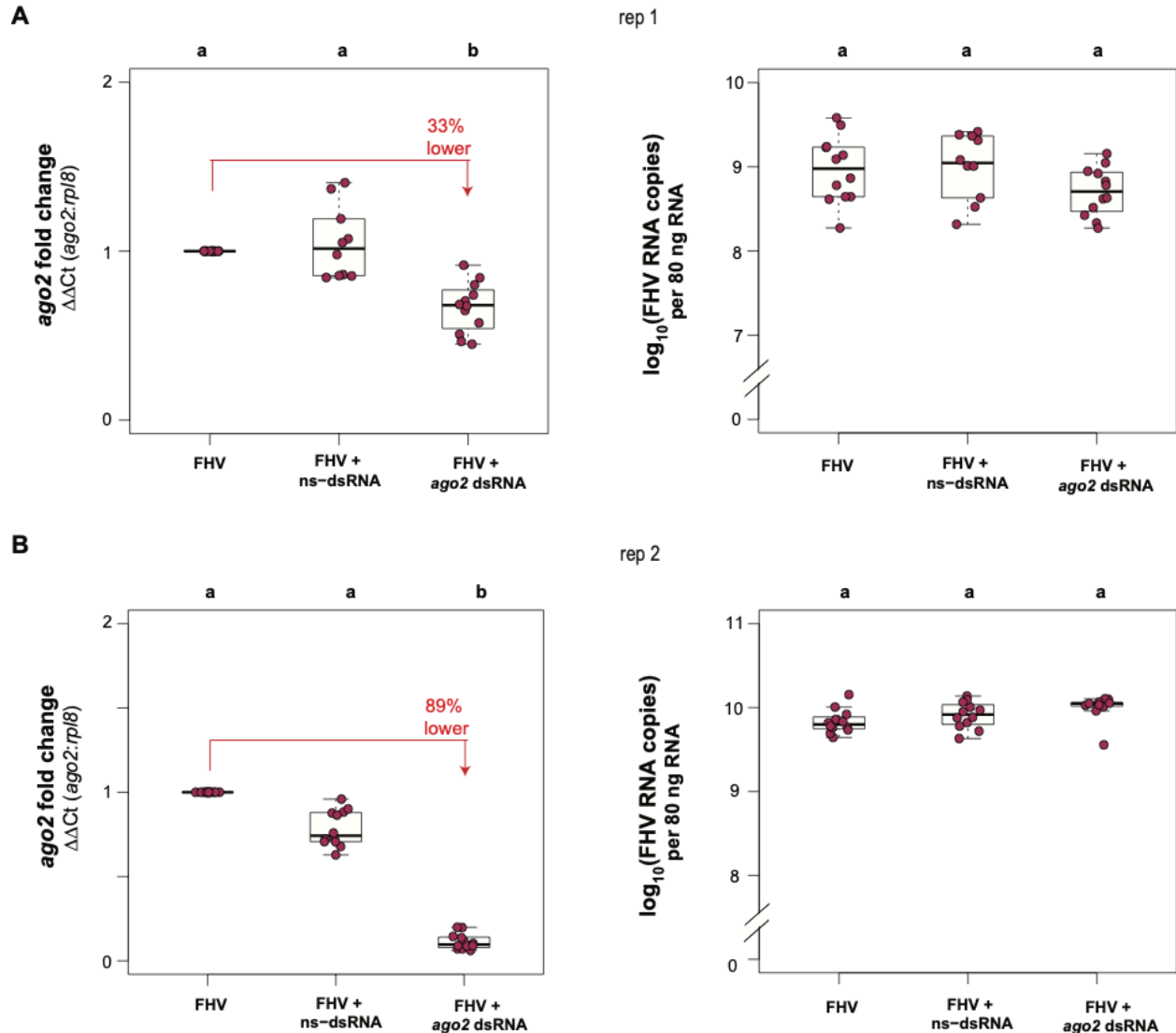
Supplemental Figure S2. Modulation in immune gene expression in FHV-infected honey bees.

The expression of select immune genes (i.e., *vitellogenin* (*vg*), *bee antiviral protein-1* (*bap1*), *dicer* (*dcr*), *argonaute-2* (*ago2*), *heat shock protein 90* (*hsp90*), and *protein lethal 2 essential for life-like* (*pl2*)) was assessed at 72 hpi in individual honey bees injected with 3.5×10^8 FHV RNA copies/bee by qPCR ($n = 9-12$ per treatment group) in each of the three experimental replicates (rep1, rep2, rep3), one of which is included in Figure 3. The data were analyzed using $\Delta\Delta C_t$ method with normalization to *rpl8*, and then the fold change values were calculated relative to mock-infected honey bees. In replicate one and three, the expression of expression of *vg* was lower and *bap1* was unchanged compared to mock-infected bees. Expression levels of *dcr*, *ago2*, *hsp90*, and *pl2* were higher in FHV-infected bees relative to mock-infected bees in both replicates, replicate one and three. In replicate two, *vg* and *bap1* expression levels were in FHV-infected bees compared to mock-infected bees. In FHV-infected honey bees, *dcr*, *ago2*, *hsp90*, and *pl2* expression was higher relative to mock-infected bees. Statistical significance was assessed using Wilcoxon Rank Sum test. Asterisks indicate a significant difference in virus abundance, significance levels: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.



Supplemental Figure S3. Reduced *dcr* expression does not impact FHV abundance in honey bees

The relative expression of *dicer* (*dcr*) was assessed by qPCR, with normalization to *rpl8* and fold changes were calculated relative to FHV-injected bees. FHV-abundance was measured by determining the FHV RNA copies using qPCR at 72 hpi in each of the two experimental replicates (rep1, rep2), one of which is included in Figure 4. **(A)** In replicate one, *dcr* expression was 18% lower ($p = 0.04$) in bees co-injected with FHV and *dcr* dsRNA, while *dcr* expression remained unchanged in bees co-injected with FHV and ns-dsRNA, relative to FHV-infected bees. FHV abundance levels were unchanged in bees co-injected with FHV and ns-dsRNA or *dcr*-dsRNA compared to FHV infected bees. **(B)** In replicate two, *dcr* expression was reduced by 69% in bees co-injected with FHV and *dcr* dsRNA ($p < 0.001$) compared to bees infected with FHV only. Honey bees co-injected with FHV and ns-dsRNA did not show appreciable changes in *dcr* expression. FHV abundance did not change in bees co-injected with FHV and either species of dsRNA (i.e., ns-dsRNA or *dcr* dsRNA) compared to FHV infected bees. Different letters above the treatment groups indicates a statistically significant difference.



Supplemental Figure S4. Reduced *ago2* expression does not impact FHV abundance in honey bees

The relative expression of *argonaute-2* (*ago2*) was assessed by qPCR, with normalization to *rp18* and fold changes were calculated relative to FHV-injected bees. FHV-abundance was measured by determining the FHV RNA copies using qPCR at 72 hpi in each of the two experimental replicates (rep1, rep2), one of which is included in Figure 4. **(A)** In replicate one, the expression of *ago2* was 33% lower ($p < 0.001$) in bee co-injected with FHV and *ago2* dsRNA compared to FHV-infected bees. *Ago2* expression was not changed in bees co-injected with FHV and ns-dsRNA. FHV abundance remained unchanged in *ago2* knockdown bees compared to bees infected with FHV only. **(B)** In replicate two, *ago2* expression was reduced by 89% in bees co-injected with FHV and *dcr* dsRNA ($p < 0.001$) compared to bees infected with FHV only. Honey bees co-injected with FHV and ns-dsRNA did not show appreciable changes in *dcr* expression. FHV abundance did not change in bees co-injected with FHV and either species of dsRNA (i.e., ns-

dsRNA or *dcr* dsRNA) compared to FHV infected bees. Different letters above the treatment groups indicates a statistically significant difference.

REFERENCES CITED

1. Gallai N, Salles J-M, Settele J, Vaissière BE. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*. 2009;68(3):810-21.
2. Eilers EJ, Kremen C, Smith Greenleaf S, Garber AK, Klein A-M. Contribution of Pollinator-Mediated Crops to Nutrients in the Human Food Supply. *PLOS ONE*. 2011;6(6):e21363.
3. Klein A-M, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, et al. Importance of pollinators in changing landscapes for world crops. *Proceedings of the royal society B: biological sciences*. 2007;274(1608):303-13.
4. Calderone NW. Insect Pollinated Crops, Insect Pollinators and US Agriculture: Trend Analysis of Aggregate Data for the Period 1992–2009. *PLOS ONE*. 2012;7(5):e37235.
5. Spleen AM, Lengerich EJ, Rennich K, Caron D, Rose R, Pettis JS, et al. A national survey of managed honey bee 2011–12 winter colony losses in the United States: results from the Bee Informed Partnership. *Journal of Apicultural Research*. 2013;52(2):44-53.
6. vanEngelsdorp D, Caron D, Hayes J, Underwood R, Henson M, Rennich K, et al. A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. *Journal of Apicultural Research*. 2012;51(1):115-24.
7. Seitz N, Traynor KS, Steinhauer N, Rennich K, Wilson ME, Ellis JD, et al. A national survey of managed honey bee 2014–2015 annual colony losses in the USA. *Journal of Apicultural Research*. 2015;54(4):292-304.
8. Traynor KS, Rennich K, Forsgren E, Rose R, Pettis J, Kunkel G, et al. Multiyear survey targeting disease incidence in US honey bees. *Apidologie*. 2016;47(3):325-47.
9. Steinhauer NA, Rennich K, Wilson ME, Caron DM, Lengerich EJ, Pettis JS, et al. A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. *Journal of Apicultural Research*. 2014;53(1):1-18.
10. McMenamin AJ, Brutscher LM, Glenny W, Flenniken ML. Abiotic and biotic factors affecting the replication and pathogenicity of bee viruses. *Current Opinion in Insect Science*. 2016;16:14-21.
11. Cornman RS, Tarpy DR, Chen Y, Jeffreys L, Lopez D, Pettis JS, et al. Pathogen Webs in Collapsing Honey Bee Colonies. *PLOS ONE*. 2012;7(8):e43562.
12. Brutscher LM, McMenamin AJ, Flenniken ML. The Buzz about Honey Bee Viruses. *PLOS Pathogens*. 2016;12(8):e1005757.

13. Forsgren E. European foulbrood in honey bees. *Journal of Invertebrate Pathology*. 2010;103:S5-S9.
14. Genersch E. American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *Journal of Invertebrate Pathology*. 2010;103:S10-S9.
15. Cornman RS, Chen YP, Schatz MC, Street C, Zhao Y, Desany B, et al. Genomic Analyses of the Microsporidian *Nosema ceranae*, an Emergent Pathogen of Honey Bees. *PLOS Pathogens*. 2009;5(6):e1000466.
16. Ravoet J, Schwarz RS, Descamps T, Yañez O, Tozkar CO, Martin-Hernandez R, et al. Differential diagnosis of the honey bee trypanosomatids *Crithidia mellificae* and *Lotmaria passim*. *Journal of Invertebrate Pathology*. 2015;130:21-7.
17. Goulson D, Nicholls E, Botías C, Rotheray EL. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*. 2015;347(6229):1255957.
18. McMenamin AJ, Flenniken ML. Recently identified bee viruses and their impact on bee pollinators. *Current Opinion in Insect Science*. 2018;26:120-9.
19. Grozinger CM, Flenniken ML. Bee Viruses: Ecology, Pathogenicity, and Impacts. *Annual Review of Entomology*. 2019;64(1):205-26.
20. 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(1):417-32.
21. Chen Y, Evans J, Feldlaufer M. Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. *J Invertebr Pathol*. 2006;92(3):152-9.
22. Yue C, Schröder M, Gisder S, Genersch E. Vertical-transmission routes for deformed wing virus of honeybees (*Apis mellifera*). *Journal of General Virology*. 2007;88(8):2329-36.
23. Ramsey SD, Ochoa R, Bauchan G, Gulbranson C, Mowery JD, Cohen A, et al. *Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph. *Proceedings of the National Academy of Sciences*. 2019;116(5):1792.
24. Annoscia D, Brown SP, Di Prisco G, De Paoli E, Del Fabbro S, Frizzera D, et al. Haemolymph removal by *Varroa* mite destabilizes the dynamical interaction between immune effectors and virus in bees, as predicted by Volterra's model. *Proceedings of the Royal Society B: Biological Sciences*. 2019;286(1901):20190331.
25. Di Prisco G, Annoscia D, Margiotta M, Ferrara R, Varricchio P, Zanni V, et al. 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(12):3203-8.

26. Francis RM, Nielsen SL, Kryger P. Patterns of viral infection in honey bee queens. *J Gen Virol.* 2013;94(Pt 3):668-76.
27. Bailey L. Honey Bee Pathology. *Annual Review of Entomology.* 1968;13(1):191-212.
28. de Miranda JR, Genersch E. Deformed wing virus. *Journal of Invertebrate Pathology.* 2010;103:S48-S61.
29. McMenamin AJ, Daughenbaugh KF, Parekh F, Pizzorno MC, Flenniken ML. Honey Bee and Bumble Bee Antiviral Defense. *Viruses.* 2018;10(8):395.
30. Evans J, Aronstein K, Chen YP, Hetru C, Imler JL, Jiang H, et al. Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect molecular biology.* 2006;15(5):645-56.
31. Ding S-W. RNA-based antiviral immunity. *Nature Reviews Immunology.* 2010;10(9):632-44.
32. Hammond SM. Dicing and slicing: The core machinery of the RNA interference pathway. *FEBS Letters.* 2005;579(26):5822-9.
33. Wang X-H, Aliyari R, Li W-X, Li H-W, Kim K, Carthew R, et al. RNA Interference Directs Innate Immunity Against Viruses in Adult *Drosophila*. *Science.* 2006;312(5772):452-4.
34. Deddouche S, Matt N, Budd A, Mueller S, Kemp C, Galiana-Arnoux D, et al. The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in *drosophila*. *Nat Immunol.* 2008;9(12):1425-32.
35. Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, Imler J-L. Essential function in vivo for Dicer-2 in host defense against RNA viruses in *drosophila*. *Nature Immunology.* 2006;7(6):590-7.
36. Zambon RA, Vakharia VN, Wu LP. RNAi is an antiviral immune response against a dsRNA virus in *Drosophila melanogaster*. *Cellular microbiology.* 2006;8(5):880-9.
37. Brutscher LM, Daughenbaugh KF, Flenniken ML. Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. *Scientific reports.* 2017;7(1):1-15.
38. Flenniken ML, Andino R. Non-specific dsRNA-mediated antiviral response in the honey bee. *Plos one.* 2013;8(10):e77263.
39. Chen YP, Pettis JS, Corona M, Chen WP, Li CJ, Spivak M, et al. Israeli Acute Paralysis Virus: Epidemiology, Pathogenesis and Implications for Honey Bee Health. *PLOS Pathogens.* 2014;10(7):e1004261.

40. Ryabov EV, Wood GR, Fannon JM, Moore JD, Bull JC, Chandler D, et al. 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(6):e1004230.
41. Ryabov EV, Christmon K, Heerman MC, Posada-Florez F, Harrison RL, Chen Y, et al. Development of a Honey Bee RNA Virus Vector Based on the Genome of a Deformed Wing Virus. *Viruses*. 2020;12(4):374.
42. Lamp B, Url A, Seitz K, Eichhorn J, Riedel C, Sinn LJ, et al. Construction and Rescue of a Molecular Clone of Deformed Wing Virus (DWV). *PLOS ONE*. 2016;11(11):e0164639.
43. Seitz K, Buczolic K, Dikunová A, Plevka P, Power K, Rümenapf T, et al. A molecular clone of Chronic Bee Paralysis Virus (CBPV) causes mortality in honey bee pupae (*Apis mellifera*). *Scientific Reports*. 2019;9(1):16274.
44. McMenamin AJ, Daughenbaugh KF, Flenniken ML. The heat shock response in the Western honey bee (*Apis Mellifera*) is antiviral. *Viruses*. 2020;12(2):245.
45. O'Neal ST, Brewster CC, Bloomquist JR, Anderson TD. Amitraz and its metabolite modulate honey bee cardiac function and tolerance to viral infection. *Journal of Invertebrate Pathology*. 2017;149:119-26.
46. O'Neal ST, Swale DR, Anderson TD. ATP-sensitive inwardly rectifying potassium channel regulation of viral infections in honey bees. *Scientific reports*. 2017;7(1):1-9.
47. Dasgupta R, Cheng L-L, Bartholomay LC, Christensen BM. Flock house virus replicates and expresses green fluorescent protein in mosquitoes. *Journal of General Virology*. 2003;84(7):1789-97.
48. Dasgupta R, Selling B, Rueckert R, editors. *Flock house virus: a simple model for studying persistent infection in cultured Drosophila cells* 1994; Vienna: Springer Vienna.
49. Dasgupta R, Free HM, Zietlow SL, Paskewitz SM, Aksoy S, Shi L, et al. Replication of Flock House Virus in Three Genera of Medically Important Insects. *Journal of Medical Entomology*. 2007;44(1):102-10.
50. Dearing SC, Scotti PD, Wigley PJ, Dhana SD. A small RNA virus isolated from the grass grub, *Costelytra zealandica* (Coleoptera: Scarabaeidae). *New Zealand Journal of Zoology*. 1980;7(2):267-9.
51. Scotti PD, Dearing S, Mossop DW. Flock house virus: A Nodavirus isolated from *Costelytra zealandica* (White) (Coleoptera: Scarabaeida). *Archives of Virology*. 1983;75(3):181-9.

52. Chao JA, Lee JH, Chapados BR, Debler EW, Schneemann A, Williamson JR. Dual modes of RNA-silencing suppression by Flock House virus protein B2. *Nature structural & molecular biology*. 2005;12(11):952-7.
53. Li H, Li WX, Ding SW. Induction and Suppression of RNA Silencing by an Animal Virus. *Science*. 2002;296(5571):1319-21.
54. Lu R, Maduro M, Li F, Li HW, Broitman-Maduro G, Li WX, et al. Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*. *Nature*. 2005;436(7053):1040-3.
55. Dasgupta R, Garcia BH, 2nd, Goodman RM. Systemic spread of an RNA insect virus in plants expressing plant viral movement protein genes. *Proceedings of the National Academy of Sciences of the United States of America*. 2001;98(9):4910-5.
56. Mcmenamin AJ, Parekh Fenali, Lawrence Verena, Flenniken Michelle. Investigating virus- host interactions in cultured primary honey bee cells. *Insects*. 2021.
57. Brutscher LM, Flenniken ML. RNAi and Antiviral Defense in the Honey Bee. *Journal of Immunology Research*. 2015;2015:941897.
58. Maori E, Garbian Y, Kunik V, Mozes-Koch R, Malka O, Kalev H, et al. A Transmissible RNA Pathway in Honey Bees. *Cell Rep*. 2019;27(7):1949-59.e6.
59. Galbraith DA, Yang X, Niño EL, Yi S, Grozinger C. Parallel Epigenomic and Transcriptomic Responses to Viral Infection in Honey Bees (*Apis mellifera*). *PLOS Pathogens*. 2015;11(3):e1004713.
60. Parker JS, Barford D. Argonaute: a scaffold for the function of short regulatory RNAs. *Trends in Biochemical Sciences*. 2006;31(11):622-30.
61. McMenamin AJ, Brutscher LM, Daughenbaugh KF, Flenniken ML. The Honey Bee Gene Bee Antiviral Protein-1 Is a Taxonomically Restricted Antiviral Immune Gene. *Frontiers in Insect Science*. 2021;1(11).
62. Nayak A, Berry B, Tassetto M, Kunitomi M, Acevedo A, Deng C, et al. Cricket paralysis virus antagonizes Argonaute 2 to modulate antiviral defense in *Drosophila*. *Nature Structural & Molecular Biology*. 2010;17(5):547-54.
63. Taylor RC, Berendzen KM, Dillin A. Systemic stress signalling: understanding the cell non-autonomous control of proteostasis. *Nature Reviews Molecular Cell Biology*. 2014;15(3):211-7.
64. Iwasaki S, Kobayashi M, Yoda M, Sakaguchi Y, Katsuma S, Suzuki T, et al. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol Cell*. 2010;39(2):292-9.

65. McKinstry M, Chung C, Truong H, Johnston BA, Snow JW. The heat shock response and humoral immune response are mutually antagonistic in honey bees. *Scientific Reports*. 2017;7(1):8850.
66. Ryabov EV, Fannon JM, Moore JD, Wood GR, Evans DJ. 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.
67. Tufail M, Takeda M. Molecular characteristics of insect vitellogenins. *J Insect Physiol*. 2008;54(12):1447-58.
68. Münch D, Amdam G, Wolschin F. Ageing in a eusocial insect: molecular and physiological characteristics of life span plasticity in the honey bee. *Functional ecology*. 2008;22(3):407-21.
69. Amdam GV, Fennern E, Havukainen H. Vitellogenin in Honey Bee Behavior and Lifespan. In: Galizia CG, Eisenhardt D, Giurfa M, editors. *Honeybee Neurobiology and Behavior: A Tribute to Randolph Menzel*. Dordrecht: Springer Netherlands; 2012. p. 17-29.
70. Seehuus S-C, Norberg K, Gimsa U, Krekling T, Amdam GV. Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(4):962-7.
71. Page RE, Rueppell O, Amdam GV. Genetics of Reproduction and Regulation of Honeybee (*Apis mellifera* L.) Social Behavior. *Annual Review of Genetics*. 2012;46(1):97-119.
72. 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-e.
73. Dainat B, Evans JD, Chen YP, Gauthier L, Neumann P. Predictive markers of honey bee colony collapse. *PLoS One*. 2012;7(2):e32151.
74. Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E, et al. IAPV, a bee-affecting virus associated with colony collapse disorder can be silenced by dsRNA ingestion. *Insect molecular biology*. 2009;18(1):55-60.
75. 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):3172-85.
76. Niu J, Smagghe G, De Coninck DI, 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;70:127-37.

77. Marshall D, Schneemann A. Specific Packaging of Nodaviral RNA2 Requires the N-Terminus of the Capsid Protein. *Virology*. 2001;285(1):165-75.
78. Saleh M-C, Tassetto M, Van Rij RP, Goic B, Gausson V, Berry B, et al. Antiviral immunity in *Drosophila* requires systemic RNA interference spread. *Nature*. 2009;458(7236):346-50.
79. de Miranda JR, Bailey L, Ball BV, Blanchard P, Budge GE, Chejanovsky N, et al. Standard methods for virus research in *Apis mellifera*. *Journal of Apicultural Research*. 2013;52(4):1-56.
80. Boncristiani HF, Di Prisco G, Pettis JS, Hamilton M, Chen YP. Molecular approaches to the analysis of deformed wing virus replication and pathogenesis in the honey bee, *Apis mellifera*. *Virology Journal*. 2009;6(1):221.
81. Yue C, Genersch E. RT-PCR analysis of Deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). *J Gen Virol*. 2005;86(Pt 12):3419-24.
82. Ongus JR, Peters D, Bonmatin JM, 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(Pt 12):3747-55.
83. Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol*. 2002;30(6):503-12.
84. Bauer DF. Constructing Confidence Sets Using Rank Statistics. *Journal of the American Statistical Association*. 1972;67(339):687-90.

CHAPTER SIX

CONCLUSIONS AND DISCUSSION

Honey Bee Host-virus Interactions At The Individual And Cellular Levels

Honey bee (*Apis mellifera*) host-virus interactions were studied in laboratory-based experiments to better understand the antiviral defense mechanisms at the individual bee and cellular levels. First, individual bee studies were carried out to elucidate the impact of chemical stimulants and stressors on honey bee immune responses and in turn, the outcome of virus infection. Then, investigations at the cellular level were carried out to demonstrate that primary cell cultures derived from honey bee larvae (i.e., hemocytes, immune cells) and pupae (i.e., mixed cell populations) support replication of a panel of RNA viruses. In response to virus infection, these primary cell cultures mounted a virus-specific transcriptional response, as assessed by evaluating the expression of key antiviral genes including *bap1*, *ago2*, and *dcr-like*. Lastly, FHV infection assays were carried out in individual bees to investigate the differential expression of antiviral genes triggered in response to FHV infection. It was determined that the immune responses elicited by FHV in primary cells can be recapitulated in adult bees, suggesting that FHV can be utilized as a tool to study host-virus interactions at the individual and cellular levels.

Honey bees have evolved strategies to deal with the adverse impacts of abiotic and biotic stressors, including pathogens (1-4). At the individual level, honey bees combat the impact of virus infections by the induction of immune pathways including the Jak/STAT, JNK, and Imd pathways, and the NF- κ B/Dorsal mediated Toll pathway, and double stranded RNA (dsRNA)-triggered immune responses (i.e., RNAi and non-sequence specific dsRNA mediated antiviral mechanisms)

(5-7) (reviewed in (1)). Herein, we demonstrate that honey bees fed sucrose syrup supplemented with 0.16 ppm thyme oil harbored 25- 87% less virus than bees fed only sucrose syrup. This trend was consistent for a panel of viruses including FHV, SINV-GFP, and DWV. Honey bees fed thyme oil exhibited greater expression of genes involved in antiviral defense including the RNAi pathway (i.e., *ago2* and *dcr-like*) and antimicrobial peptide genes regulated by Toll and Imd pathways (i.e., *abaecin* and *hymenoptaecin*), as well as a gene involved in immune competence and a marker of the overall bee health, *vitellogenin*. Together these results indicate that thyme oil stimulates honey bee immune responses that mitigated virus infections. Our results, coupled with previous studies are promising and may indicate that thyme oil augmented diets, either naturally acquired or fed, enhance individual bee immunocompetence and can be further developed as a strategy that beekeepers could use to mitigate virus-associated colony deaths (8). Additional studies are required to further investigate the precise mechanisms of action, while results to date suggest that thymol stimulates the expression of key honey bee immune genes. The signal transduction cascades, sensors, transcription factors, and effector proteins involved have yet to be fully elucidated.

The impact of agrochemicals, including neonicotinoid insecticides on honey bee health and colony longevity is an important area of research that includes a large body of literature (9-12). We determined that honey bees fed sucrose containing clothianidin (1 ppb) had reduced expression of key immune genes (i.e., *ago2*, *dcr-like*, *abaecin*, *hymenoptaecin*, and *vitellogenin*), which resulted in more severe virus infections, using virus abundance as a proxy for infection severity. Together our results coupled with those from previous studies, suggest that sublethal doses of clothianidin may increase the overall virus burden in a colony. However, honey bees fed sucrose

syrup containing 10 ppb clothianidin had virus levels that were similar to, or lower than, levels in bees fed only sucrose syrup. Although additional studies are needed, we hypothesize that bees fed 10 ppb clothianidin may have been less suitable hosts for viruses, which are obligate intracellular pathogens and, thus, rely on healthy host cells for replication. In addition to insecticides, honey bees are exposed to beekeeper applied in hive treatments, including miticides and the antifungal compound fumagillin, which is used to mitigate *Nosema* spp. infections. To further investigate the impacts of fumagillin treatment on the honey bee immune system and the outcome of virus infection, honey bees were fed sucrose containing the recommended dose of 25 ppm, or a greater dose of 75 ppm. We determined that virus abundance was higher in bees fed sucrose syrup containing 25 ppm or 75 ppm fumagillin, relative to bees fed sucrose syrup only. The greater virus abundance is likely due to immune suppression, as the expression of select honey bee immune genes (i.e., *ago2*, *dcr-like*, *abaecin*, *hymenoptaecin*, and *vitellogenin*) were reduced in fumagillin fed bees. Although future studies are warranted before adjusting recommendations for honey bee colony management, our results suggest that beekeepers should consider trade-off of reducing *Nosema* infections, but increasing virus abundance. While the focus of our experiments was to evaluate the impact of dual stressors on individual honey bee health, we determined that honey bees exposed to a single stressor, i.e., fumagillin (25 ppm or 75 ppm) or clothianidin (1 ppb) had reduced levels of key immune genes (i.e., *ago2*, *dcr-like*, *abaecin*, *hymenoptaecin*, and *vitellogenin*) compared to bees fed sucrose syrup alone. The reduced expression of *vitellogenin* in fumagillin or clothianidin (1 ppb) fed bees indicates poor health status of the host while the reduced expression of key immune genes suggests hindered immune responses. Collectively, this work demonstrates that thyme oil serves as an immunostimulant and results in reduced virus abundance

while chemical stressors such as fumagillin and clothianidin suppress the honey bee immune response resulting in more severe viral infections. Future studies should focus on investigating the impact of simultaneous exposure to chemical stimulant (i.e., thyme oil) and chemical stressor (i.e., clothianidin) at the colony level that may help beekeeping operations to reduce virus associated colony losses.

Investigating honey bee host-virus interactions at the cellular level has been challenging since robust cell culture systems are currently unavailable. The currently available honey bee fibroblast-type cell line, AmE-711 is difficult to maintain, making it challenging to use for studying honey bee host-virus interactions (13, 14). Therefore, the work described herein focuses on establishing primary cell cultures from two honey bee developmental stages- i.e., larvae and pupae. Hemocytes, the primary immune cells that phagocytose or encapsulate pathogens, were isolated from honey bee larvae and maintained in culture (15). We demonstrated that hemocytes can be productively infected by a model virus (i.e., FHV) as well as a honey bee infecting virus (i.e., SBV). In addition, we demonstrated that hemocytes extracted from larvae were naturally infected by SBV. The observation that honey bee hemocytes are infected by viruses has important implications for viral pathogenesis, since viruses are known to destabilize cellular processes and impair cell function. Moreover, the ability of hemocytes to support FHV replication provides tools to study honey bee hemocyte-virus interactions more precisely since FHV is structurally and genetically well-studied, and easy to propagate and purify. Additionally, we demonstrated productive replication of SBV, FHV, and DWV in mixed cell cultures (including epithelial cells, adipocytes, muscle cells, hemocytes) derived from honey bee pupae. Infected honey bee pupal cell cultures exhibited virus specific differential expression profiles of *bap1*, *ago2*, and *dcr-like* in

response to SBV, FHV, and DWV, suggesting that these cultures provide useful tools for studying honey bee transcriptional responses to viral infection. Furthermore, we demonstrated that FHV-infected honey bee hemocytes and FHV-infected pupal cells elicit similar immune response profiles, i.e., higher expression of *ago2* and *dcr-like* and no change in *bap1* expression. Antiviral immune response elicited by honey bee pupal cell cultures in response to DWV infection also recapitulate data obtained from transcriptome level studies in honey bee pupae (16).

To assess the transcriptional regulation of honey bee immune genes in response to FHV infection, we experimentally inoculated adult honey bees with FHV. Similar to immune responses mounted in honey bee hemocytes and pupal cells, FHV-infected adult honey bees exhibited higher levels of *ago2* and *dcr-like* expression, and no change in *bap1* expression. In addition, the expression of select heat shock protein (HSP) expressing genes was evaluated in FHV-infected bees since previous research determined that the heat shock response pathway is induced by various stressors, including virus infection (17, 18). The expression of *hsp90*, a core heat shock response gene and *p12*, a small heat shock protein was higher in FHV-infected bees compared to mock-infected bees, suggesting that FHV infection may trigger the heat shock response. In summary, these data suggest that FHV can serve as an effective model to investigate basic immunology questions important to better understand the honey bee antiviral immunity at both the cellular and individual bee levels.

Herein, the impact of phytochemicals and chemical stressors on honey bee immunity and consequent impact on outcome of virus infection was evaluated, primary honey bee cell culture tools were developed, and the honey bee immune response to FHV infection at individual bee and cellular level was investigated. This work focused on elucidating honey bee host-virus interactions

that may help develop strategies to mitigate virus associated honey bee losses at the colony level. The development of cell culture tools provides opportunities to identifying specific antiviral genes. Future work at the individual bee level should focus on investigating the impact of a mixture of chemical stimulant and stressor on the outcome of virus infection. At the cellular level, RNAi-mediated gene knockdown experiments will facilitate the study of honey bee host–virus interactions by directly demonstrating the importance of specific genes in limiting virus infection. In the future, it may be that bee breeders select for bees that express optimal levels of antiviral genes. In addition, better understanding of honey bee antiviral defense mechanisms and antiviral gene expression in the context of additional abiotic stressors, including agrochemical exposures, may prove important in modifications to current regulations and usage. Together, the studies described herein may aid in the development of strategies that reduce virus associated honey bee colony losses.

REFERENCES CITED

1. McMenamin AJ, Daughenbaugh KF, Parekh F, Pizzorno MC, Flenniken ML. Honey Bee and Bumble Bee Antiviral Defense. *Viruses*. 2018;10(8):395.
2. Spivak M, Reuter GS. Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. *Apidologie*. 2001;32(6):555-65.
3. Di Pasquale G, Salignon M, Le Conte Y, Belzunces LP, Decourtye A, Kretzschmar A, et al. Influence of Pollen Nutrition on Honey Bee Health: Do Pollen Quality and Diversity Matter? *PLOS ONE*. 2013;8(8):e72016.
4. Pritchard DJ. Grooming by honey bees as a component of varroa resistant behavior. *Journal of Apicultural Research*. 2016;55(1):38-48.
5. Kingsolver MB, Hardy RW. Making connections in insect innate immunity. *Proceedings of the National Academy of Sciences*. 2012;109(46):18639-40.
6. Kingsolver MB, Huang Z, Hardy RW. Insect Antiviral Innate Immunity: Pathways, Effectors, and Connections. *Journal of Molecular Biology*. 2013;425(24):4921-36.
7. Brutscher LM, Daughenbaugh KF, Flenniken ML. Antiviral defense mechanisms in honey bees. *Current Opinion in Insect Science*. 2015;10:71-82.
8. Palmer-Young EC, Tozkar CÖ, Schwarz RS, Chen Y, Irwin RE, Adler LS, et al. Nectar and Pollen Phytochemicals Stimulate Honey Bee (Hymenoptera: Apidae) Immunity to Viral Infection. *Journal of Economic Entomology*. 2017;110(5):1959-72.
9. Sandrock C, Tanadini M, Tanadini LG, Fauser-Misslin A, Potts SG, Neumann P. Impact of Chronic Neonicotinoid Exposure on Honeybee Colony Performance and Queen Supersedure. *PLOS ONE*. 2014;9(8):e103592.
10. Wade A, Lin C-H, Kurkul C, Regan ER, Johnson RM. Combined toxicity of insecticides and fungicides applied to California almond orchards to honey bee larvae and adults. *Insects*. 2019;10(1):20.
11. Shi J, Liao C, Wang Z, Zeng Z, Wu X. Effects of sublethal acetamiprid doses on the lifespan and memory-related characteristics of honey bee (*Apis mellifera*) workers. *Apidologie*. 2019;50(4):553-63.
12. Johnson RM, Ellis MD, Mullin CA, Frazier M. Pesticides and honey bee toxicity – USA*. *Apidologie*. 2010;41(3):312-31.
13. Carrillo-Tripp J, Dolezal AG, Goblirsch MJ, Miller WA, Toth AL, Bonning BC. In vivo and in vitro infection dynamics of honey bee viruses. *Scientific Reports*. 2016;6(1):22265.

14. Goblirsch MJ, Spivak MS, Kurtti TJ. A Cell Line Resource Derived from Honey Bee (*Apis mellifera*) Embryonic Tissues. *PLOS ONE*. 2013;8(7):e69831.
15. Lavine MD, Strand MR. Insect hemocytes and their role in immunity. *Insect Biochem Mol Biol*. 2002;32(10):1295-309.
16. Ryabov EV, Fannon JM, Moore JD, Wood GR, Evans DJ. 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.
17. McMenamin AJ, Daughenbaugh KF, Flenniken ML. The heat shock response in the Western honey bee (*Apis Mellifera*) is antiviral. *Viruses*. 2020;12(2):245.
18. Merklings SH, Overheul GJ, van Mierlo JT, Arends D, Gilissen C, van Rij RP. The heat shock response restricts virus infection in *Drosophila*. *Scientific Reports*. 2015;5(1):12758.

ALL REFERENCES CITED

- Abdelkader FB, Kairo G, Bonnet M, Barbouche N, Belzunces LP, Brunet JL. Effects of clothianidin on antioxidant enzyme activities and malondialdehyde level in honey bee drone semen. *Journal of Apicultural Research* (2019) 58(5):740-5. doi: 10.1080/00218839.2019.1655182.
- Aizen MA, Garibaldi LA, Cunningham SA, Klein AM. Long-Term Global Trends in Crop Yield and Production Reveal No Current Pollination Shortage but Increasing Pollinator Dependency. *Current Biology*. 2008;18(20):1572-5.
- Aizen MA, Harder LD. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. *Curr Biol*. 2009;19(11):915-8.
- Alaux C, Ducloz F, Crauser D, Le Conte Y. Diet effects on honeybee immunocompetence. *Biology letters* (2010) 6(4):562-5.
- 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.
- Alburaki M, Boutin S, Mercier P-L, Loublier Y, Chagnon M, Derome N. Neonicotinoid-Coated Zea mays Seeds Indirectly Affect Honeybee Performance and Pathogen Susceptibility in Field Trials. *PLOS ONE* (2015) 10(5):e0125790. doi: 10.1371/journal.pone.0125790.
- 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.
- Amdam GV, Fennern E, Havukainen H. Vitellogenin in Honey Bee Behavior and Lifespan. In: Galizia CG, Eisenhardt D, Giurfa M, editors. *Honeybee Neurobiology and Behavior: A Tribute to Randolph Menzel*. Dordrecht: Springer Netherlands (2012). p. 17-29.
- Amdam GV, Omholt SW. The Regulatory Anatomy of Honeybee Lifespan. *Journal of Theoretical Biology* (2002) 216(2):209-28. doi: <https://doi.org/10.1006/jtbi.2002.2545>.
- 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.
- Annoscia D, Brown SP, Di Prisco G, De Paoli E, Del Fabbro S, Frizzera D, et al. Haemolymph removal by Varroa mite destabilizes the dynamical interaction between immune effectors and virus in bees, as predicted by Volterra's model. *Proceedings of the Royal Society B: Biological Sciences* (2019) 286(1901):20190331. doi: doi:10.1098/rspb.2019.0331.
- Annoscia D, Del Piccolo F, Covre F, Nazzi F. Mite infestation during development alters the in-hive behaviour of adult honeybees. *Apidologie* (2015) 46(3):306-14.
- Annoscia D, Di Prisco G, Becchimanzi A, Caprio E, Frizzera D, Linguadoca A, et al. Neonicotinoid Clothianidin reduces honey bee immune response and contributes to Varroa mite proliferation. *Nature Communications* (2020) 11(1):5887. doi: 10.1038/s41467-020-19715-8.
- 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.
- Antúnez K, Anido M, Branchiccela B, Harriet J, Campa J, Invernizzi C, et al. Seasonal Variation of Honeybee Pathogens and its Association with Pollen Diversity in Uruguay. *Microbial Ecology* (2015) 70(2):522-33. doi: 10.1007/s00248-015-0594-7.
- Aquino DA, Peng D, Lopez C, Farooq M. The Constitutive Heat Shock Protein-70 Is Required for Optimal Expression of Myelin Basic Protein During Differentiation of Oligodendrocytes. *Neurochemical Research* (1998) 23(3):413-20. doi: 10.1023/A:1022473904335.
- 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.

- 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.
- Arras G, Grella GE. Wild thyme, *Thymus capitatus*, essential oil seasonal changes and antimycotic activity. *Journal of Horticultural Science* (1992) 67(2):197-202. doi: 10.1080/00221589.1992.11516237.
- 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.
- Bailey L. Honey Bee Pathology. *Annual Review of Entomology*. 1968;13(1):191-212.
- Bailey L. The Treatment of Nosema Disease with Fumagillin. *Bee World* (1953) 34(7):136-7. doi: 10.1080/0005772X.1953.11094806.
- Bailey, L. Acute bee-paralysis virus in adult honey bees injected with sacbrood virus. *Virology* 33, (2), 368.
- 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.

- Bauer DF. Constructing Confidence Sets Using Rank Statistics. *Journal of the American Statistical Association* (1972) 67(339):687-90. doi: 10.1080/01621459.1972.10481279.
- 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, USA, 2008; pp. 391–424.
- Beaurepaire A, Piot N, Doublet V, Antunez K, Campbell E, Chantawannakul P, et al. Diversity and Global Distribution of Viruses of the Western Honey Bee, *Apis mellifera*. *Insects* (2020) 11(4):239. PubMed PMID: 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.
- Bernklau E, Bjostad L, Hogeboom A, Carlisle A, H. S. A. Dietary Phytochemicals, Honey Bee Longevity and Pathogen Tolerance. *Insects*. 2019;10(1):14.
- 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.
- 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 *Sinivirus* phylogeny. *J. Gen. Virol.* 2017, 98, 2864–2875, doi:10.1099/jgv.0.000957.
- Blacqui re T, Smagghe G, van Gestel CAM, Mommaerts V. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology* (2012) 21(4):973-92. doi: 10.1007/s10646-012-0863-x.
- 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.

- Boecking O, Genersch E. Varroosis – the Ongoing Crisis in Bee Keeping. *Journal für Verbraucherschutz und Lebensmittelsicherheit* (2008) 3(2):221-8. doi: 10.1007/s00003-008-0331-y.
- 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.
- Bonmatin JM, Giorio C, Girolami V, Goulson D, Kreutzweiser DP, Krupke C, et al. Environmental fate and exposure; neonicotinoids and fipronil. *Environmental Science and Pollution Research*. 2015;22(1):35-67.
- 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.
- Botías C, David A, Horwood J, Abdul-Sada A, Nicholls E, Hill E, et al. Neonicotinoid Residues in Wildflowers, a Potential Route of Chronic Exposure for Bees. *Environmental Science & Technology* (2015) 49(21):12731-40. doi: 10.1021/acs.est.5b03459.
- 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.
- Bradbeer, N. World Distribution of Major Honeybee Diseases and Pests. *Bee World* 1988, 69, 15–39, doi:10.1080/0005772X.1988.11098943.
- Brandt A, Gorenflo A, Siede R, Meixner M, Büchler R. The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (*Apis mellifera* L.). *Journal of Insect Physiology* (2016) 86:40-7. doi: https://doi.org/10.1016/j.jinsphys.2016.01.001.
- 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.
- Brodschneider R, Crailsheim K. Nutrition and health in honey bees*. *Apidologie* (2010) 41(3):278-94.
- 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.

- Brosi, B. J. Pollinator specialization: from the individual to the community. *New Phytol.* 2016, 210, (4), 1190-1194.
- 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).
- Brutscher LM, Daughenbaugh KF, Flenniken ML. Antiviral defense mechanisms in honey bees. *Current Opinion in Insect Science.* 2015;10:71-82.
- Brutscher LM, Daughenbaugh KF, Flenniken ML. Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. *Scientific reports* (2017) 7(1):1-15.
- Brutscher LM, Daughenbaugh KF, Flenniken ML. Virus and dsRNA-triggered transcriptional responses reveal key components of honey bee antiviral defense. *Scientific reports.* 2017;7(1):1-15.
- Brutscher LM, Flenniken ML. RNAi and Antiviral Defense in the Honey Bee. *Journal of Immunology Research.* 2015;2015:941897.
- Brutscher LM, McMenamin AJ, Flenniken ML. The Buzz about Honey Bee Viruses. *PLOS Pathogens.* 2016;12(8):e1005757.
- 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.
- Brutscher, L. M.; Flenniken, M. L. RNAi and Antiviral Defense in the Honey Bee. *Journal of Immunology Research* 2015, 2015, (1), 941897-10.
- 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.
- 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.

- Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology* (2004) 94(3):223-53. doi: <https://doi.org/10.1016/j.ijfoodmicro.2004.03.022>.
- Cabirol A, Haase A. The Neurophysiological Bases of the Impact of Neonicotinoid Pesticides on the Behaviour of Honeybees. *Insects* (2019) 10(10):344. PubMed PMID: doi:10.3390/insects10100344.
- 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.
- 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, (12).
- 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 AG, Goblirsch MJ, Miller WA, Toth AL, Bonning BC. In vivo and in vitro infection dynamics of honey bee viruses. *Scientific Reports* (2016) 6(1):22265. doi: 10.1038/srep22265.
- 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.
- 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.
- 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.
- 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.
- 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, Evans J, Feldlaufer M. Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. *J Invertebr Pathol*. 2006;92(3):152-9.
- Chen Y, Evans J, Feldlaufer M. Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. *J Invertebr Pathol*. 2006;92(3):152-9.
- Chen Y, Evans JD, Smith IB, Pettis JS. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. *Journal of Invertebrate Pathology* (2008) 97(2):186-8. doi: <https://doi.org/10.1016/j.jip.2007.07.010>.
- Chen YP, Pettis JS, Corona M, Chen WP, Li CJ, Spivak M, et al. Israeli Acute Paralysis Virus: Epidemiology, Pathogenesis and Implications for Honey Bee Health. *PLOS Pathogens* (2014) 10(7):e1004261. doi: 10.1371/journal.ppat.1004261.
- Chen, Y. P.; Siede, R., Honey Bee Viruses. In *Adv Virus Res*, Elsevier: 2007; Vol. 70, pp 33-80.
- 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.; Evans, J.; Feldlaufer, M. Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. *J Invertebr Pathol* 2006, 92, (3), 152-159.
- 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.
- 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.
- Cohen E. Pesticide-mediated homeostatic modulation in arthropods. *Pesticide Biochemistry and Physiology* (2006) 85(1):21-7. doi: <https://doi.org/10.1016/j.pestbp.2005.09.002>.
- Collison E, Hird H, Cresswell J, Tyler C. Interactive effects of pesticide exposure and pathogen infection on bee health - a critical analysis. *Biol Rev Camb Philos Soc* (2016) 91(4):1006-19. Epub 2015/07/08. doi: 10.1111/brev.12206. PubMed PMID: 26150129.
- 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.

- Cornman RS, Chen YP, Schatz MC, Street C, Zhao Y, Desany B, et al. Genomic Analyses of the Microsporidian *Nosema ceranae*, an Emergent Pathogen of Honey Bees. *PLOS Pathogens*. 2009;5(6):e1000466.
- 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.
- 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.
- Corona M, Velarde RA, Remolina S, Moran-Lauter A, Wang Y, Hughes KA, et al. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proceedings of the National Academy of Sciences* (2007) 104(17):7128-33. doi: 10.1073/pnas.0701909104.
- Cosentino S, Tuberoso CIG, Pisano B, Satta M, Mascia V, Arzedi E, et al. In-vitro antimicrobial activity and chemical composition of Sardinian thymus essential oils. *Letters in applied microbiology* (1999) 29(2):130-5.
- Costa C, Lodesani M, Maistrello L. Effect of thymol and resveratrol administered with candy or syrup on the development of *Nosema ceranae* and on the longevity of honeybees (*Apis mellifera* L.) in laboratory conditions*. *Apidologie* (2010) 41(2):141-50.
- Coulon M, Schurr F, Martel A-C, Cougoule N, Bégau A, Mangoni P, et al. Influence of chronic exposure to thiamethoxam and chronic bee paralysis virus on winter honey bees. *PLOS ONE* (2019) 14(8):e0220703. doi: 10.1371/journal.pone.0220703.
- 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.
- 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.
- Cutler CG, Scott-Dupree CD. Exposure to Clothianidin Seed-Treated Canola Has No Long-Term Impact on Honey Bees. *Journal of Economic Entomology* (2007) 100(3):765-72. doi: 10.1093/jee/100.3.765.
- Cutler GC, Rix RR. Can poisons stimulate bees? Appreciating the potential of hormesis in bee–pesticide research. *Pest management science* (2015) 71(10):1368-70.

- Cutler GC. Insects, Insecticides and Hormesis: Evidence and Considerations for Study. *Dose-Response* (2013) 11(2):dose-response.12-008.Cutler. doi: 10.2203/dose-response.12-008.Cutler. PubMed PMID: 23930099.
- 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.
- Dainat B, Evans JD, Chen YP, Gauthier L, Neumann P. Predictive markers of honey bee colony collapse. *PLoS One.* 2012;7(2):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.
- 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>.
- 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.
- 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 LC, Christensen BM. Flock house virus replicates and expresses green fluorescent protein in mosquitoes. *Journal of General Virology.* 2003;84(7):1789-97.
- Dasgupta R, Free HM, Zietlow SL, Paskewitz SM, Aksoy S, Shi L, et al. Replication of Flock House Virus in Three Genera of Medically Important Insects. *Journal of Medical Entomology* (2007) 44(1):102-10. doi: 10.1093/jmedent/41.5.102.
- Dasgupta R, Garcia BH, 2nd, Goodman RM. Systemic spread of an RNA insect virus in plants expressing plant viral movement protein genes. *Proceedings of the National Academy of Sciences of the United States of America.* 2001;98(9):4910-5.
- Dasgupta R, Selling B, Rueckert R, editors. *Flock house virus: a simple model for studying persistent infection in cultured Drosophila cells* 1994; Vienna: Springer Vienna.
- 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.; 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.
- 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.
- de D'Aubeterre JP, Myrold DD, Royce LA, Rossignol PA. A scientific note of an application of isotope ratio mass spectrometry to feeding by the mite, *Varroa jacobsoni* Oudemans, on the honeybee, *Apis mellifera* L. *Apidologie* (1999) 30(4):351-2.
- 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, Bailey L, Ball BV, Blanchard P, Budge GE, Chejanovsky N, et al. Standard methods for virus research in *Apis mellifera*. *Journal of Apicultural Research*. 2013;52(4):1-56.
- De Miranda JR, Cornman RS, Evans JD, Semberg E, Haddad N, Neumann P, et al. Genome Characterization, Prevalence and Distribution of a Macula-Like Virus from *Apis mellifera* and *Varroa destructor*. *Viruses*. 2015;7(7):3586-602.
- de Miranda JR, Genersch E. Deformed wing virus. *Journal of Invertebrate Pathology*. 2010;103:S48-S61.
- 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.
- 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.
- 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, doi:10.1101/2020.09.15.298042.

- Decourtye A, Devillers J. Ecotoxicity of Neonicotinoid Insecticides to Bees. In: Thany SH, editor. *Insect Nicotinic Acetylcholine Receptors*. New York, NY: Springer New York; 2010. p. 85-95.
- 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.
- DeGrandi-Hoffman G, Chen Y, Simonds R. The Effects of Pesticides on Queen Rearing and Virus Titers in Honey Bees (*Apis mellifera* L.). *Insects* (2013) 4(1):71-89. PubMed PMID: doi:10.3390/insects4010071.
- DeGrandi-Hoffman, G.; Chen, Y. Nutrition, immunity and viral infections in honey bees. *Curr Opin Insect Sci* 2015, 10, 170-176.
- Desai S, Eu YJ, Whyard S, Currie R. 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(4):446-55.
- 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.
- Di Pasquale G, Salignon M, Le Conte Y, Belzunces LP, Decourtye A, Kretzschmar A, et al. Influence of Pollen Nutrition on Honey Bee Health: Do Pollen Quality and Diversity Matter? *PLOS ONE* (2013) 8(8):e72016. doi: 10.1371/journal.pone.0072016.
- Di Prisco G, Annoscia D, Margiotta M, Ferrara R, Varricchio P, Zanni V, et al. 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(12):3203-8.
- Di Prisco G, Cavaliere V, Annoscia D, Varricchio P, Caprio E, Nazzi F, et al. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences*. 2013;110(46):18466-71.
- 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.
- 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.

- 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.
- Didier ES. Effects of albendazole, fumagillin, and TNP-470 on microsporidial replication in vitro. *Antimicrobial Agents and Chemotherapy* (1997) 41(7):1541-6. doi: 10.1128/AAC.41.7.1541.
- Ding, S.-W. RNA-based antiviral immunity. *Nat Rev Immunol* 2010, 10, (9), 632-644.
- Dolezal AG, Carrillo-Tripp J, Judd TM, Allen Miller W, Bonning BC, Toth AL. Interacting stressors matter: diet quality and virus infection in honeybee health. *Royal Society open science* (2019) 6(2):181803.
- 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.
- 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.
- Doublet V, Labarussias M, de Miranda JR, Moritz RF, Paxton RJ. Bees under stress: sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle. *Environmental microbiology*. 2015;17(4):969-83.
- 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.
- Drescher N, Klein A-M, Schmitt T, Leonhardt SD. A clue on bee glue: New insight into the sources and factors driving resin intake in honeybees (*Apis mellifera*). *PLOS ONE* (2019) 14(2):e0210594. doi: 10.1371/journal.pone.0210594.
- 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.
- 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.
- Eilers EJ, Kremen C, Smith Greenleaf S, Garber AK, Klein A-M. Contribution of Pollinator-Mediated Crops to Nutrients in the Human Food Supply. *PLOS ONE*. 2011;6(6):e21363.
- 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.
- 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.
- Erez, T.; Chejanovsky, N. Infection of a Lepidopteran Cell Line with Deformed Wing Virus. *Viruses* 2020, 12, 739, doi:10.3390/v12070739.
- Evans J, Aronstein K, Chen YP, Hetru C, Imler JL, Jiang H, et al. Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect molecular biology*. 2006;15(5):645-56.
- 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.
- 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.; Mougél, 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.
- 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, Glenney W, Daughenbaugh KF, McMenamin AJ, Burkle LA, Flenniken ML. 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(9):e0237544. doi: 10.1371/journal.pone.0237544.
- Fine JD, Cox-Foster DL, Mullin CA. An Inert Pesticide Adjuvant Synergizes Viral Pathogenicity and Mortality in Honey Bee Larvae. *Scientific Reports* (2017) 7(1):40499. doi: 10.1038/srep40499.
- 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.; Andino, R. Non-specific dsRNA-mediated antiviral response in the honey bee. *PLoS ONE* 2013, 8, (10), e77263.
- 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.
- Floris I, Satta A, Cabras P, Garau VL, Angioni A. Comparison Between Two Thymol Formulations in the Control of *Varroa destructor*: Effectiveness, Persistence, and Residues. *Journal of Economic Entomology* (2004) 97(2):187-91. doi: 10.1093/jee/97.2.187.
- Forsgren E. European foulbrood in honey bees. *Journal of Invertebrate Pathology*. 2010;103:S5-S9.
- 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.
- 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.

- 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.
- Gábor, E.; Cinege, G.; Csordás, G.; Török, T.; Folkl-Medzihradsky, K.; Darula, Z.; Andó, I.; Kurucz, É. Hemolymph 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.
- 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.
- 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.
- 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. *Nat. Immunol.* 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 BE. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics* (2009) 68(3):810-21. doi: <https://doi.org/10.1016/j.ecolecon.2008.06.014>.
- 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.
- 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.; Dudenhofer, 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.; Tscharnkte, 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.

- Gätschenberger H, Azzami K, Tautz J, Beier H. Antibacterial Immune Competence of Honey Bees (*Apis mellifera*) Is Adapted to Different Life Stages and Environmental Risks. *PLOS ONE* (2013) 8(6):e66415. doi: 10.1371/journal.pone.0066415.
- 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, Gisder S, Hedtke K, Hunter WB, Möckel N, Müller U. Standard methods for cell cultures in *Apis mellifera* research. *Journal of Apicultural Research*. 2013;52(1):1–8.
- Genersch E, von der Ohe W, Kaatz H, Schroeder A, Otten C, Büchler R, et al. The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies*. *Apidologie* (2010) 41(3):332–52.
- Genersch E. American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *Journal of Invertebrate Pathology*. 2010;103:S10–S9.
- 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. *J. Apic. Res.* 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.; 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.
- 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.
- 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.
- Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol* (2002) 30(6):503–12. Epub 2002/06/14. doi: 10.1016/s0301-472x(02)00806-8. PubMed PMID: 12063017.
- 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 KF, Radford R, Kegley SE, Flenniken ML. Honey bee (*Apis mellifera*) colony health and pathogen composition in migratory beekeeping operations involved in California almond pollination. *PLoS One* (2017) 12(8):e0182814. Epub 2017/08/18. doi: 10.1371/journal.pone.0182814. PubMed PMID: 28817641; PubMed Central PMCID: PMC5560708.
- Glotzer JB, Saltik M, Chiocca S, Michou AI, Moseley P, Cotten M. Activation of heat-shock response by an adenovirus is essential for virus replication. *Nature* (2000) 407(6801):207-11. Epub 2000/09/23. doi: 10.1038/35025102. PubMed PMID: 11001061.
- Goblirsch MJ, Spivak MS, Kurtti TJ. A Cell Line Resource Derived from Honey Bee (*Apis mellifera*) Embryonic Tissues. *PLOS ONE*. 2013;8(7):e69831.
- 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.
- Goulson D, Nicholls E, Botías C, Rotheray EL. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* (2015) 347(6229):1255957. doi: 10.1126/science.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.; 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.
- 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),

- 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.
- Gregorc A, Planinc I. The control of Varroa destructor in honey bee colonies using the thymol-based acaricide-Apiguard. *American Bee Journal* (2005) 145(8):672-5.
- Grozinger CM, Flenniken ML. Bee Viruses: Ecology, Pathogenicity, and Impacts. *Annual Review of Entomology* (2019) 64(1):205-26. doi: 10.1146/annurev-ento-011118-111942. PubMed PMID: 30629896.
- Guedes RNC, Cutler GC. Insecticide-induced hormesis and arthropod pest management. *Pest Management Science* (2014) 70(5):690-7.
- 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.
- Guruceaga X, Perez-Cuesta U, Abad-Diaz de Cerio A, Gonzalez O, Alonso RM, Hernando FL, et al. Fumagillin, a Mycotoxin of *Aspergillus fumigatus*: Biosynthesis, Biological Activities, Detection, and Applications. *Toxins* (Basel). 2019;12(1):7.
- 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.
- 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.
- Hammond SM. Dicing and slicing: The core machinery of the RNA interference pathway. *FEBS Letters*. 2005;579(26):5822-9.
- 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. *Nat. Rev. Immunol.* 2021, 21, 441–453, doi:10.1038/s41577-020-00474-y.
- 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.

- Higes M, Nozal MJ, Alvaro A, Barrios L, Meana A, Martín-Hernández R, et al. The stability and effectiveness of fumagillin in controlling *Nosema ceranae* (Microsporidia) infection in honey bees (*Apis mellifera*) under laboratory and field conditions. *Apidologie* (2011) 42(3):364-77. doi: 10.1007/s13592-011-0003-2.
- Highfield AC, Nagar AE, Mackinder LCM, Noël LM-LJ, Hall MJ, Martin SJ, et al. Deformed Wing Virus Implicated in Overwintering Honeybee Colony Losses. *Applied and Environmental Microbiology* (2009) 75(22):7212-20. doi: doi:10.1128/AEM.02227-09.
- Hoffmann, J. A. The immune response of *Drosophila*. *Nature* 2003, 426, (6962), 33-38.
- 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.
- Huang W-F, Solter LF, Yau PM, Imai BS. *Nosema ceranae* Escapes Fumagillin Control in Honey Bees. *PLOS Pathogens* (2013) 9(3):e1003185. doi: 10.1371/journal.ppat.1003185.
- 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.
- Hunter WB. Medium for development of bee cell cultures (*Apis mellifera*: Hymenoptera: Apidae). *In Vitro Cellular & Developmental Biology-Animal*. 2010;46(2):83-6.
- 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). *Vitr. Cell. Dev. Biol. Anim.* 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.
- Iwasa T, Motoyama N, Ambrose JT, Roe RM. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Protection*. 2004;23(5):371-8.
- Iwasaki S, Kobayashi M, Yoda M, Sakaguchi Y, Katsuma S, Suzuki T, et al. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol Cell*. 2010;39(2):292-9.
- 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.
- 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.
- Jeschke P, Nauen R. Neonicotinoids—from zero to hero in insecticide chemistry. *Pest Management Science: formerly Pesticide Science* (2008) 64(11):1084-98.
- Johnson RM, Ellis MD, Mullin CA, Frazier M. Pesticides and honey bee toxicity – USA*. *Apidologie*. 2010;41(3):312-31.
- 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.
- Juliano C, Mattana A, Usai M. Composition and in vitro Antimicrobial Activity of the Essential Oil of *Thymus herba-barona* Loisel Growing Wild in Sardinia. *Journal of Essential Oil Research* (2000) 12(4):516-22. doi: 10.1080/10412905.2000.9699578.
- Kampmueller KM, Miller DJ. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in *Drosophila* cells. *J Virol* (2005) 79(11):6827-37. Epub 2005/05/14. doi: 10.1128/jvi.79.11.6827-6837.2005. PubMed PMID: 15890922; PubMed Central PMCID: PMC1112161.
- 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.
- Kemp C, Imler J-L. Antiviral immunity in *drosophila*. *Current opinion in immunology* (2009) 21(1):3-9.
- 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.

- Kingsolver MB, Hardy RW. Making connections in insect innate immunity. *Proceedings of the National Academy of Sciences*. 2012;109(46):18639-40.
- Kingsolver MB, Huang Z, Hardy RW. Insect Antiviral Innate Immunity: Pathways, Effectors, and Connections. *Journal of Molecular Biology*. 2013;425(24):4921-36.
- Klee J, Besana AM, Genersch E, Gisder S, Nanetti A, Tam DQ, et al. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. *Journal of Invertebrate Pathology* (2007) 96(1):1-10. doi: <https://doi.org/10.1016/j.jip.2007.02.014>.
- Klein A-M, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, et al. Importance of pollinators in changing landscapes for world crops. *Proceedings of the royal society B: biological sciences*. 2007;274(1608):303-13.
- 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.
- Krupke CH, Holland JD, Long EY, Eitzer BD. Planting of neonicotinoid-treated maize poses risks for honey bees and other non-target organisms over a wide area without consistent crop yield benefit. *Journal of Applied Ecology* (2017) 54(5):1449-58. doi: <https://doi.org/10.1111/1365-2664.12924>.
- Krupke CH, Hunt GJ, Eitzer BD, Andino G, Given K. Multiple Routes of Pesticide Exposure for Honey Bees Living Near Agricultural Fields. *PLOS ONE* (2012) 7(1):e29268. doi: 10.1371/journal.pone.0029268.
- Kulhanek K, Steinhauer N, Rennich K, Caron DM, Sagili RR, Pettis JS, et al. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. *Journal of Apicultural Research* (2017) 56(4):328-40. doi: 10.1080/00218839.2017.1344496.
- 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.
- 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 LJ, et al. Construction and Rescue of a Molecular Clone of Deformed Wing Virus (DWV). *PLOS ONE* (2016) 11(11):e0164639. doi: 10.1371/journal.pone.0164639.
- 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.
- Laurino D, Manino A, Patetta A, Porporato M. Toxicity of neonicotinoid insecticides on different honey bee genotypes. (2013).
- Lavine MD, Strand MR. Insect hemocytes and their role in immunity. *Insect Biochem Mol Biol.* 2002;32(10):1295-309.
- 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. 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.
- 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.
- Lemaitre, B.; Hoffmann, J. The Host Defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.* 2007, 25, (1), 697-743.
- Lens-Lisbonne C, Cremieux A, Maillard C, Balansard G. Methodes d'évaluation de l'activité antibactérienne des huiles essentielles: application aux essences de thym et de cannelle. *J Pharm Belg* (1987) 42(5):297-302.
- Levin S, Galbraith D, Sela N, Erez T, Grozinger CM, Chejanovsky N. Presence of *Apis* Rhabdovirus-1 in Populations of Pollinators and Their Parasites from Two Continents. *Frontiers in Microbiology.* 2017;8(2482).
- 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 H, Li WX, Ding SW. Induction and Suppression of RNA Silencing by an Animal Virus. *Science.* 2002;296(5571):1319-21.
- Li Z, Zhang S, Liu Q. Vitellogenin Functions as a Multivalent Pattern Recognition Receptor with an Opsonic Activity. *PLOS ONE* (2008) 3(4):e1940. doi: 10.1371/journal.pone.0001940.

- Li Z, Zhang S, Zhang J, Liu M, Liu Z. Vitellogenin is a cidal factor capable of killing bacteria via interaction with lipopolysaccharide and lipoteichoic acid. *Molecular Immunology* (2009) 46(16):3232-9. doi: <https://doi.org/10.1016/j.molimm.2009.08.006>.
- 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 CH, Sponsler DB, Richardson RT, Watters HD, Glinski DA, Henderson WM, et al. Honey bees and neonicotinoid-treated corn seed: contamination, exposure, and effects. *Environmental toxicology and chemistry* (2021) 40(4):1212-21.
- 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 T, Daniels CK, Cao S. Comprehensive review on the HSC70 functions, interactions with related molecules and involvement in clinical diseases and therapeutic potential. *Pharmacology & Therapeutics* (2012) 136(3):354-74. doi: <https://doi.org/10.1016/j.pharmthera.2012.08.014>.
- 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.
- 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.
- Lopez MI, Pettis JS, Smith IB, Chu P-S. Multiclass Determination and Confirmation of Antibiotic Residues in Honey Using LC-MS/MS. *Journal of Agricultural and Food Chemistry* (2008) 56(5):1553-9. doi: 10.1021/jf073236w.
- Lourenço AP, Florecki MM, Simões ZLP, Evans JD. Silencing of *Apis mellifera* dorsal genes reveals their role in expression of the antimicrobial peptide defensin-1. *Insect molecular biology* (2018) 27(5):577-89.
- Lourenço AP, Guidugli-Lazzarini KR, Freitas FCP, Bitondi MMG, Simões ZLP. Bacterial infection activates the immune system response and dysregulates microRNA expression in honey bees. *Insect Biochemistry and Molecular Biology* (2013) 43(5):474-82. doi: <https://doi.org/10.1016/j.ibmb.2013.03.001>.
- 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.
- Lundgren, J. G.; Duan, J. J. RNAi-Based Insecticidal Crops: Potential Effects on Nontarget Species. *Bioscience* 2013, 63, (8), 657-665.

- 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.
- Mao W, Schuler MA, Berenbaum MR. A dietary phytochemical alters caste-associated gene expression in honey bees. *Science Advances.* 2015;1(7):e1500795.
- Maori E, Garbian Y, Kunik V, Mozes-Koch R, Malka O, Kalev H, et al. A Transmissible RNA Pathway in Honey Bees. *Cell Rep.* 2019;27(7):1949-59.e6.
- Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E, et al. IAPV, a bee-affecting virus associated with colony collapse disorder can be silenced by dsRNA ingestion. *Insect molecular biology.* 2009;18(1):55-60.
- 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.; 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.
- Marco Antonio DS, Guidugli-Lazzarini KR, do Nascimento AM, Simões ZLP, Hartfelder K. RNAi-mediated silencing of vitellogenin gene function turns honeybee (*Apis mellifera*) workers into extremely precocious foragers. *Naturwissenschaften* (2008) 95(10):953-61. doi: 10.1007/s00114-008-0413-9.
- 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.
- 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.

- Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends in Pharmacological Sciences*. 2001;22(11):573-80.
- Matsumoto T. Reduction in homing flights in the honey bee *Apis mellifera* after a sublethal dose of neonicotinoid insecticides. *Bulletin of Insectology*. 2013;66(1):1-9.
- 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.
- McGimpsey JA, Douglas MH, Van Klink JW, Beauregard DA, Perry NB. Seasonal variation in essential oil yield and composition from naturalized *Thymus vulgaris* L. in New Zealand. *Flavour and Fragrance Journal* (1994) 9(6):347-52.
- McKinstry M, Chung C, Truong H, Johnston BA, Snow JW. The heat shock response and humoral immune response are mutually antagonistic in honey bees. *Scientific Reports*. 2017;7(1):8850.
- 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 AJ, Brutscher LM, Daughenbaugh KF, Flenniken ML. The Honey Bee Gene Bee Antiviral Protein-1 Is a Taxonomically Restricted Antiviral Immune Gene. *Frontiers in Insect Science*. 2021;1(11).
- McMenamin AJ, Brutscher LM, Glenn W, Flenniken ML. Abiotic and biotic factors affecting the replication and pathogenicity of bee viruses. *Current Opinion in Insect Science*. 2016;16:14-21.
- McMenamin AJ, Daughenbaugh KF, Flenniken ML. The heat shock response in the Western honey bee (*Apis Mellifera*) is antiviral. *Viruses* (2020) 12(2):245.
- McMenamin AJ, Daughenbaugh KF, Parekh F, Pizzorno MC, Flenniken ML. Honey Bee and Bumble Bee Antiviral Defense. *Viruses* (2018) 10(8):395. PubMed PMID: doi:10.3390/v10080395.
- McMenamin AJ, Flenniken ML. Recently identified bee viruses and their impact on bee pollinators. *Current Opinion in Insect Science*. 2018;26:120-9.
- Mcmenamin AJ, Parekh Fenali, Lawrence Verena, Flenniken Michelle. Investigating virus-host interactions in cultured primary honey bee cells. *Insects*. 2021.

- 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.
- 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.
- 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.
- Merkling SH, Overheul GJ, van Mierlo JT, Arends D, Gilissen C, van Rij RP. The heat shock response restricts virus infection in *Drosophila*. *Scientific Reports* (2015) 5(1):12758. doi: 10.1038/srep12758.
- 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.
- Michener C. The bees of the world, The Johns Hopkins University Press: Baltimore, USA. 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).
- 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.

- 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.
- 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.
- 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, 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.
- Morfin N, Goodwin PH, Correa-Benitez A, Guzman-Novoa E. Sublethal exposure to clothianidin during the larval stage causes long-term impairment of hygienic and foraging behaviours of honey bees. *Apidologie* (2019) 50(5):595-605. doi: 10.1007/s13592-019-00672-1.
- Morfin N, Goodwin PH, Guzman-Novoa E. Interaction of field realistic doses of clothianidin and Varroa destructor parasitism on adult honey bee (*Apis mellifera* L.) health and neural gene expression, and antagonistic effects on differentially expressed genes. *PLOS ONE*. 2020;15(2):e0229030.
- Morfin N, Goodwin PH, Hunt GJ, Guzman-Novoa E. Effects of sublethal doses of clothianidin and/or V. destructor on honey bee (*Apis mellifera*) self-grooming behavior and associated gene expression. *Scientific Reports*. 2019;9(1):5196.
- Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, vanEngelsdorp D, et al. High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. *PLOS ONE* (2010) 5(3):e9754. doi: 10.1371/journal.pone.0009754.
- Münch D, Amdam G, Wolschin F. Ageing in a eusocial insect: molecular and physiological characteristics of life span plasticity in the honey bee. *Functional ecology*. 2008;22(3):407-21.
- 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. *Proc. Natl. Acad. Sci. USA* 2008, 105, 19938, doi:10.1073/pnas.0803408105.
- 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 SP, Annoscia D, Del Piccolo F, Di Prisco G, Varricchio P, et al. Synergistic Parasite-Pathogen Interactions Mediated by Host Immunity Can Drive the Collapse of Honeybee Colonies. *PLOS Pathogens* (2012) 8(6):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(1):417-32.
- 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). Epub 2018/03/31. doi: 10.3390/v10040159. PubMed PMID: 29601473; PubMed Central PMCID: PMCPMC5923453.
- 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.
- 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.
- Nazzi, F.; Pennacchio, F. Disentangling multiple interactions in the hive ecosystem. *Trends Parasitol.* 2014, 30, (12), 556-561.
- 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).
- Negri P, Ramirez L, Quintana S, Szawarski N, Maggi MD, Eguaras MJ, et al. Immune-related gene expression of *Apis mellifera* larvae in response to cold stress and Abscissic Acid (ABA) dietary supplementation. *Journal of Apicultural Research* (2020) 59(4):669-76. doi: 10.1080/00218839.2019.1708653.

- Negri P, Villalobos E, Szawarski N, Damiani N, Gende L, Garrido M, et al. Towards Precision Nutrition: A Novel Concept Linking Phytochemicals, Immune Response and Honey Bee Health. *Insects* (2019) 10(11):401. PubMed PMID: doi:10.3390/insects10110401.
- 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.
- 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, Smagghe G, De Coninck DI, 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) 70:127-37. Epub 2015/12/30. doi: 10.1016/j.ibmb.2015.12.006. PubMed PMID: 26711439.
- 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. *Curr. Opin. Insect Sci.* 2014, 6, 22–27, doi:10.1016/j.cois.2014.09.014.
- 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.
- 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.
- Nozal MJ, Bernal JL, Jiménez JJ, González MJ, Higes M. Extraction of thymol, eucalyptol, menthol, and camphor residues from honey and beeswax: Determination by gas chromatography with flame ionization detection. *Journal of Chromatography A* (2002) 954(1):207-15. doi: [https://doi.org/10.1016/S0021-9673\(02\)00153-X](https://doi.org/10.1016/S0021-9673(02)00153-X).
- 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.

- 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.
- 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.
- 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.
- 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.
- Page RE, Rueppell O, Amdam GV. Genetics of Reproduction and Regulation of Honeybee (*Apis mellifera* L.) Social Behavior. *Annual Review of Genetics.* 2012;46(1):97-119.
- Pajuelo AG, Torres C, Bermejo FJO. Colony losses: a double blind trial on the influence of supplementary protein nutrition and preventative treatment with fumagillin against *Nosema ceranae*. *Journal of Apicultural Research* (2008) 47(1):84-6.
- Palmer-Young EC, Sadd BM, Stevenson PC, Irwin RE, Adler LS. Bumble bee parasite strains vary in resistance to phytochemicals. *Scientific Reports* (2016) 6(1):37087. doi: 10.1038/srep37087.
- Palmer-Young EC, Tozkar CÖ, Schwarz RS, Chen Y, Irwin RE, Adler LS, et al. Nectar and Pollen Phytochemicals Stimulate Honey Bee (Hymenoptera: Apidae) Immunity to Viral Infection. *Journal of Economic Entomology.* 2017;110(5):1959-72.
- 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.

- 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.
- Parker JS, Barford D. Argonaute: a scaffold for the function of short regulatory RNAs. *Trends in Biochemical Sciences*. 2006;31(11):622-30.
- 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.
- 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.
- 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*. *Dev Comp Immunol* 2008, 32, (3), 191-197.
- 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.
- Pluta P, Sokół R. Changes in the expression of antimicrobial peptide genes in honey bees (*Apis mellifera*) under the influence of various pathogens. *Ann Parasitol* (2020) 66(4):457-65. Epub 2020/01/01. doi: 10.17420/ap6604.286. PubMed PMID: 33646735.
- 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.
- 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.
- Pritchard DJ. Grooming by honey bees as a component of varroa resistant behavior. *Journal of Apicultural Research.* 2016;55(1):38-48.
- 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.
- Rada V, Máchová M, Huk J, Marounek M, Dušková D. Microflora in the honeybee digestive tract: counts, characteristics and sensitivity to veterinary drugs. *Apidologie* (1997) 28(6):357-65.
- Ramsey SD, Ochoa R, Bauchan G, Gulbranson C, Mowery JD, Cohen A, et al. *Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph. *Proceedings of the National Academy of Sciences* (2019) 116(5):1792. doi: 10.1073/pnas.1818371116.
- 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, Schwarz RS, Descamps T, Yañez O, Tozkar CO, Martin-Hernandez R, et al. Differential diagnosis of the honey bee trypanosomatids *Crithidia mellificae* and *Lotmaria passim*. *Journal of Invertebrate Pathology.* 2015;130:21-7.
- 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.
- Rennich K, Pettis J, VanEngelsdorp D, Bozarth R, Eversole H, Roccasecca K, et al. 2011-2012 National Honey Bee Pests and Diseases Survey Report. USDA Animal Plant and Health Inspection (2012).
- 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.
- Rice CM, Levis R, Strauss JH, Huang HV. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. *Journal of virology* (1987) 61(12):3809-19. doi: 10.1128/JVI.61.12.3809-3819.1987. PubMed PMID: 3479621.

- Richardson LL, Adler LS, Leonard AS, Andicoechea J, Regan KH, Anthony WE, et al. Secondary metabolites in floral nectar reduce parasite infections in bumblebees. *Proceedings of the Royal Society B: Biological Sciences* (2015) 282(1803):20142471.
- 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.
- Ronald, P. C.; Beutler, B. Plant and Animal Sensors of Conserved Microbial Signatures. *Science* 2010, 330, (6007), 1061-1064.
- 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.
- 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, (6), e20656.
- Rundlöf M, Andersson GKS, Bommarco R, Fries I, Hederström V, Herbertsson L, et al. Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature*. 2015;521(7550):77-80.
- Ryabov EV, Christmon K, Heerman MC, Posada-Florez F, Harrison RL, Chen Y, et al. Development of a Honey Bee RNA Virus Vector Based on the Genome of a Deformed Wing Virus. *Viruses* (2020) 12(4):374. doi: 10.3390/v12040374. PubMed PMID: 32231059.
- Ryabov EV, Fannon JM, Moore JD, Wood GR, Evans DJ. 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.
- Ryabov EV, Wood GR, Fannon JM, Moore JD, Bull JC, Chandler D, et al. 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(6):e1004230.

- 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.; Jermin, 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.; Aqrabi, 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, Tassetto M, Van Rij RP, Goic B, Gausson V, Berry B, et al. Antiviral immunity in *Drosophila* requires systemic RNA interference spread. *Nature* (2009) 458(7236):346-50.
- 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.
- Salmela H, Amdam GV, Freitak D. Transfer of Immunity from Mother to Offspring Is Mediated via Egg-Yolk Protein Vitellogenin. *PLOS Pathogens* (2015) 11(7):e1005015. doi: 10.1371/journal.ppat.1005015.
- Samson-Robert O, Labrie G, Chagnon M, Fournier V. Planting of neonicotinoid-coated corn raises honey bee mortality and sets back colony development. *PeerJ* (2017) 5:e3670.

- 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-Bayo F, Goka K. Pesticide Residues and Bees – A Risk Assessment. *PLOS ONE* (2014) 9(4):e94482. doi: 10.1371/journal.pone.0094482.
- 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.
- Sandrock C, Tanadini M, Tanadini LG, Fauser-Misslin A, Potts SG, Neumann P. Impact of Chronic Neonicotinoid Exposure on Honeybee Colony Performance and Queen Supersedure. *PLOS ONE* (2014) 9(8):e103592. doi: 10.1371/journal.pone.0103592.
- 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.
- 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.
- Schoonvaere, K.; Smaghe, 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.
- 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.
- Scotti PD, Dearing S, Mossop DW. Flock house virus: A Nodavirus isolated from *Costelytra zealandica* (White) (Coleoptera: Scarabaeida). *Archives of Virology* (1983) 75(3):181-9. doi: 10.1007/BF01315272.
- Seehuus S-C, Norberg K, Gimsa U, Krekling T, Amdam GV. Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(4):962-7.
- Šegvić Klarić M, Kosalec I, Mastelić J, Piecková E, Pepeljnak S. Antifungal activity of thyme (*Thymus vulgaris* L.) essential oil and thymol against moulds from damp dwellings. *Letters in applied microbiology* (2007) 44(1):36-42.
- Seitz K, Buczolic K, Dikunová A, Plevka P, Power K, Rümenapf T, et al. A molecular clone of Chronic Bee Paralysis Virus (CBPV) causes mortality in honey bee pupae (*Apis mellifera*). *Scientific Reports* (2019) 9(1):16274. doi: 10.1038/s41598-019-52822-1.

- Seitz N, Traynor KS, Steinhauer N, Rennich K, Wilson ME, Ellis JD, et al. A national survey of managed honey bee 2014–2015 annual colony losses in the USA. *Journal of Apicultural Research*. 2015;54(4):292-304.
- 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.
- 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.
- Shi J, Liao C, Wang Z, Zeng Z, Wu X. Effects of sublethal acetamiprid doses on the lifespan and memory-related characteristics of honey bee (*Apis mellifera*) workers. *Apidologie* (2019) 50(4):553-63. doi: 10.1007/s13592-019-00669-w.
- Sim, C.; Hong, Y. S.; Tssetsarkin, 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.; 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.
- 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.
- 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.
- Smart M, Pettis J, Rice N, Browning Z, Spivak M. Linking Measures of Colony and Individual Honey Bee Health to Survival among Apiaries Exposed to Varying Agricultural Land Use. *PLOS ONE* (2016) 11(3):e0152685. doi: 10.1371/journal.pone.0152685.
- 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.

- Spivak M, Reuter GS. Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. *Apidologie* (2001) 32(6):555-65.
- 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.
- Steinhauer NA, Rennich K, Wilson ME, Caron DM, Lengerich EJ, Pettis JS, et al. A national survey of managed honey bee 2012–2013 annual colony losses in the USA: results from the Bee Informed Partnership. *Journal of Apicultural Research*. 2014;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).
- 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.
- Strachecka A, Krauze M, Olszewski K, Borsuk G, Paleolog J, Merska M, et al. Unexpectedly strong effect of caffeine on the vitality of western honeybees (*Apis mellifera*). *Biochemistry (Moscow)* (2014) 79(11):1192-201. doi: 10.1134/S0006297914110066.
- Takeuchi, O.; Akira, S. RIG-I-like antiviral protein in flies. *Nat Immunol* 2008, 9, (12), 1327-1328.
- Taning CNT, Christiaens O, Li X, Swevers L, Casteels H, Maes M, et al. Engineered Flock House Virus for Targeted Gene Suppression Through RNAi in Fruit Flies (*Drosophila melanogaster*) in Vitro and in Vivo. *Frontiers in Physiology* (2018) 9(805). doi: 10.3389/fphys.2018.00805.
- 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.
- Tauber JP, Collins WR, Schwarz RS, Chen Y, Grubbs K, Huang Q, et al. Natural Product Medicines for Honey Bees: Perspective and Protocols. *Insects* (2019) 10(10):356. doi: 10.3390/insects10100356. PubMed PMID: 31635365.
- Taylor RC, Berendzen KM, Dillin A. Systemic stress signalling: understanding the cell non-autonomous control of proteostasis. *Nature Reviews Molecular Cell Biology*. 2014;15(3):211-7.

- 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 ME, et al. Prevalence and Seasonal Variations of Six Bee Viruses in *Apis mellifera* L. and *Varroa destructor* Mite Populations in France. *Applied and Environmental Microbiology* (2004) 70(12):7185-91. doi: 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.; Grimmeliikhuijzen, 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.
- 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.
- Tison L, Duer A, Púčiková V, Greggers U, Menzel R. Detrimental effects of clothianidin on foraging and dance communication in honey bees. *PLOS ONE* (2020) 15(10):e0241134. doi: 10.1371/journal.pone.0241134.
- 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.
- 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 KS, Rennich K, Forsgren E, Rose R, Pettis J, Kunkel G, et al. Multiyear survey targeting disease incidence in US honey bees. *Apidologie* (2016) 47(3):325-47. doi: 10.1007/s13592-016-0431-0.
- Tsvetkov N, Samson-Robert O, Sood K, Patel HS, Malena DA, Gajiwala PH, et al. Chronic exposure to neonicotinoids reduces honey bee health near corn crops. *Science* (2017) 356(6345):1395-7. doi: 10.1126/science.aam7470.
- Tufail M, Takeda M. Molecular characteristics of insect vitellogenins. *J Insect Physiol.* 2008;54(12):1447-58.
- 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.
- van den Heever JP, Thompson TS, Otto SJG, Curtis JM, Ibrahim A, Pernal SF. The effect of dicyclohexylamine and fumagillin on *Nosema ceranae*-infected honey bee (*Apis mellifera*) mortality in cage trial assays. *Apidologie* (2016) 47(5):663-70. doi: 10.1007/s13592-015-0411-9.
- 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 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.
- Van Rij RP, Saleh M-C, Berry B, Foo C, Houk A, Antoniewski C, et al. The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in *Drosophila melanogaster*. *Genes & development* (2006) 20(21):2985-95.
- vanEngelsdorp D, Caron D, Hayes J, Underwood R, Henson M, Rennich K, et al. A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. *Journal of Apicultural Research.* 2012;51(1):115-24.
- vanEngelsdorp D, Meixner MD. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *Journal of Invertebrate Pathology* (2010) 103:S80-S95. doi: <https://doi.org/10.1016/j.jip.2009.06.011>.
- 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.

- 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.; 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 AD, Tooker JF, Grozinger CM, Patch HM. Bee nutrition and floral resource restoration. *Current Opinion in Insect Science* (2015) 10:133-41. doi: <https://doi.org/10.1016/j.cois.2015.05.008>.
- 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.
- Wade A, Lin C-H, Kurkul C, Regan ER, Johnson RM. Combined toxicity of insecticides and fungicides applied to California almond orchards to honey bee larvae and adults. *Insects* (2019) 10(1):20.
- Wang X-H, Aliyari R, Li W-X, Li H-W, Kim K, Carthew R, et al. RNA Interference Directs Innate Immunity Against Viruses in Adult *Drosophila*. *Science*. 2006;312(5772):452-4.
- 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.
- 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 merguensis*. *Dis. Aquat. Organ.* 2002, 52, 249–259, doi:10.3354/dao052249.

- Webster TC. Fumagillin Affects *Nosema apis* and Honey Bees (Hymenoptera: Apidae). *Journal of Economic Entomology* (1994) 87(3):601-4. doi: 10.1093/jee/87.3.601.
- 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.
- Wiese N, Fischer J, Heidler J, Lewkowski O, Degenhardt J, Erler S. The terpenes of leaves, pollen, and nectar of thyme (*Thymus vulgaris*) inhibit growth of bee disease-associated microbes. *Scientific Reports* (2018) 8(1):14634. doi: 10.1038/s41598-018-32849-6.
- Wilfert L, Long G, Leggett H, Schmid-Hempel P, Butlin R, Martin S, et al. Deformed wing virus is a recent global epidemic in honeybees driven by *Varroa* mites. *Science* (2016) 351(6273):594-7.
- 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.
- Williams GR, Sampson MA, Shutler D, Rogers REL. Does fumagillin control the recently detected invasive parasite *Nosema ceranae* in western honey bees (*Apis mellifera*)? *Journal of Invertebrate Pathology* (2008) 99(3):342-4. doi: <https://doi.org/10.1016/j.jip.2008.04.005>.
- Williams GR, Shutler D, Little CM, Burgher-Maclellan KL, Rogers REL. The microsporidian *Nosema ceranae*, the antibiotic Fumagilin-B®, and western honey bee (*Apis mellifera*) colony strength. *Apidologie* (2011) 42(1):15-22. doi: 10.1051/apido/2010030.
- 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.
- Wu JY, Anelli CM, Sheppard WS. Sub-Lethal Effects of Pesticide Residues in Brood Comb on Worker Honey Bee (*Apis mellifera*) Development and Longevity. *PLOS ONE* (2011) 6(2):e14720. doi: 10.1371/journal.pone.0014720.
- 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.
- 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.
- 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.
- Yue C, Genersch E. RT-PCR analysis of Deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*). *J Gen Virol.* 2005;86(Pt 12):3419-24.
- Yue C, Schröder M, Gisder S, Genersch E. Vertical-transmission routes for deformed wing virus of honeybees (*Apis mellifera*). *Journal of General Virology.* 2007;88(8):2329-36.
- Zambon RA, Vakharia VN, Wu LP. RNAi is an antiviral immune response against a dsRNA virus in *Drosophila melanogaster*. *Cellular microbiology* (2006) 8(5):880-9.
- Zamore, P. D. Ancient pathways programmed by small RNAs. *Science* 2002, 296, (5571), 1265-9.
- Zanni V, Galbraith DA, Annoscia D, Grozinger CM, Nazzi F. Transcriptional signatures of parasitization and markers of colony decline in *Varroa*-infested honey bees (*Apis mellifera*). *Insect Biochemistry and Molecular Biology* (2017) 87:1-13. doi: <https://doi.org/10.1016/j.ibmb.2017.06.002>.
- Zhao R, Houry WA. Hsp90: a chaperone for protein folding and gene regulation. *Biochemistry and Cell Biology* (2005) 83(6):703-10. doi: 10.1139/o05-158 %M 16333321.
- Zhu YC, Adameczyk J, Rinderer T, Yao J, Danka R, Luttrell R, et al. Spray Toxicity and Risk Potential of 42 Commonly Used Formulations of Row Crop Pesticides to Adult Honey Bees (Hymenoptera: Apidae). *Journal of Economic Entomology* (2015) 108(6):2640-7. doi: 10.1093/jee/fov269.
- 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.