Replication of Invertebrate Iridescent Virus 6 (IIV-6) in European Honey Bees - Potential Involvement in Colony Collapse Disorder?

Joseph L. McInnis[1], Trevor Williams[2], Ya-Chen Chuang[3], and Douglas A. Gregg[4]

Abstract. Colony collapse disorder of honey bees, *Apis mellifera* L., is a global problem with no conclusive cause yet accepted. A previous U.S. Army study identified a DNA virus, invertebrate iridescent virus (IIV-6), and two microsporian pathogens, *Nosema apis* Zander 1909 and *Nosema ceranae* Fries et al. 1996, in bee samples from a collapsing hive. A PCR-based study using limited samples failed to confirm IIV-6 in collapsing colonies, causing the finding to be questioned. Here we demonstrate that honey bees are very susceptible to the virus. We observed viral inclusion bodies in the cytoplasm of honey bee brood previously inoculated with IIV-6. Electron microscopy revealed massive numbers of viral particles in cells at 3 days post-inoculation. Viral factories and paracrystalline arrays of particles ~125 nm in diameter were observed. Few cells were spared infection, indicating that honey bee larvae are very susceptible to IIV-6 and most tissues are infected.

Introduction

Pollinators, including honey bees, *Apis mellifera* Linnaeus 1758, are critical to human survival, because 40% of the food supply of the world depends on the action of pollinators. Colony collapse disorder is a serious global problem resulting in 23% or more death of honey bee colonies each year (Kulhanek et al. 2017). The multiple theories for the cause of colony collapse disorder include proliferation of the parasitic Varroa mite (Le Conte et al. 2010), pathogens (Le Conte et al. 2010), pesticides such as neonicotinoids (Iwasa et al. 2004, Zhang and Nieh 2015), stress induced by climate change (Le Conte and Navajas 2008), shipping stress, malnutrition (Dhruba 2009), and even stray electromagnetic waves (Sainudeen et al. 2011). However, none is a satisfactory explanation, because colony collapse is seen in areas such as the Swiss Alps that are free of many of the factors. Collapsed colonies typically have a queen, nurse bees, and adequate honey, but few remaining worker bees. There often is no obvious cause for loss of worker bees, and dead bees are not found in the vicinity.

In 2010, a study by the U.S. Army to train bees to detect land mines provided a clue (Bromenshenk et al. 2010). Because the colonies being trained collapsed, a novel proteomics approach based on mass spectrometry of trypsin-digested sample from honey bees was used to study bees from both healthy and collapsing hives. After subtracting normal from abnormal spectral spikes, computer analysis identified the signature of a DNA virus from the family Iridoviridae, invertebrate iridescent virus

[1]Corresponding author, Dartmouth College. me@joemcinnis.com
[2]Instituto de Ecología AC, Xalapa, Mexico.
[3]Stony Brook University, ThINC facility, Advanced Energy Center, Stony Brook, NY.
[4]Plum Island Animal Disease Center, NY, retired.

6 (IIV-6), and two microsporidian pathogens: *Nosema apis* (Zander 1909) and *Nosema ceranae* (Fries et al. 1996), common intestinal pathogens similar to cryptosporidiosis in animals.

Invertebrate iridescent viruses such as IIV-6 can adversely affect larval development and shorten life span of some insects (Marina et al. 1999). Many iridescent virus infections seem covert in nature and can be detected only by using molecular methods or direct observation by electron microscopy of virus particles in insect cells (Kleespies et al. 2000). Infection by iridescent virus also might affect susceptibility to other common pests and pathogens because the viruses can modulate insect immune response (Williams et al. 2009, Ahlers et al. 2016). In bees, these could include *Nosema* sp. Pests in the hive, including wax moths, *Galleria mellonella* (L.), and Varroa mites are common opportunists and can weaken collapsing colonies. Larvae of the wax moth are susceptible to infection by IIV-6, and electron microscopy showed iridescent virus-like particles in Varroa mites (Kleespies et al. 2000). Still, attempts to use PCR failed to confirm IIV-6 in collapsing colonies (Tokarz et al. 2011). This might have been because of insufficient sampling or departure of infected bees from the colony. In this study, we studied susceptibility of bee larvae to infection by IIV-6 and the range of tissues infected by the virus, to determine whether honey bees were a susceptible host.

Materials and Methods

Using methods developed for studying African swine fever virus, a morphologically similar virus (Gregg 1995), we investigated susceptibility of honey bees to IIV-6 infection by inoculating bee larvae and using histology and electron microscopy to demonstrate viral inclusion bodies in infected cells. A frame of freshly capped larvae from a honey-producing hive was acquired from a local beekeeper at Cutchogue, NY. A sample of IIV-6 was purified from patently infected wax moth larvae at 5 days post-inoculation by using a characterized strain of IIV-6 (Williams and Cory 1994). Virus was purified from the wax moth larvae by centrifugation at 5,000 *g* in a microfuge, resulting in an opalescent pellet of virus. The pellet was resuspended in 1 ml saline and diluted 1:100 to inoculate into bee larvae through the comb cell cap with a 30-gauge needle delivering approximately 1 µl into each larva in the comb. The bioassay method by Constantino et al. (2001) was used to titer virus in wax moth larvae. Patent infection of *G. mellonella* was at an injected dose 50% of 10^6 particles. Each larva was inoculated with 10^3 virus particles.

Check larvae were inoculated identically with saline solution alone. Cell caps were resealed with beeswax. Thirty check and inoculated larvae in the comb were incubated at 37°C in a moist chamber. At 3, 5, and 7 days post-inoculation, inoculated and check larvae were fixed in 10% buffered formalin with caps removed in the comb. After 48 hours, combs were transferred to 2.5% glutaraldehyde for 48 hours before dissecting the larvae from the comb (Fig. 1). No Varroa mites were on the 100 larvae from the comb. Each larva was divided in half by sagittal section for histopathology and electron microscopy.

Half of each larva was processed routinely into paraffin, under vacuum in graded ethanol at 80, 95, 100, 100% for 1 hour each, followed by three stages of undiluted xylene, and finally, three stages of paraffin. Histologic sections were cut in the mid-sagittal plane at 3-µm thickness for staining by hematoxylin and eosin. Because the larva had no sclerotized cuticle, it was soft, and sectioning was routine.

Following examination of histologic sections, a few samples were selected from the head, thorax, and abdomen of matching halves of virus-inoculated and check larvae for processing into epoxy resin for transmission electron microscopy. Standard procedures were used for embedding, sectioning, and staining by osmium tetroxide for electron microscopy.

Fig. 1. Bee pupa check.

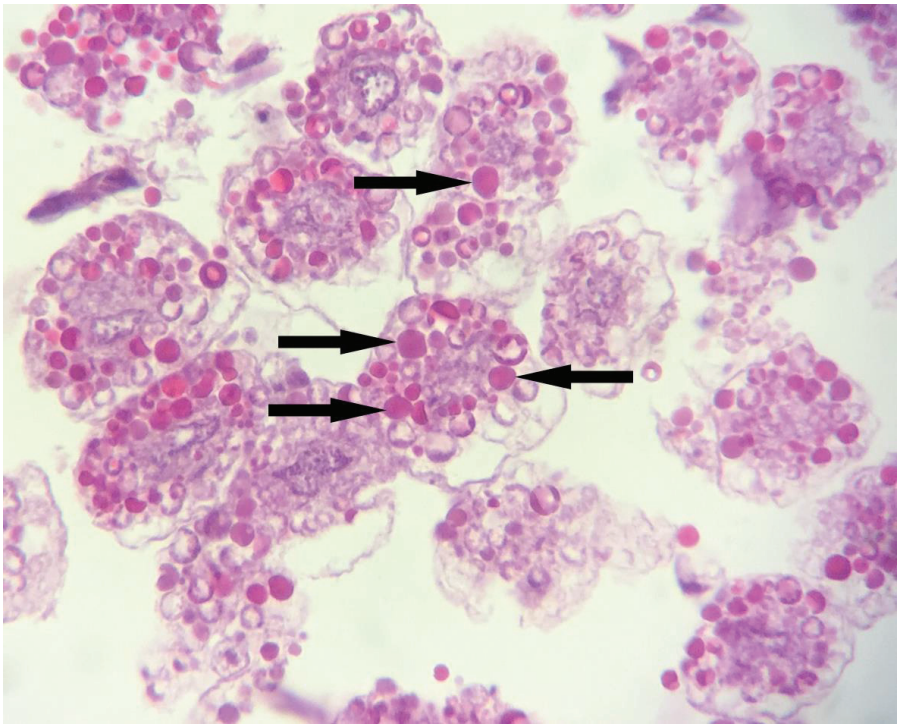

Fig. 2. Fat body cells from a virus-infected larva with multiple magenta inclusion bodies (H&E stained).

Results

When sections were examined by light microscopy, some inoculated 3-day samples and all 5- and 7-day samples were autolyzed, indicating most larvae died at Day 3 or 4. A few of the 3-day inoculated samples were well preserved and had distinct cytoplasmic viral inclusion bodies in a few of the gut cells.

Most larvae were filled with actively dividing fat body cells. The cells were filled with eosinophilic granules of various sizes, making identification of inclusion bodies difficult. Many granules in the inoculated samples seemed to be larger bodies that were slightly more magenta in color (Fig. 2).

Remarkably, by electron microscopy, almost every cell in each examined section of the virus-inoculated larva had hexagonal virus particles of ~125 nm diameter, characteristic of iridescent virus in the cytoplasm (Fig. 3). Many cells had distinct viral factories or paracrystalline arrays of virions in the cytoplasm. Crystalline arrays were very abundant and obvious in infected cells and corresponded to the larger magenta granules observed in the H&E-stained sections.

Systemic infection apparently spread rapidly in bee larvae and by 3 days post-infection, almost all cell types were infected, especially the predominant fat body cells. Death occurred by 4 days in all inoculated larvae, confirming that honey bee larvae are susceptible to lethal infection by IIV-6. It is noteworthy that when the IIV-6 virus was titrated into wax moth larvae with 10 larvae each per log dilution of virus from -1 to -10 logs, visible virus pellets were obtained from 60% of the larvae, but not a single larva died until 6 days post inoculation.

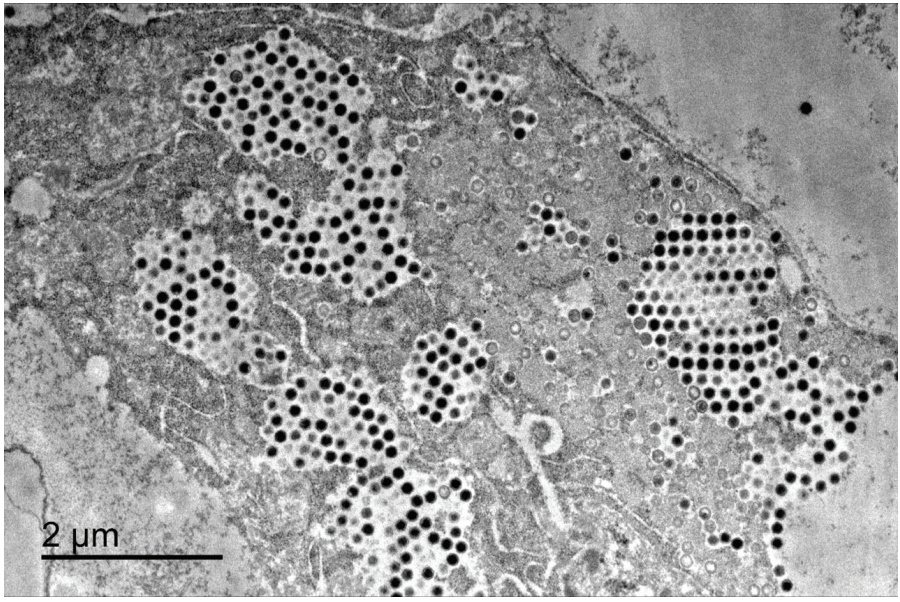

Fig. 3. Electron micrograph of fat body cell with multiple paracrystalline arrays of iridescent virus particles.

Discussion

The study demonstrated that IIV-6 is very pathogenic to honey bee larvae. The finding supports the demonstration of experimental infection of adult honey bees with iridescent virus in a caged colony along with rapid colony loss following co-infection with IIV-6 and *Nosema* (Bromenshenk et al. 2010). Honey bees are very susceptible to the virus, which should be considered one of the pathogens potentially contributing to colony collapse disorder. Further controlled studies are required to establish if multiple agents are involved in colony collapse disorder. If IIV-6 persists naturally in bee colonies, two additional hive inhabitants might be involved in the persistence and transmission of the pathogen. Covertly infected wax moths might pass non-lethal infection through the egg to developing larva as suggested by transovarial transmission of IIV-6 in the citrus aphid (Hunter et al. 2001). If covert infection is triggered into a patent lethal disease, the virus load in the wax moth larva can exceed 10 logs (Constantino et al. 2001). Such large quantities of virus produced in a single wax moth larva, could contaminate the hive with IIV particles and expose healthy bees to abundant viral inoculum. Varroa mites could also serve as a vector for IIV-6 from worker bees to honey bee larvae on which they prefer to feed. Feeding by Varroa mites on larvae in a capped cell of the comb involves piercing the larval tegument and might be a route for introduction of virus particles into the larval hemolymph, which is the most efficient route for transmission of the viruses. Because covert IIV infection has debilitating effects on development and adult survival of some insects (Marina et al. 1999) and seemed to be lethal to honey bee larvae in this study, the relationship between IIV infection and the health of honey bee colonies should be studied further. At least, IIV-6 might lead to attrition of the worker bees in a hive. Wax moths and Varroa mites might be more than a nuisance and might be vectors of disease in the honey bee colony.

We were struck by similarities to African swine fever virus, a morphologically similar virus rampant in Russia and China today. The African swine fever virus is very infectious and transmitted by direct contact as well as by soft ticks. African swine fever causes a specific immunodeficiency by infecting antigen-presenting cells, resulting in failure of immunity to African swine fever virus and any other novel agent (Gregg et al. 1995ab, Franzoni et al. 2018).

Declaration of Conflicting Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

Acknowledgment

Thanks to Salt Air Farms, Robert Jester, David Gamberg, Luis Chiriboga, New York State Histotechnological Society, Rainbow Mealworms, Chung-Chueh Chang, Tony Brown, and Mary Kathryn Baker. We thank the following donors for their kind and generous support: Uncle Stanley's Apiary, Robert Jester, Prudence Heston, David Gamberg, Sarah Phillips, Lynn Summers, Mary Herrick, Robert Christensen, David Abatelli, Ian Wile, Doug Roberts, Margaret Lauber, and the GoFundMe Team.

References Cited

Ahlers, L. R. H., R. G. Bastos, A. Hiroyasu, and A. G. Goodman. 2016. Invertebrate iridescent virus 6, a DNA virus, stimulates a mammalian innate immune response through RIG-I-like receptors. PLOS ONE 11: e0166088.

Bromenshenk, J. J., C. B. Henderson, C. H. Wick, M. F. Stanford, A. W. Zulich, et al. 2010. Iridovirus and microsporidian linked to honey bee colony decline. PLOS ONE 5: e13181. _Constantino, M., P. Christian, C. F. Marina, and T. Williams. 2001. A comparison of techniques for detecting Invertebrate iridescent virus 6. J. Virological Methods 98: 109-118.

Dhruba, N. 2009. Nutritional stress due to habitat loss may explain recent honeybee colony collapses. Biological Conservation 142: 2369-2372.

Franzoni, G., S. D. Giudici, and A. Oggiano. 2018. Infection, modulation and responses of antigen-presenting cells to African swine fever viruses. Virus Research 258.

Gregg, D. A., D. H. Schlafer, and C. A. Mebus. 1995a. African swine fever virus infection of skin-derived dendritic cells in vitro causes interference with subsequent foot-and-mouth disease virus infection. J. Vet. Diagn. Invest. 7: 44-51.

Gregg, D. A., C. A. Mebus, and D. H. Schlafer. 1995b. Early infection of interdigitating dendritic cells in the pig lymph node with African swine fever viruses of high and low virulence: immunohistochemical and ultrastructural studies. J. Vet. Diagn. Invest. 7: 23-30.

Hunter, W. B., et al. 2001. Iridovirus infection and vertical transmission in citrus aphids. Proc. Fla. State Hort. Soc. 114: 70-72.

Iwasa, T., N. Motoyama, J. T. Ambrose, R. M. Roe, et al. 2004. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. Crop Protection 23: 371-378.

Kleespies, R. G., J. Radtke, and K. Bielefeld. 2000. Virus-like particles found in the ectoparasitic bee mite *Varroa jacobsoni* Oudemans. J. Invert. Path. 75: 87-90.

Kulhanek, K., N. Steinhauer, K. Rennich, D. M. Caron, R. R. Sagili, J. S. Pettis, et al. 2017. A national survey of managed honey bee 2015-2016 annual colony losses in the USA. J. Apic. Res. 56: 328-340.

Le Conte, Y., and M. Navajas. 2008. Climate change: impact on honey bee populations and diseases. Revue Scientifique et Technique (International Office of Epizootics) 27: 485-497, 499.

Le Conte, Y., M. Ellis, and W. Ritter. 2010. Varroa mites and honey bee health: can Varroa explain part of the colony losses? Apidologie 41: 353-363.

Marina, C., J. I. Arredondo-Jiménez, A. Castillo, and T. Williams. 1999. Sublethal effects of iridovirus in a mosquito. Oecologia 119: 383-388.

Sainudeen, S., et al. 2011. Impact of mobile phones on the density of honeybees. J. Pub. Admin. Pol. Res. 3: 131-117.

Tokarz, R., C. Firth, C. Street, D. L. Cox-Foster, and W. I. Lipkin. 2011. Lack of evidence for an association between iridovirus and colony collapse disorder. PLOS ONE 6: e21844.

Williams, T., and J. S. Cory. 1994. Proposals for a new classification of iridescent viruses. J. Gen. Virol. 75: 1291-1301.

Williams, T., N. S. Chitnis, and S. L. Bilimoria. 2009. Invertebrate iridovirus modulation of apoptosis. Virologica Sinica 24: 295-304.

Zhang, E., and J. C. Nieh. 2015. The neonicotinoid imidacloprid impairs honey bee aversive learning of simulated predation. J. Exp. Biol. 218: 3199-3205.