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Novel Role of a Cypovirus in Polydnavirus-Parasitoid-Host Relationship

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I am a Kentucky native from Clark County. I attended George Rogers Clark High School in Winchester, Kentucky. It was here that I first became interested in pursuing a career in science, and found my love for learning. I am now a second-year student on the way toward a degree in Agricultural Biotechnology, a major that I chose for its diverse coverage of scientific fields and its focus on research. In the summer prior to my arrival at the University of Kentucky, I was captivated by the concepts of Dr. Bruce Webb's research into the polydnaviruses (PDV) that symbiotically assist their parasitoid wasp host in overcoming the immune defenses of caterpillar hosts. I have been working as an undergraduate researcher in his lab ever since, and have encountered many new techniques and concepts throughout this experience. This opportunity has provided me with a first-hand encounter of scientific research and the enormous community behind this field of expertise.

My laboratory research has convinced me of my desire and ability to seek out a career in scientific research. Thus, upon graduating from the University of Kentucky, I desire, and plan, to pursue even higher education in a Ph.D program, most likely in the field of entomology. I am also very interested in other subjects such as chemistry, particularly that deal with alternative energy sources, and art history, the wonders of which I was introduced to in my high school AP Art History course. Even if I do not pursue a career in these areas, I will continue to be fascinated by the second-hand knowledge that I can acquire from such fields.

Presently, I am continuing my personal education through striving for excellence in all of my courses, and will continue to assimilate and savor the knowledge that I am provided with and the skills that I shall develop. More importantly, my continued research in the Department of Entomology will improve my qualities as a scientist and researcher by strengthening and widening my range of laboratory techniques and sharpening my ability to develop relevant, important questions, to collect and interpret data, and, most importantly, to accurately and convincingly present the importance of my research to the scientific community.

Overview:

Viruses, and their interactions with eukaryotes, have caused disease since the beginnings of life and have been an important topic for research since their discovery. Most commonly, viruses have been studied and dealt with as pernicious, infectious agents that cause illness and potentially death in exposed animals and plants. Historically, these baneful characteristics have focused virus studies to emphasize research in the medical field. In recent years, however, viruses have been discovered in new roles, as an increasing number of viral infections are described that have few, if any, repercussions on their hosts. In fact, some fascinating virus infections have even been discovered that have beneficial effects for the primary host organism.

Among these benign virus systems is that of the polydnaviruses (PDV). These double-strand DNA viruses are intrinsic and essential to the lifecycles of thousands of species of parasitoid wasps In fact, PDV genomes are integrated into the genomes of their primary hymenopteran host. Specialized cells in female parasitoids, the calyx cells, manufacture virus particles, which are then injected, along with the progeny of the wasp, into a suitable host, which is always a lepidopteran caterpillar. Although PDVs are harmless within the wasp, they express genes that result in the suppression of the immune system and block maturation of the wasp's host. Thus, PDVs play a crucial role in protecting parasitoid progeny from the defenses of a host, and the virus has become

intimately integrated into the lifecycle of these wasps, forming a fascinating symbiosis between virus and host. In the course of my undergraduate research, I have studied the behavior and peculiarities of the PDV associated with the ichneumonid wasp, *Campoletis sonorensis*, which uses tobacco budworm caterpillars as a host organism.

Recently, I co-discovered a second virus, apparently a double-stranded RNA virus, in this parasitoid-host system. Surprisingly, the biology of this virus acting in the context of this system appears to mirror the PDV in that it seems to have little effect on its caterpillar host, but causes considerable mortality in the parasitoid wasp larvae that become exposed to this second virus when feeding on the caterpillar. Through preliminary studies, I have been able to identify this new virus as a double-stranded RNA cypovirus. The activity of this cypovirus in the PDV lifecycle is of great importance in providing a chance to study the unique, complex relationship that exists between these viruses and their development as biological weapons in their respective, primary hosts.

I propose that this understudied cypovirus system should be explored to determine the complete nature of this virus' association with its tobacco budworm host and their parasitoid predators. Cypoviruses have yet to be studied in the context of parasitoid-host networks, and this particular system provides a nearly unique opportunity to study the characteristics of these viruses in a system with a sequenced polydnavirus genome. Through a series of experiments, I seek to test the hypothesis that this cypovirus replicates in both caterpillar and wasp tissues, but has significantly more adverse consequences for wasp larvae, which often die from infection, sparing the budworm host.

Viruses are well-known for the diseases that they cause in eukaryotic organisms across the world, but the study of symbiotic viral systems that impart beneficial results for hosts provides an opportunity to observe viruses in a new perspective. Viruses are important components in the lives of many organisms, both as pathogens and as harmless, and occasionally essential, factors. Indeed, beneficial viruses have even been described in humans. Parasites are similarly ubiquitous and have a wide range of interactions with human societies as agents of disease, nuisance, or biological control. Both of these topics deserve attention, as the perceived position of viruses and parasites in society shifts to accommodate those systems that are directly or indirectly auspicious to our interests, and may, through future study, be innovatively harnessed to attain agricultural, environment, and medical goals.

Introduction:

Complex relationships often exist between viruses and their hosts. Viruses have most often been of great medical concern because of their detrimental, sometimes deadly interactions with humans and other organisms. For instance, recent mutations of influenza virus A have become the source of great health-related fears. The impressive mutability of viruses is a serious concern for physicians around the globe, who witness, firsthand the dangers of unforeseen virus outbreaks. These grave issues alone justify intensive research into viral systems. More recently, viruses having less pronounced effects, no effect, or even beneficial effects for their host organism have become a research topic of great interest to scientists. In human studies, for example, many chronic viral infections (including infection of germline cells) have been identified that have a variety of consequences for human hosts. Some of these viruses and retroviral elements

may even play a symbiotic role by stimulating the "normal" activity of the human immune system (Herbert W. Virgin, 2009).

One, particularly fascinating benign viral system is associated with parasitoid wasps, many of which make use of polydnaviruses that are essential for their lifecycle. The genetic material of PDVs is, interestingly, encoded in the wasp genome where it replicates in specialized, calyx cells located near the ovarioles of female wasps. These particular cells then manufacture large quantities of virus particles, which are released through either budding or lysis of the cell membrane. The PDVs are not pathogenic in the wasp, but are crucial in the suppression of the immune system of the wasp's caterpillar host, into which the virus is injected, along with the eggs of the wasp. Thus, the virus requires the wasp for replication and, in turn, the wasp needs the virus to protect its offspring as they mature inside a dynamic host organism (see Fig.1).

During my recent studies of a parasitoid wasp and its PDV, I co-discovered a second virus, which we refer to as the *Heliothis virescens* cypovirus (*H.v.*CPV), within this system. Curiously, the *H.v.*CPV seems to mirror that of the polydnavirus in that it appears to have little or no effects on the caterpillar (*Heliothis virescens*), but has significant, even lethal effects on the parasitic wasp larvae (*Campoletis sonorensis*). Further research is desirable in this complex system that may represent the evolution of two independent symbiotic viruses. To follow up on these initial observations, we have formulated the hypothesis that the *H.v.*CPV resides in *H. virescens* larvae midgut tissue, where it serves as a proto-biological weapon that can protect the caterpillar from parasitoid infestations.

To test this hypothesis, I propose two lines of research. First, we must analyze the affect that *H.v.*CPV has in both the wasp and its lepidopteran host by comparing the growth rate and any mortality with and without the presence of cypovirus infection. Parasitization efficiency of the wasp will also be analyzed for both clean and cypovirus infected hosts, as well as a non-host system using artificial media in a controlled environment. A second set of experiments will be performed to examine the *H.v.*CPV genome and its expression in *H. virescens* and *C. sonorensis*. Finally, the caterpillar's immune response to *H.v.*CPV infection will be analyzed, and comparisons will be made of the immune responses of the caterpillar to PDV infection with and without *H.v.*CPV.

If we demonstrate that *H.v.*CPV is indeed a pathogenic virus used by the caterpillar as a defense against parasitization by a wasp, it would provide a unique example of two viruses with host-specific pathogenesis. In this case, we will have the opportunity to study the actions of viruses sharing similar purposes for their primary symbiotic host and acting to have negative effects on their secondary host in a network of parasitoid-host biological warfare. The characterization of beneficial virus systems can contribute to a novel view of viruses as infectious agents that may serve to impart disease or to improve the well-being of their hosts.

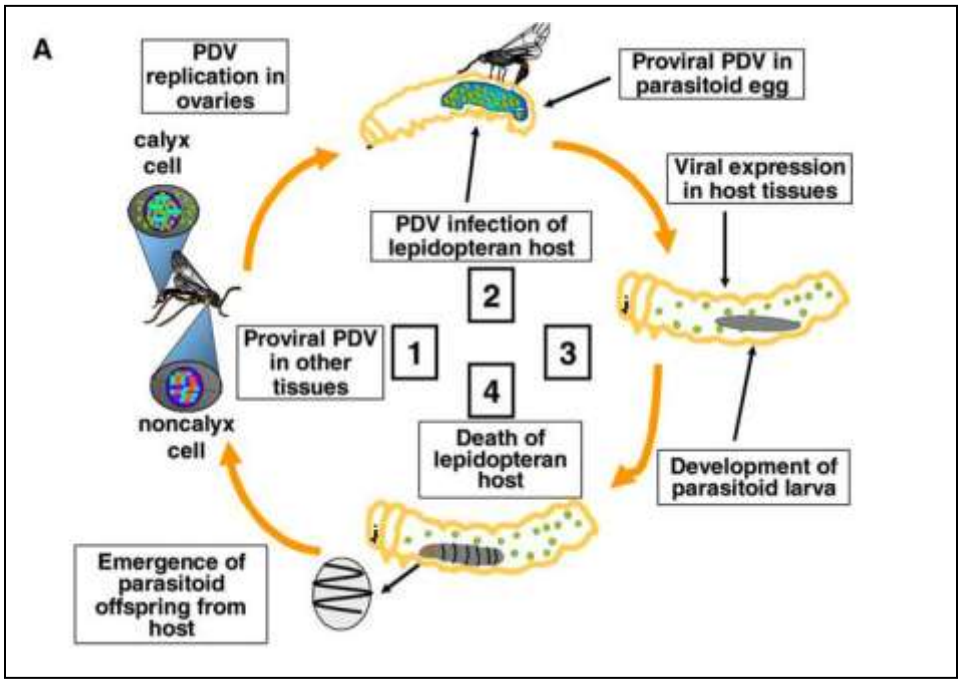

Fig. 1: PDV life cycle integration into that of parasitoid wasps. **1:** PDV genes are integrated into wasp chromosomes and therefore present in all cells, but replicate only in female wasp calyx cells. **2:** Female wasp injects eggs and PDV into host. **3:** PDV is expressed in wasp host, suppressing the immune system and facilitating parasitoid development. **4:** Parasitoid wasp larva kills its host during emergence.

Background:

Complex, and occasionally mutualistic associations between viruses and insects have been observed in other host-parasitoid relationships as well. Earlier examples of viruses that improve resistance to parasitization include studies of the defenses of certain aphids against parasitoid attack. These aphids make use of a bacterial endosymbiont, known as *Hamiltonella defensa*, which is supplemented by bacteriophages. The phages impart a gene to *H. defensa* that allows the bacteria to produce a toxin that attacks eukaryotic parasites that attempt to infest the aphid. (Nancy A. Moran, 2005).

Cypoviruses are a genus of insect-infecting viruses in the family Reoviridae. Within this family, they are unique in that each virion has only a single protein capsid layer and that they commonly are found occluded in protein-based polyhedra. These viruses often infect insects orally, and the polyhedra are dissolved under the alkaline conditions in the midgut, releasing the virus particles to infect epithelial cells (Hill, 1999). Cypovirus capsids themselves appear to be very resilient to alkaline conditions, although it is sensitive to low pH environments, urea, and detergents, which cause the capsid to break open (Zhang, 2002). Cypovirus infections may be observed as occlusion bodies via light microscopy (Inglis, 2003). They have been studied for their potential use in pest-control, as several are lethal to the lepidopteran larvae that they infect (Zeddam, 2003).

Preliminary Data:

Our first experience with *H.v.*CPV was the observation of unexpected and prominent nucleic acid bands in genomic DNA preparations that were extracted from fifth-instar *C. sonorensis* larvae. Roughly nine distinct bands of nucleic acid were observed (Fig. 2a). To examine the identity of these bands, I digested the nucleic acids with several nuclease enzymes. The bands were unaffected by DNase I digestion, but were completely degraded when treated with RNase A, indicating that the unexpected genetic material was RNA, and not DNA (Fig. 2a). Complete digestion with RNase III treatment and no degradation with RNase H revealed that the RNA was double-stranded (dsRNA), which suggested that it was likely originating from an RNA virus (such as a reovirus). Several reoviruses have been characterized from insects, and some from parasitic wasps, but never from parasitic wasp larvae. Furthermore, the banding pattern likely represents separate segments of a virus genome (Fig. 2b). Because the observation of this RNA was unexpected, and could interact with the functions of PDV, we decided to continue to explore the nature of these unexpected nucleic acids.

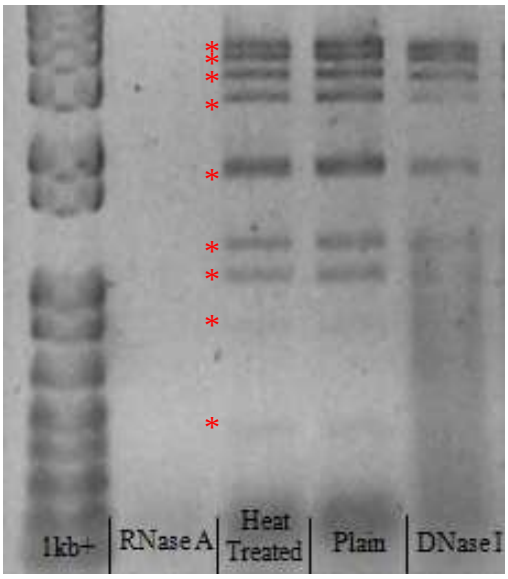

Fig. 2a: Nucleic acid was isolated from fifth-instar *C. sonorensis* larvae and prominent bands were observed (*). The identity of these bands was determined using nuclease treatments. Bands were completely digested with RNase A treatment and not degraded by DNase I digestion, indicating that the nucleic acids are RNA. **RNase A** is the product of RNase A digestion of larval wasp nucleic acids. **Heat Treated** is the larval wasp nucleic acids after heat treatment (at nuclease incubation temperature). **Plain** is untreated larval wasp nucleic acids. **DNase I** is the product of larval wasp nucleic acids after digestion with DNase I.

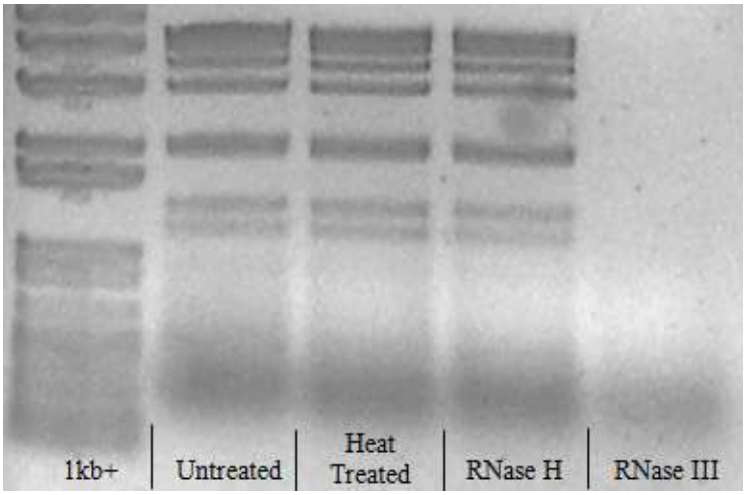

Fig. 2b: RNA extracted from fifth-instar *C. sonorensis* larvae displayed distinct RNA bands, which were then more completely characterized by further nuclease treatment. Complete digestion of RNA bands with RNase III, and no digestion when treated with RNase H, indicates that the RNA bands are double-stranded. Double-strand RNAs are commonly associated with the presence of RNA viruses. **Untreated** is untreated larval wasp RNA. **Heat Treated** is larval wasp RNA that has been subjected to nuclease activity incubation temperatures. **RNase H** is larval wasp RNA after treatment with RNase H. **RNase III** is larval wasp RNA that has been treated with RNase III.

Virtually all fifth-instar *C. sonorensis* larvae that I have tested displayed the characteristic dsRNA segments in extracted genetic material. However, we still did not know where the RNA originated from, or if it was present in any other life stages of the wasp. Therefore, I decided to screen for the dsRNAs in various life stages of the parasitic *C. sonorensis* larvae, surgically extracted from parasitized *H. virescens* hosts. Genetic material isolated from first-instar wasp larvae was the only other larval stage that contained a visible quantity of the characteristic dsRNA bands. I further tested the *C. sonorensis* life stages by extracting and screening nucleic acids from adult wasps, wasp pupae, and fifth-instar larva, removed from cocoons. All dsRNA bands were greatly reduced or absent in the adult wasps and pupae, although the larvae continue to possess the presumably viral genomic segments after spinning a cocoon. These results suggested that the wasp larvae likely acquire the virus during their parasitic life-stage and then shed most of the virus upon pupation. This finding led me to isolate and screen genetic material from ordinary *H. virescens* caterpillars, (under the assumption that they contain virus), the *C. sonorensis* gut waste that is expelled prior to pupation (meconium), and the gut tissue of fifth-instar wasp larvae (separated from other bodily tissues). Reovirus segments were observed in extremely high concentration in *C. sonorensis* meconium, the waste material released from insects after pupation, and were also found to be concentrated in gut tissue in fifth-instar larvae (see Fig. 3). Reovirus bands were also observed in low concentration in *H. virescens* larvae. The results support the idea that virus is shed by the wasp during pupation, and likely introduced into the wasp while the parasitoids are inside of their lepidopteran hosts.

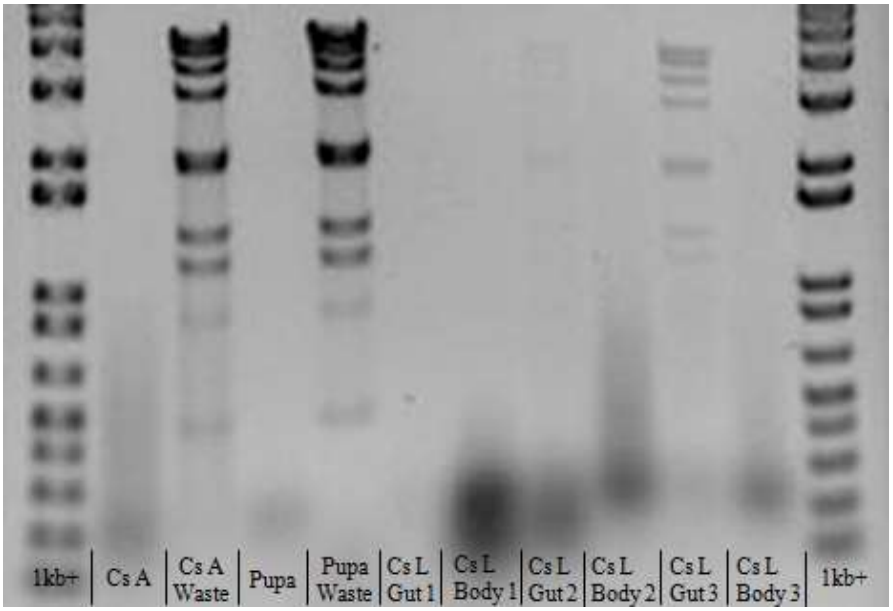

Fig. 3: *H.v.*CPV screening of genetic material from various *C. sonorensis* life stages. This gel reveals characteristic CPV segments concentrated in wastes expelled by the wasp just prior to pupation. *H.v.*CPV is absent in the adult and wasp pupa, suggesting that it is completely shed in waste. CPV appears to be restricted to gut tissue of fifth-instar wasp larvae. **Cs A** and **Cs A Waste** are genetic material extracted from an adult wasp and its pupation waste, respectively. **Pupa** and **Pupa Waste** are nucleic acids extracted from a wasp pupa and its waste, respectively. **Cs L Gut** samples are nucleic acids extracted from the guts of each of three fifth-instar wasp larvae, while **Cs L Body** samples were extracted from the remaining tissues of the three dissected larvae.

We then developed a method to amplify reovirus genetic material using reverse-transcription and PCR with primers designed from a conserved reovirus gene. We then cloned amplified portions of the viral genome into *E. coli* and sequenced the amplified, cloned DNA. The resulting sequence showed a close relation of our dsRNA virus to other insect-infecting reoviruses, notably to the cypoviruses. To confirm this we sent a sample of our reovirus, extracted from fifth-instar *C. sonorensis* larvae gut tissue by homogenization in insect Ringer's solution and 0.2µm filtration, to an expert, Don Stoltz, who subjected the sample to electron microscopy and concluded that there were reovirus virions present, likely belonging to the cypovirus genus.

In summary, we discovered the *Heliothis virescens* cypovirus as a nearly ubiquitous viral infection of our *C. sonorensis* colony. The virus seems particularly abundant at an important developmental time for the insects, during which we have observed a high mortality rate. We suspect that this mortality is causally associated with *H.v.*CPV and propose to test this hypothesis in this study.

Specific Aims/Approach:

We hypothesize that *H. virescens* larvae benefit from the presence of *H.v.*CPV. We believe that the virus functions to combat parasitism by *C. sonorensis* wasps. If this is the case, then this system is an example "virological warfare" between parasitic wasps and their hosts, in which symbiotic viruses are deployed by both the host to combat the parasite and the parasite to suppress host immune responses. We theorize that the CPV is capable of producing a significant degree of mortality in parasitoid wasps before or during the crucial period of wasp metamorphosis. To test this hypothesis, I will use the following approaches to investigate the system.

First, we must be able to conclusively detect the presence of *H.v.*CPV in our insect colonies under normal, laboratory rearing conditions. Then, I will investigate whether or not the CPV actually replicates in the caterpillar and/or the wasp. The

replication and lifecycle of the CPV in this system is not yet known. To supplement our observations, we must quantitatively document the effects of *H.v.*CPV on *C. sonorensis* and *H. virescens* within our colonies. Presently, we know that there is a high degree of mortality in *C. sonorensis* during metamorphosis that can possibly attributable to the cypovirus, but the studies to show that it causes the mortality have not been done

We will determine whether *H.v.*CPV is capable of infecting and replicating in *C. sonorensis* and *H. virescens* gut tissue with the use of quantitative reverse transcription PCR (qRTPCR) and SDS-PAGE/Western Blotting analysis. We will use a structural protein gene, and its expression, as our target for assessing whether or not the virus is replicating in the wasp tissue. If the virus is truly replicating inside the caterpillar or the wasp, then there should be an increase in the quantity of structural proteins, and the genes that encode them, as the insects mature. These experiments can be performed to document the capacity of *H.v.*CPV to infect *C. sonorensis* and *H. virescens*, and to further implicate the virus in the high death rate of developing wasps.

To further test our hypothesis, I propose to manipulate the presence and evaluate the effects of the *H.v.*CPV in *C. sonorensis* and *H. virescens* life systems. I predict that *H.v.*CPV presence has little or no ill-effects on the caterpillar, but will result in significant parasite mortality in exposed wasp larvae.

*H.v.*CPV-free colonies of *H. virescens* will be acquired from the USDA Biocontrol lab at Mississippi State and maintained by careful monitoring of the environment and diet. Passaging wasps through this virus-free colony should enable me to produce a CPV-free wasp line. The infected and non-infected caterpillars will be separated as experimentally manipulated hosts for the wasps. We will observe and compare the health and mortality of *H.v.*CPV infected and *H.v.*CPV-free *H. virescens*, as well as the parasitization efficiency of *C. sonorensis* on infected and non-infected hosts. We will also screen the hosts and parasitoids for the presence and proliferation of CPV using the techniques previously mentioned. This experiment will provide data to conclusively describe the effect of *H.v.*CPV in this parasitoid-host system. From the results, we will be able to conclude whether or not this virus is being used by *H. virescens* as a defensive weapon against parasitization.

Significance:

Systems involving viruses that are benign and even beneficial to their host organisms are a relatively new branch of study that deserves greater attention. Research into the relationships between parasitoid wasps, their host organisms, and the respective viruses associated with both will offer unique insight into this successful and surprising symbiotic system. If *H.v.*CPV does indeed display characteristics that mimic those of the PDV, by protecting a host organism to the disadvantage of another insect, this system could be extremely useful in studying convergent evolution of symbiotic relationships between viruses and arthropod hosts. This research will provide further insight into the potentially beneficial effects of viruses in sophisticated virus-host relationships. Now, we are able to study the systems in which viruses improve the success of their hosts by supplementing defensive capabilities of their hosts. These studies have the potential to lead to important innovations in pest management, biological control, and biotechnology, through a more complete understanding and subtle manipulation of virus activities.

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