EVOLUTION AND FUNCTION OF A PARASITOID FACULTATIVE SYMBIONT

by

KELSEY ANNE COFFMAN

(Under the Direction of Gaelen R. Burke)

ABSTRACT

Parasitoid wasps are insects that survive as obligate parasites that feed from and eventually kill their insect hosts. One of the most spectacular biological innovations that has repeatedly arisen in parasitoid wasp lineages is the evolution of heritable associations with viruses. Parasitoid wasps use these viruses as biological weapons that they introduce into host insects in order to subdue host defenses and alter host physiology to promote successful parasitism. Most known beneficial viruses share many features due to convergent evolution, which implies that these characteristics are important for the formation and persistence of parasitoid-virus associations. However, there is currently a major gap in knowledge pertaining to parasitoid viruses of diverse viral ancestry. Here, I conducted four studies to functionally and genetically characterize a novel virus inherited by *Diachasmimorpha longicaudata* wasps, known as Diachasmimorpha longicaudata entomopoxvirus (DlEPV). I first determined the replication and transmission dynamics of DlEPV within *D. longicaudata* wasps and their fruit fly hosts, then investigated the effects of the virus on wasp and fly health. My results indicated that DlEPV shares many features with other parasitoid viruses due to convergent evolution, but is also unique in aspects that are likely due to its poxvirus ancestry. Second, I sequenced the DlEPV genome and analyzed differences in viral activity between wasps and fly hosts. I found that, contrary to all other known beneficial viruses, DlEPV

maintains a largely autonomous viral genome, and must use novel means to perpetuate its relationship with *D. longicaudata*. Third, I explored additional means of DlEPV transmission among wasps given the unique status of its genome. I determined that DlEPV utilizes post-hatch transmission that allows for highly efficient spread of the virus and its beneficial phenotype to wasps. Lastly, I investigated the possible effects of DlEPV on the host range of *D. longicaudata* by measuring DlEPV activity in fly species with varying permissiveness to *D. longicaudata* parasitism. I showed that DlEPV replication and virulence capabilities within fly hosts were strongly correlated with *D. longicaudata* parasitism ability, suggesting that DlEPV plays a major role in the success of *D. longicaudata* as a generalist parasitoid species.

INDEX WORDS: insects, parasitoid wasp, virus, symbiosis, parasitism, genomics, evolution

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Dedication

To my loving cat, Heidi. Please don’t rip this to shreds.

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CHAPTER 1

Introduction and Literature Review

The role of microbial symbionts in shaping the lives and evolution of their eukaryotic hosts has gained a deeper appreciation in recent decades. Animals are now known to be inextricably linked to their symbiotic microbes, as they have strongly influenced animal evolution in vast ways [1]. Microbial symbionts allow animal hosts to adapt quickly and provide novel phenotypes to hosts, which in turn, can foster diversification of symbiont-associated organisms [2,3]. The most successful group of animals on Earth are the insects, which comprise millions of species and have conquered most known ecological habitats [4,5]. The interactions between microbes and insects have been of wide scale interest to both basic and applied scientific fields, and study of insect-microbe symbiosis has uncovered countless intriguing examples of symbiotic relationships that enable insects to thrive [6]. Symbiotic bacteria, in particular, are pivotal for the survival and fitness of diverse insect lineages and serve as a model for many facets of microbial symbiosis [7].

Bacterial symbionts have extremely varied types of associations with insects, in terms of the consequence of the relationship for insect fitness, the necessity of the relationship for either partner, the phenotype provided to the insect host, the ways in which symbionts are transmitted among insects, symbiont interactions with other microbes, and symbiont effects on insect ecology [8]. Specifically, endosymbiotic bacteria, namely those that are restricted to live within the insect

body, have been extensively studied to reveal complex dynamics with insect hosts that can require a highly regulated interplay between both partners [9]. Endosymbionts can cause varying effects on overall insect fitness, ranging from those that are detrimental to those that provide immense benefit for the insect partner. Often associated with the fitness outcome of an association is the degree of insect dependence on the symbiont. Bacteria that are required for the survival of the insect, known as obligate symbionts, are almost always mutualistic, while those microbes that are not required but can induce various phenotypes to the insect when present, known as facultative symbionts, can be detrimental or beneficial for the insect [9].

Obligate symbionts represent many of the most intricate known relationships, and well-known examples are those bacteria that provision nutrients needed by insects that are not supplied by the insect diet [10]. The presence of an obligate symbiont is a common occurrence for insects that survive by feeding exclusively on relatively nutrient-poor substances, such as plant sap or vertebrate blood [11,12]. For instance, pea aphids (*Acyrthosiphon pisum*) do not obtain all of the essential amino acids they require from their plant sap diet alone; therefore, the obligate symbiont *Buchnera aphidicola* produces the remaining needed amino acids for aphid survival [13]. Obligate symbionts are often restricted within the insect to a specific symbiont-housing organ, known as the bacteriome, and are exclusively transmitted through internal incorporation into insect eggs, a process known as transovarial transmission [11,14,15]. The perpetual host restriction often exhibited by obligate symbionts, while useful from the insect's perspective for control of bacterial populations and faithful transmission of symbionts to the next generation, has unusual effects on symbiont genomes compared to free-living bacteria [9,16]. Endosymbiont genomes have a tendency to erode over time, due to a severe manifestation of a phenomenon known as Muller's ratchet [17]. Extreme bacterial population bottlenecks experienced during vertical transmission to

future generations leads to relaxed natural selection on symbionts and a stronger influence from genetic drift that allows for random fixation of deleterious mutations within symbiont populations. Additionally, the inaccessibility of horizontal transmission causes these deleterious mutations to accumulate in symbiont genomes over time, resulting in the inactivation of bacterial genes and eventual degeneration of these genomes [18,19]. Insects with symbiont decay, like the pea aphid, have evolved to compensate for functional losses experienced by symbionts such as *Buchnera* through complementation of essential biochemical pathways using both insect and symbiont intermediates [20,21]. However, genome reduction may not be stable for the association in the long term and could ultimately lead to extinction of both partners [22,23].

Facultative symbionts are similar to obligate symbionts in many ways: facultative symbionts are often vertically transmitted, and they have evolved to live within insect hosts. However, symbionts with a facultative role are known to provide a wider range of phenotypes for insect hosts, including protection from pathogens and parasites or tolerance to environmental stressors [24,25]. The bacterium *Spiroplasma*, for instance, infects *Drosophila* flies and protects them from pathogenic nematodes, parasitic wasps, and pathogenic fungi [26–28]. A secondary category of facultative symbionts is those that are not beneficial for the insect host *per se*, but instead exist as reproductive parasites of infected insects. The bacterium *Wolbachia*, which is nearly ubiquitous among insects, causes various forms of reproductive manipulation, such as cytoplasmic incompatibility, male-killing, parthenogenesis, and feminization, that restrict the mating capacity of the insect host in order to drive transmission of the bacteria within insect populations [29,30]. Insects without the bacterium are therefore at a disadvantage compared to those that are infected, imposing a net loss in fitness for uninfected individuals. Moreover, *Wolbachia* has been shown to contribute to speciation within insects, such as *Drosophila* and *Nasonia* wasps, through the

promotion of reproductive isolation [31,32]. In many instances, facultative symbiont dynamics resemble those of a pathogen more than they do an obligate symbiont, due to the nonessential role of these symbionts for insect survival or reproduction and the comparably robust condition of their genomes. Whereas obligate symbionts are heavily controlled by the associated insect, facultative symbionts often display more autonomous characteristics within insect hosts, including symbiont-driven vertical and horizontal transmission strategies and localization throughout the insect (e.g. within insect hemolymph) [33]. These features also offer a greater diversity in the resources that facultative symbionts can impart on infected insects [34]. Therefore, study of facultative symbionts has been instrumental for expanding our overall understanding regarding the multitude of ways that bacteria contribute to insect biology and evolution.

The increasing availability of high throughput genetic sequencing technologies has facilitated a large expansion in the study of insect microbial symbionts following the transition into the new millennium. Bacterial associations remain the predominant focus of current research, although fungal, archaeal, and protozoan symbionts have also been described in insects [35,36]. The study of beneficial viruses, in contrast, is limited to isolated examples across the tree of life [37]. Perhaps a large reason for this relative dearth of information pertaining to beneficial viruses is the lack of conservation among viral genomes, due to the polyphyletic nature of independently derived virus lineages. Common methods currently used to explore microbial diversity in animal systems, such as amplicon sequencing of the bacterial 16S ribosomal RNA gene or the fungal internal transcribed spacer (ITS) gene, are not feasible for identifying resident viruses, which do not contain universally-conserved genes. Furthermore, viruses exhibit a much faster rate of evolution that, when coupled with the lack of gene conservation, can make virus identification via genetic sequence data a difficult or impossible feat [38]. Metagenomic sequencing surveys have more

recently offered a promising alternative for the identification of viral diversity in eukaryotes [39,40], although most of these efforts have focused on RNA viruses and of those directed toward arthropods, many are limited to insect vectors of human importance [41,42] (but see [43,44]). Moreover, functional data on novel viruses discovered from metagenomic sequencing is currently limited [45]. An additional factor that may contribute to the scarcity of research on beneficial viruses is the widely held conception that viruses are solely pathogenic entities. However, considerable evidence now suggests that viruses evolve in ways that favor persistence within hosts rather than a strictly antagonistic existence [46,47]. Several instances of viral interactions within bacterial symbiont systems have proven to be fundamental for establishment of the symbiont-induced phenotype. For example, *Wolbachia* strains that cause cytoplasmic incompatibility (CI) in insects are infected with a bacteriophage, known as WO [48]. The causative genes of CI, *cifA* and *cifB*, are both encoded within the prophage WO genome, indicating that CI is a phenotype provided by a phage rather than *Wolbachia* itself [49,50]. In addition, the bacterium *Hamiltonella defensa*, a facultative symbiont in pea aphids, protects aphids from parasitism by parasitoid wasps [51,52]. However, the protective phenotype is dependent on the infection of *H. defensa* with a bacteriophage, known as Acyrthosiphon pisum secondary endosymbiont (APSE), which causes mortality of wasp eggs deposited within the aphid hemolymph [53,54]. Both of these examples highlight the impact that viruses can have on symbiosis, and continued study will likely illuminate more examples in which viruses contribute to symbiotic systems. Even still, an important exception to the paucity of knowledge regarding beneficial viruses in insects can be found within the parasitoid wasps, which have repeatedly established heritable associations with viruses throughout evolutionary time.

The Hymenoptera represent one of the largest orders of insects, likely surpassing other megadiverse insect lineages in species-richness, such as the Coleoptera [55–57]. The parasitoid wasps constitute the majority of members within this order and are distinguished by their obligately parasitic lifestyle. Parasitoid wasps oviposit on or within other arthropod hosts, and wasp offspring feed from host tissue during development [58,59]. Successful parasitism almost inevitably kills the host insect, although hosts that produce an effective defense can instead avoid oviposition or kill an invading immature wasp [60]. The inherently lethal interactions caused by the parasitoid life cycle drives coevolution between parasitoids and their hosts, leading to a multitude of adaptations for exploitation of the host by the parasitoid and in return, resistance by the host. Endoparasitoids (i.e. those that develop within the host hemocoel), which are the main focus of this work and hereafter will be referred to simply as 'parasitoids', must contend with the immune system of the host and have thus evolved a number of biological innovations to either actively combat or avoid detection by host immunological defenses [61]. Additionally, parasitoids have evolved tactics to manipulate host physiology to optimize the nutritional availability of the host [62]. These adaptations generally take the form of biological factors introduced into hosts during oviposition and continuously throughout parasitism to alter host physiology in ways that promote successful parasitism by the wasp. Host manipulation factors can include venoms and ovarian fluids produced by adult female wasps that are injected into hosts during oviposition, specialized cells of the wasp embryo called teratocytes that dissociate from parasitoid eggs after oviposition and release factors into the hemolymph of the parasitized host, and secretions emitted by wasp larvae during feeding [60,63,64]. The reproductive tissues of female wasps, such as the venom gland and the ovaries, are also the sites in which numerous beneficial viruses have been

discovered, which has launched intensive study on the evolutionary and functional characteristics of these virus-derived entities over the better half of the last century [65].

The best studied examples of beneficial viral elements within parasitoids are named polydnaviruses (PDVs), which represent ancient associations that play a crucial role in successful parasitoid development. PDVs have an estimated presence within tens of thousands of parasitoid wasp species and for those inherited by braconid wasps, known as bracoviruses (BVs), were initially acquired by an ancestral wasp approximately 100 million years ago [66–69]. PDVs are subdivided between BVs and those associated with ichneumonid wasps, known as ichnoviruses (IVs) [70]. BVs and IVs share many characteristics regarding their life cycle and genomic architecture, but many lines of evidence suggest they arose from at least two independent viral acquisitions that have experienced extreme evolutionary convergence [71,72]. Yet both associations likely established heritability through invasion and viral genome integration of wasp germline cells. Presently, PDV genomes exist in two forms: (1) as a remnant provirus within the genome of all wasp cells, and (2) as double-stranded DNA (dsDNA) virus particles, or virions, that are deposited into host insects that contain an encapsidated DNA genome [73]. PDV genes involved in forming new virions, referred to as replication genes, are scattered across multiple loci within wasp genomes and are transcribed in wasp ovaries beginning during pupal development [71,72,74,75]. The resulting viral gene products amplify and package specific proviral DNA segments into PDV virions continuously throughout adulthood [76,77]. These encapsidated segments contain virulence genes, which function to disrupt host development and immunity during parasitism, but do not include viral replication genes [78]. Consequently, PDV particles delivered to hosts express virulence genes that are required for wasp survival but are unable to further reproduce. This contrasts starkly with PDV activity in wasp ovaries, where virus replication

is prioritized and virulence genes are not expressed [74,79]. Thus, the dispersal of viral replication genes within wasp genomes is thought to be an important adaptation in the evolution of PDV associations, because it helped establish a dichotomy of viral function, in which replication occurs exclusively in wasps and immunosuppression is restricted to hosts. The basis for the lack of virulence gene expression in wasps is unknown, but this characteristic likely preserves wasp health by enabling non-pathogenic PDV replication. In addition, the evolution of replicatively defective PDV virions has permanently linked the fate of these viruses to that of their wasps [80]. Collectively, these features, facilitated by the genomic integration events of PDV ancestors, have maintained stable heritable associations.

Because of the endogenous nature of PDV genomes, they represent unusual examples of endogenous viral elements (EVEs), in which the ancestral function of virus particle production has been preserved [81]. Additional instances of EVEs have been characterized more recently within the wasps *Venturia canescens* and *Fopius arisanus* [82,83]. Both wasp species have independently acquired EVEs derived from the same virus family as BVs, known as the nudiviruses. These newly reported EVEs, known as Venturia canescens endogenous nudivirus (VcENV) and Fopius arisanus endogenous nudivirus (FaENV), display the same dispersed viral genome architecture as PDVs, and exhibit specific viral gene losses common to all nudivirus-derived EVEs [84]. Additionally, the virus-like particles (VLPs) produced by VcENV and FaENV both occur within the same region of female wasp ovaries where PDVs are produced, known as the calyx [82,83]. The function of FaENV-produced VLPs remains unclear, although VcENV VLPs coat wasp eggs during oviposition and prevent egg encapsulation by the host immune system [85–87]. The recurrent evolution of the genomic architecture displayed by all known EVEs strongly supports the importance of viral genome endogenization and reorganization for establishing these long-term

associations. It also raises the question of whether non-endogenous mutualistic viruses exist. The repeated observation of nudiviruses as the ancestor for multiple independent associations suggests that the shared genomic architecture could, in part, be a product of the shared ancestry among these systems rather than the necessity of this feature for maintaining long term wasp-virus mergers. Various types of heritable viruses have been described within parasitoids that are not PDVs, including DNA viruses like the ascovirus found within *Diadromus pulchellus* wasps, along with RNA viruses, such as the iflaviruses within *V. canescens* and *Dinocampus coccinellae*, and reoviruses found in multiple wasp species [88–91]. However, few of these non-PDV examples have been functionally and genetically characterized. The relative absence of comparative data from parasitoid viruses of diverse ancestry is an important problem, because there are likely alternative mechanisms through which beneficial viruses can arise and are maintained that are dictated by viral origin but remain unexplored. Therefore, the goal of my doctoral research was to characterize a virus of divergent viral ancestry in order to ascertain the shared properties of heritable viruses from a more inclusive standpoint.

Diachasmimorpha longicaudata is a braconid larval-pupal parasitoid that has been widely used as a biological control agent within tropical and subtropical areas for the suppression of several tephritid fruit fly pest species. These include the Caribbean fruit fly (*Anastrepha suspensa*) in the southern United States and Central America, as well as the Mediterranean fruit fly (*Ceratitis capitata*) and the oriental fruit fly (*Bactrocera dorsalis*) in Hawaii and throughout the Pacific Islands [92,93]. *D. longicaudata* belongs to the braconid subfamily Opiinae, which primarily comprises parasitoids that attack cyclorrhaphous Diptera in the family Tephritidae [94]. Opiine wasps ancestrally lack PDVs, although several non-PDV viruses or VLPs have been identified within wasps in this subfamily, including *F. arisanus*, *Psyttalia concolor*, and *D. longicaudata*

[83,95,96]. In addition to FaENV-produced VLPs that were recently discovered within *F. arisanus*, VLPs were also reported in the venom gland of *P. concolor* wasps, although more recent venom analysis does not support the presence of VLPs in this species [95,97]. Two viruses, a rhabdovirus and a poxvirus, were both identified within *D. longicaudata* wasps, named Diachasmimorpha longicaudata rhabdovirus (DlRhV) and Diachasmimorpha longicaudata entomopoxvirus (DlEPV), respectively [96,98]. DlRhV and DlEPV were both initially described using microscopy, localized within the female wasp venom gland, as well as *A. suspensa* fly hosts during parasitism by *D. longicaudata* [96,99]. The function of DlRhV has remained largely elusive, although its genome encodes a 24 kDa protein that was detected during parasitism and had a hypothesized role in wasp survival [100–102]. Furthermore, the sudden disappearance of DlRhV within a *D. longicaudata* laboratory colony demonstrated that it must have a facultative relationship to wasps [102]. DlEPV, in contrast, has been observed within *A. suspensa* hemocytes during *D. longicaudata* parasitism, suggesting that DlEPV is involved in host immune suppression, similar to PDVs [103]. Early efforts to sequence several DlEPV genes confirmed morphological findings that suggested DlEPV was a poxvirus [104–106]. Taken together, the initial molecular and genetic characterization of DlEPV has provided a starting point for further investigation of this virus, but more in-depth analysis of the DlEPV system is needed to ascertain its function within *D. longicaudata* wasps and fruit fly hosts, in addition to the evolutionary standing of DlEPV compared to parasitoid EVEs and other microbial symbionts.

Within this dissertation, I describe my research characterizing the evolution and function of DlEPV, a novel viral mutualist inherited by parasitoid wasps. I used a combination of molecular biology, manipulative genetics, and genomic sequencing techniques to establish a foundational understanding of the DlEPV system. The collective findings from these efforts, detailed in the

following chapters, substantiate a more comprehensive definition of beneficial virus evolution in the context of other heritable microbes within insects.

Chapter 2 catalogues my initial work to establish basic DlEPV dynamics within *D. longicaudata* wasps and *A. suspensa* fruit fly hosts, including virus replication and transmission strategies of DlEPV, as well as the consequences of DlEPV activity for parasitoid and host survival and fitness. My results provided strong evidence that DlEPV shares many features with PDVs due to convergent evolution between independent parasitoid-virus associations of diverse origins in order to provide a similar beneficial role to wasps during parasitism. I also developed a novel method in this chapter to eliminate the resident DlEPV population from *D. longicaudata* wasps using RNA interference (RNAi) technology, which will continue to be a useful tool for increased understanding of DlEPV activity and the functions of specific DlEPV genes.

In Chapter 3, I sequenced the DlEPV genome, then used comparative and functional genomic approaches to assess the evolution of DlEPV. I showed that DlEPV is not integrated within the *D. longicaudata* genome, indicating that DlEPV is not an EVE but instead, the first exogenous virus with a demonstrated mutualistic role within parasitoid wasps. A close relative to DlEPV was also identified in this chapter, suggesting that DlEPV arose from a fly pathogen. Additionally, I showed that DlEPV transcriptional activity is vastly different within wasps compared to fly hosts, supporting a novel mechanism in which DlEPV maintains its relationship with *D. longicaudata*.

Given the newfound classification of DlEPV as an exogenous viral symbiont, I investigated additional modes of transmission that DlEPV utilizes in Chapter 4. I established a stable colony of uninfected wasps using previously developed RNAi methods, which confirmed that DlEPV is a highly beneficial but facultative virus for *D. longicaudata*. Furthermore, I showed that uninfected wasps could reacquire a full viral load of DlEPV by developing within a virus-infected host. These

results revealed that DlEPV can be efficiently transferred among wasps, resulting in vertical or horizontal transmission via an external route during parasitism. Moreover, the beneficial phenotype that DlEPV provides to wasp offspring was immediately restored after reacquisition of the virus. DlEPV is therefore the first known beneficial virus to display post-hatch transmission within insects.

Finally, Chapter 5 explores the role of DlEPV in determining the host range of *D. longicaudata*. I investigated the effects of DlEPV on three tropical fruit fly species: two of which are permissive hosts for *D. longicaudata*, while the third is a non-permissive host. I showed that DlEPV replication and virulence activities were strongly correlated with host permissiveness, as the two permissive host species were also the most susceptible to the virus, while the non-permissive species demonstrated competent immunity against viral infection. My findings suggest that DlEPV is a major contributing factor to *D. longicaudata* success as a generalist species and more broadly, as a highly effective biological control agent against fruit fly pests. Therefore, DlEPV could represent a novel means of niche expansion for *D. longicaudata*, which has not been demonstrated for a viral symbiont to date.

This combined body of work ultimately offers a transformative example of viral symbiosis that expands upon current notions of microbial symbiont evolution and challenges existing conceptions of the evolutionary processes that govern mutualistic viruses.

1.1 REFERENCES

1. McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Lošo T, Douglas AE, et al. Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci. 2013;110: 3229–3236.

2. Margulis L, Fester R, editors. Symbiosis as a Source of Evolutionary Innovation: Speciation and Morphogenesis. Cambridge, MA: MIT Press; 1991.

3. Moran NA. Symbiosis as an adaptive process and source of phenotypic complexity. Proc Natl Acad Sci. 2007;104: 8627–8633.

4. Foottit RG, Adler PH, editors. Insect Biodiversity. Chichester, UK: John Wiley & Sons, Ltd; 2017.

5. Stork NE. How many species of insects and other terrestrial arthropods are there on Earth? Annu Rev Entomol. 2018;63: 31–45.

6. Bourtzis K, Miller TA, editors. Insect Symbiosis. Boca Raton, FL: CRC Press; 2003.

7. Buchner P. Endosymbiosis of Animals with Plant Microorganisms. New York: John Wiley & Sons; 1965.

8. Douglas AE. Multiorganismal insects: diversity and function of resident microorganisms. Annu Rev Entomol. 2015;60: 17–34.

9. Moran NA, McCutcheon JP, Nakabachi A. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet. 2008;42: 165–190.

10. Douglas AE. The microbial dimension in insect nutritional ecology. Funct Ecol. 2009;23: 38–47.

11. Baumann P. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microbiol. 2005;59: 155–189.

12. Rio RVM, Attardo GM, Weiss BL. Grandeur alliances: symbiont metabolic integration and obligate arthropod hematophagy. Trends Parasitol. 2016;32: 739–749.

13. Douglas AE. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. Annu Rev Entomol. 1998;43: 17–37.

14. Douglas AE. Mycetocyte symbiosis in insects. Biol Rev Camb Philos Soc. 1989;64: 409–434.

15. Wilkinson TL, Fukatsu T, Ishikawa H. Transmission of symbiotic bacteria *Buchnera* to parthenogenetic embryos in the aphid *Acyrthosiphon pisum* (Hemiptera: Aphidoidea). Arthropod Struct Dev. 2003;32: 241–245.

16. Wernegreen JJ. Genome evolution in bacterial endosymbionts of insects. Nat Rev Genet. 2002;3: 850–861.

17. Moran NA. Accelerated evolution and Muller's rachet in endosymbiotic bacteria. Proc Natl Acad Sci. 1996;93: 2873–2878.

18. McCutcheon JP, Moran NA. Extreme genome reduction in symbiotic bacteria. Nat Rev Microbiol. 2012;10: 13–26.

19. Moran NA, Bennett GM. The tiniest tiny genomes. Annu Rev Microbiol. 2014;68: 195–215.

20. Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. Nature. 2000;407: 81–86.

21. Hansen AK, Moran NA. Aphid genome expression reveals host–symbiont cooperation in the production of amino acids. Proc Natl Acad Sci. 2011;108: 2849–2854.

22. Bennett GM, Moran NA. Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole. Proc Natl Acad Sci. 2015;112: 10169–10176.

23. Wernegreen JJ. In it for the long haul: evolutionary consequences of persistent endosymbiosis. Curr Opin Genet Dev. 2017;47: 83–90.

24. Haine ER. Symbiont-mediated protection. Proc R Soc Lond B Biol Sci. 2008;275: 353–361.

25. Brownlie JC, Johnson KN. Symbiont-mediated protection in insect hosts. Trends Microbiol. 2009;17: 348–354.

26. Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ. Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. Science. 2010;329: 212–215.

27. Xie J, Vilchez I, Mateos M. *Spiroplasma* bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma*. PLoS One. 2010;5: e12149.

28. Łukasik P, van Asch M, Guo H, Ferrari J, Godfray HCJ. Unrelated facultative endosymbionts protect aphids against a fungal pathogen. Ecol Lett. 2013;16: 214–218.

29. Werren JH, Zhang W, Guo LR. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc R Soc Lond B Biol Sci. 1995;261: 55–63.

30. Werren JH, Baldo L, Clark ME. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008;6: 741–751.

31. Shoemaker DD, Katju V, Jaenike J. *Wolbachia* and the evolution of reproductive isolation between *Drosophila recens* and *Drosophila subquinaria*. Evolution. 1999;53: 1157–1164.

32. Bordenstein SR, O'Hara FP, Werren JH. *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. Nature. 2001;409: 707–710.

33. Perlmutter JI, Bordenstein SR. Microorganisms in the reproductive tissues of arthropods. Nat Rev Microbiol. 2020;18: 97–111.

34. Oliver KM, Degnan PH, Burke GR, Moran NA. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annu Rev Entomol. 2010;55: 247–266.

35. Gibson CM, Hunter MS. Extraordinarily widespread and fantastically complex: comparative biology of endosymbiotic bacterial and fungal mutualists of insects. Ecol Lett. 2010;13: 223–234.

36. Brune A. Symbiotic digestion of lignocellulose in termite guts. Nat Rev Microbiol. 2014;12: 168–180.

37. Roossinck MJ. The good viruses: viral mutualistic symbioses. Nat Rev Microbiol. 2011;9: 99–108.

38. Duffy S, Shackelton LA, Holmes EC. Rates of evolutionary change in viruses: patterns and determinants. Nat Rev Genet. 2008;9: 267–276.

39. Koonin EV, Dolja VV. Metaviromics: a tectonic shift in understanding virus evolution. Virus Res. 2018;246: A1–A3.

40. Zhang Y-Z, Shi M, Holmes EC. Using metagenomics to characterize an expanding virosphere. Cell. 2018;172: 1168–1172.

41. Junglen S, Drosten C. Virus discovery and recent insights into virus diversity in arthropods. Curr Opin Microbiol. 2013;16: 507–513.

42. Bolling BG, Weaver SC, Tesh RB, Vasilakis N. Insect-specific virus discovery: significance for the arbovirus community. Viruses. 2015;7: 4911–4928.

43. Shi M, Lin X-D, Tian J-H, Chen L-J, Chen X, Li C-X, et al. Redefining the invertebrate RNA virosphere. Nature. 2016;540: 539–543.

44. Wallace MA, Coffman KA, Gilbert C, Ravindran S, Albery GF, Abbott J, et al. The discovery, distribution and diversity of DNA viruses associated with *Drosophila melanogaster* in Europe. bioRxiv [Preprint]. 2020; 2020.10.16.342956.

45. Obbard DJ. Expansion of the metazoan virosphere: progress, pitfalls, and prospects. Curr Opin Virol. 2018;31: 17–23.

46. Villarreal LP. Virus-host symbiosis mediated by persistence. Symbiosis. 2007;44: 1–9.

47. Roossinck MJ, Bazán ER. Symbiosis: viruses as intimate partners. Annu Rev Virol. 2017;4: 123–139.

48. Kent BN, Bordenstein SR. Phage WO of *Wolbachia*: lambda of the endosymbiont world. Trends Microbiol. 2010;18: 173–181.

49. LePage DP, Metcalf JA, Bordenstein SR, On J, Perlmutter JI, Shropshire JD, et al. Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. Nature. 2017;543: 243–247.

50. Beckmann JF, Ronau JA, Hochstrasser M. A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. Nat Microbiol. 2017;2: 17007.

51. Oliver KM, Russell JA, Moran NA, Hunter MS. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc Natl Acad Sci. 2003;100: 1803–1807.

52. Oliver KM, Moran NA, Hunter MS. Variation in resistance to parasitism in aphids is due to symbionts not host genotype. Proc Natl Acad Sci. 2005;102: 12795–12800.

53. Oliver KM, Degnan PH, Hunter MS, Moran NA. Bacteriophages encode factors required for protection in a symbiotic mutualism. Science. 2009;325: 992–994.

54. Brandt JW, Chevignon G, Oliver KM, Strand MR. Culture of an aphid heritable symbiont demonstrates its direct role in defence against parasitoids. Proc R Soc Lond B Biol Sci. 2017;284. doi:10.1098/rspb.2017.1925

55. LaSalle J, Gauld ID. Hymenoptera: their biodiversity, and their impact on the diversity of other organisms. In: LaSalle J, Gauld ID, editors. Hymenoptera and Biodiversity. Wallingford, UK: CAB International; 1993. pp. 1–26.

56. Huber JT. Biodiversity of Hymenoptera. In: Foottit RG, Adler PH, editors. Insect Biodiversity. Chichester, UK: John Wiley & Sons, Ltd; 2017. pp. 419–461.

57. Forbes AA, Bagley RK, Beer MA, Hippee AC, Widmayer HA. Quantifying the unquantifiable: why Hymenoptera, not Coleoptera, is the most speciose animal order. BMC Ecol. 2018;18: 21.

58. Godfray HCJ. Parasitoids: Behavioral and Evolutionary Ecology. Princeton, NJ: Princeton University Press; 1994.

59. Quicke DLJ. Parasitic wasps. London, UK: Chapman & Hall; 1997.

60. Strand MR, Pech LL. Immunological basis for compatibility in parasitoid host relationships. Annu Rev Entomol. 1995;40: 31–56.

61. Vinson SB. How parasitoids deal with the immune system of their host: an overview. Arch Insect Biochem Physiol. 1990;13: 3–27.

62. Pennacchio F, Strand MR. Evolution of developmental strategies in parasitic Hymenoptera. Annu Rev Entomol. 2006;51: 233–258.

63. Vinson SB, Iwantsch GF. Host regulation by insect parasitoids. Q Rev Biol. 1980;55: 143–165.

64. Asgari S, Rivers DB. Venom proteins from endoparasitoid wasps and their role in host-parasite interactions. Annu Rev Entomol. 2011;56: 313–335.

65. Strand MR, Burke GR. Polydnaviruses: from discovery to current insights. Virology. 2015;479: 393–402.

66. Murphy N, Banks JC, Whitfield JB, Austin AD. Phylogeny of the microgastroid complex of subfamilies of braconid parasitoid wasps (Hymenoptera) based on sequence data from seven genes, with an improved estimate of the time of origin of the lineage. Mol Phylogenet Evol. 2008;47: 378–395.

67. Quicke DLJ, Laurenne NM, Fitton MG, Broad GR. A thousand and one wasps: a 28S rDNA and morphological phylogeny of the Ichneumonidae (Insecta: Hymenoptera) with an investigation into alignment parameter space and elision. J Nat Hist. 2009;43: 1305–1421.

68. Whitfield JB. Estimating the age of the polydnavirus/braconid wasp symbiosis. Proc Natl Acad Sci. 2002;99: 7508–7513.

69. Rodriguez JJ, Fernández-Triana JL, Alex Smith M, Janzen DH, Hallwachs W, Erwin TL, et al. Extrapolations from field studies and known faunas converge on dramatically increased estimates of global microgastrine parasitoid wasp species richness (Hymenoptera: Braconidae). Insect Conserv Divers. 2013;6: 530–536.

70. Strand MR, Drezen JM. Polydnaviridae. In: King AMQ, Lefkowitz E, Adams MJ, Carstens EB, editors. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier; 2012. pp. 237–248.

71. Bézier A, Annaheim M, Herbinière J, Wetterwald C, Gyapay G, Bernard-Samain S, et al. Polydnaviruses of braconid wasps derive from an ancestral nudivirus. Science. 2009;323: 926–930.

72. Volkoff A-N, Jouan V, Urbach S, Samain S, Bergoin M, Wincker P, et al. Analysis of virion structural components reveals vestiges of the ancestral ichnovirus genome. PLoS Pathog. 2010;6: e1000923.

73. Strand MR. Polydnaviruses. In: Asgari S, Johnson KN, editors. Insect Virology. Norfolk, UK: Caister Academic Press; 2010. pp. 171–197.

74. Burke GR, Strand MR. Deep sequencing identifies viral and wasp genes with potential roles in replication of Microplitis demolitor bracovirus. J Virol. 2012;86: 3293–3306.

75. Burke GR, Walden KKO, Whitfield JB, Robertson HM, Strand MR. Widespread genome reorganization of an obligate virus mutualist. PLoS Genet. 2014;10: e1004660.

76. Burke GR, Thomas SA, Eum JH, Strand MR. Mutualistic polydnaviruses share essential replication gene functions with pathogenic ancestors. PLoS Pathog. 2013;9: e1003348.

77. Lorenzi A, Ravallec M, Eychenne M, Jouan V, Robin S, Darboux I, et al. RNA interference identifies domesticated viral genes involved in assembly and trafficking of virus-derived particles in ichneumonid wasps. PLoS Pathog. 2019;15: e1008210.

78. Webb BA, Strand MR, Dickey SE, Beck MH, Hilgarth RS, Barney WE, et al. Polydnavirus genomes reflect their dual roles as mutualists and pathogens. Virology. 2006;347: 160–174.

79. Bitra K, Zhang S, Strand MR. Transcriptomic profiling of Microplitis demolitor bracovirus reveals host, tissue and stage-specific patterns of activity. J Gen Virol. 2011;92: 2060–2071.

80. Strand MR, Burke GR. Polydnaviruses: nature's genetic engineers. Annu Rev Virol. 2014;1: 333–354.

81. Drezen J-M, Leobold M, Bézier A, Huguet E, Volkoff A-N, Herniou EA. Endogenous viruses of parasitic wasps: variations on a common theme. Curr Opin Virol. 2017;25: 41–48.

82. Pichon A, Bézier A, Urbach S, Aury JM, Jouan V, Ravallec M, et al. Recurrent DNA virus domestication leading to different parasite virulence strategies. Sci Adv. 2015;1: e1501150.

83. Burke GR, Simmonds TJ, Sharanowski BJ, Geib SM. Rapid viral symbiogenesis via changes in parasitoid wasp genome architecture. Mol Biol Evol. 2018;35: 2463–2474.

84. Burke GR. Common themes in three independently derived endogenous nudivirus elements in parasitoid wasps. Curr Opin Insect Sci. 2019;32: 28–35.

85. Salt G. Experimental studies in insect parasitism XIII. The haemocytic reaction of a caterpillar to eggs of its habitual parasite. Proc R Soc Lond B Biol Sci. 1965;162: 303–318.

86. Rotheram S. Immune surface of eggs of a parasitic insect. Nature. 1967;214: 700.

87. Feddersen I, Sander K, Schmidt O. Virus-like particles with host protein-like antigenic determinants protect an insect parasitoid from encapsulation. Experientia. 1986;42: 1278–1281.

88. Bigot Y, Rabouille A, Sizaret PY, Hamelin MH, Periquet G. Particle and genomic characteristics of a new member of the *Ascoviridae*: Diadromus pulchellus ascovirus. J Gen Virol. 1997;78 : 1139–1147.

89. Reineke A, Asgari S. Presence of a novel small RNA-containing virus in a laboratory culture of the endoparasitic wasp *Venturia canescens* (Hymenoptera: Ichneumonidae). J Insect Physiol. 2005;51: 127–135.

90. Dheilly NM, Maure F, Ravallec M, Galinier R, Doyon J, Duval D, et al. Who is the puppet master? Replication of a parasitic wasp-associated virus correlates with host behaviour manipulation. Proc R Soc Lond B Biol Sci. 2015;282: 20142773.

91. Renault S, Stasiak K, Federici B, Bigot Y. Commensal and mutualistic relationships of reoviruses with their parasitoid wasp hosts. J Insect Physiol. 2005;51: 137–148.

92. Ovruski S, Aluja M, Sivinski J, Wharton R. Hymenopteran parasitoids on fruit-infesting Tephritidae (Diptera) in Latin America and the Southern United States: diversity, distribution, taxonomic status and their use in fruit fly biological control. Integr Pest Manage Rev. 2000;5: 81–107.

93. Vargas RI, Leblanc L, Harris EJ, Manoukis NC. Regional suppression of *Bactrocera* fruit flies (Diptera: Tephritidae) in the Pacific through biological control and prospects for future introductions into other areas of the world. Insects. 2012;3: 727–742.

94. Wharton RA, Marsh PM. New World Opiinae (Hymenoptera: Braconidae) parasitic on Tephritidae (Diptera). J Wash Acad Sci. 1978;68: 147–167.

95. Jacas JA, Budia F, Rodríguez-Cerezo E, Vinuela E. Virus-like particles in the poison gland of the parasitic wasp *Opius concolor*. Ann Appl Biol. 1997;130: 587–592.

96. Lawrence PO, Akin D. Virus-like particles from the poison glands of the parasitic wasp *Biosteres longicaudatus* (Hymenoptera: Braconidae). Can J Zool. 1990;68: 539–546.

97. Mathé-Hubert H, Colinet D, Deleury E, Belghazi M, Ravallec M, Poulain J, et al. Comparative venomics of *Psyttalia lounsburyi* and *P. concolor*, two olive fruit fly parasitoids: a hypothetical role for a GH1 β-glucosidase. Sci Rep. 2016;6: 35873.

98. Edson KM, Barlin MR, Vinson SB. Venom apparatus of braconid wasps: comparative ultrastructure of reservoirs and gland filaments. Toxicon. 1982;20: 553–562.

99. Lawrence PO, Matos LF. Transmission of the Diachasmimorpha longicaudata rhabdovirus (DlRhV) to wasp offspring: an ultrastructural analysis. J Insect Physiol. 2005;51: 235–241.

100. Shi X, Gomez SP, Lawrence PO. A 24 kDa parasitism-specific protein from the Caribbean fruit fly, *Anastrepha suspensa*: cDNA and deduced amino acid sequence. Insect Biochem Mol Biol. 1999;29: 749–755.

101. Khoo CCH, Lawrence PO. Hagen's glands of the parasitic wasp *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae): ultrastructure and the detection of entomopoxvirus and parasitism-specific proteins. Arthropod Struct Dev. 2002;31: 121–130.

102. Simmonds TJ, Carrillo D, Burke GR. Characterization of a venom gland-associated rhabdovirus in the parasitoid wasp *Diachasmimorpha longicaudata*. J Insect Physiol. 2016;91-92: 48–55.

103. Lawrence PO. Morphogenesis and cytopathic effects of the Diachasmimorpha longicaudata entomopoxvirus in host haemocytes. J Insect Physiol. 2005;51: 221–233.

104. Lawrence PO. Purification and partial characterization of an entomopoxvirus (DlEPV) from a parasitic wasp of tephritid fruit flies. J Insect Sci. 2002;2: 10.

105. Mwaengo DM, Lawrence PO. A putative DNA helicase and novel oligoribonuclease in the Diachasmimorpha longicaudata entomopoxvirus (DlEPV). Arch Virol. 2003;148: 1431–1444.

106. Lawrence PO, Dillard BE. A homolog of the vaccinia virus D13L rifampicin resistance gene is in the entomopoxvirus of the parasitic wasp, *Diachasmimorpha longicaudata*. J Insect Sci. 2008;8: 8.

CHAPTER 2

A Mutualistic Poxvirus Exhibits Convergent Evolution with Other Heritable Viruses in Parasitoid Wasps[1]

[1] Coffman KA, Harrell TC, Burke GR. *Journal of Virology*. 2020;94: e02059–19. Reprinted here with permission of the publisher.

2.1 ABSTRACT

For insects known as parasitoid wasps, successful development as a parasite results in the death of the host insect. As a result of this lethal interaction, wasps and their hosts have coevolved strategies to gain an advantage in this evolutionary arms race. Although normally considered to be strict pathogens, some viruses have established persistent infections within parasitoid wasp lineages and are beneficial to wasps during parasitism. Heritable associations between viruses and parasitoid wasps have evolved independently multiple times, but most of these systems remain largely understudied with respect to viral origin, transmission and replication strategies of the virus, and interactions between the virus and host insects. Here, we report a detailed characterization of Diachasmimorpha longicaudata entomopoxvirus (DlEPV), a poxvirus found within the venom gland of *Diachasmimorpha longicaudata* wasps. Our results show that DlEPV exhibits similar but distinct transmission and replication dynamics compared to those of other parasitoid viral elements, including vertical transmission of the virus within wasps, as well as virus replication in both female wasps and fruit fly hosts. Functional assays demonstrate that DlEPV is highly virulent within fly hosts, and wasps without DlEPV have severely reduced parasitism success compared to those with a typical viral load. Taken together, the data presented in this study illustrate a novel case of beneficial virus evolution, in which a virus of unique origin has undergone convergent evolution with other viral elements associated with parasitoid wasps to provide an analogous function throughout parasitism.

2.2 IMPORTANCE

Viruses are generally considered to be disease-causing agents, but several instances of beneficial viral elements have been identified in insects called parasitoid wasps. These virus-

derived entities are passed on through wasp generations and enhance the success of the wasps' parasitic life cycle. Many parasitoid-virus partnerships studied to date exhibit common features among independent cases of this phenomenon, including a mother-to-offspring route of virus transmission, a restricted time and location for virus replication, and a positive effect of virus activity on wasp survival. Our characterization of Diachasmimorpha longicaudata entomopoxvirus (DlEPV), a poxvirus found in *Diachasmimorpha longicaudata* parasitoid wasps, represents a novel example of beneficial virus evolution. Here, we show that DlEPV exhibits functional similarities to known parasitoid viral elements that support its comparable role during parasitism. Our results also demonstrate unique differences that suggest DlEPV is more autonomous than other long-term viral associations described in parasitoid wasps.

Keywords: DNA virus, endogenous viral elements, evolution, parasitism, parasitoid wasp, poxvirus, symbiosis

2.3 INTRODUCTION

In parasitic relationships between species, coevolutionary arms races often lead to the emergence of innovative adaptations that allow the host organism to defend against the parasite and, conversely, the parasite to evade host defenses. Insects known as parasitoid wasps (order Hymenoptera), whose larvae develop by feeding from and eventually killing other arthropod hosts, have evolved a number of strategies to exploit their hosts and escape detection and/or destruction by the host immune system [1]. These tactics commonly involve the introduction of maternally derived factors into the host during wasp egg laying (oviposition), including various combinations of venomous proteins, as well as heritable viruses and virus-like particles (VLPs) [2]. The virus

particles (virions) and VLPs carried by many parasitoid wasps serve as vectors that deliver virulence genes and/or proteins to subdue host defenses and promote survival of wasp progeny during parasitism [3,4]. The best-studied examples of viruses associated with parasitoid wasps are the polydnaviruses (PDVs), which are derived from at least two different viral ancestors that were independently acquired by wasp lineages and have since evolved convergently to share many characteristics [5,6]. PDVs are endogenous viral elements (EVEs) that are vertically transmitted within the genomes of successive wasp generations and produce infectious virions within wasp ovaries [7]. PDV particles are incapable of replication once delivered to hosts but instead produce virulence gene products that are responsible for multiple forms of physiological manipulation, including alteration of host development and suppression of host immunity, that are required for successful parasitism by wasps [8,9].

Recent studies have shed light on two additional independent cases of EVEs in the wasp species *Venturia canescens* and *Fopius arisanus*, named VcENV and FaENV, respectively. These EVEs are responsible for the production of VLPs within female wasps and are distinguished from traditional virions by the absence of nucleic acid within the viral capsid. VcENV and FaENV share several features with PDVs, including an ovarian localization of VLP production beginning during the pupal stage of development, as well as a viral genome architecture that restricts replication to occur only within wasp tissues [10,11]. Many additional examples of heritable viruses have been identified in parasitoid wasps, although few have been genetically and functionally characterized [12]. Furthermore, most instances for which genomic data exist, such as VcENV, FaENV, and PDVs produced by wasps in the family Braconidae, are all derived from the same family of pathogenic insect viruses, known as nudiviruses [13]. The second group that comprises PDVs, namely, those carried by wasps in the family Ichneumonidae, has also been thoroughly

characterized but does not yet have a known viral ancestor [6,14]. Therefore, study of parasitoid viruses with varied ancestry and biology is imperative for understanding the common evolutionary processes that have repeatedly given rise to this phenomenon.

Diachasmimorpha longicaudata is a parasitoid species that has been widely introduced to tropical and subtropical areas for the biological control of tephritid fruit fly pests, such as the Caribbean fruit fly *Anastrepha suspensa* [15]. *D. longicaudata* wasps belong to a braconid lineage (subfamily Opiinae) that is not associated with PDVs [16]. Instead, *D. longicaudata* has been observed to harbor both a rhabdovirus and a poxvirus, or in different populations, an uncharacterized rod-shaped virus [17–19]. Diachasmimorpha longicaudata rhabdovirus (DlRhV) and Diachasmimorpha longicaudata entomopoxvirus (DlEPV) virions were first identified via transmission electron microscopy (TEM) surveys of the female wasp venom gland, which is responsible for the production and secretion of venomous fluid during oviposition [17,18]. Both DlRhV and DlEPV were also shown to infect the cells of parasitized *A. suspensa* host flies following oviposition [18,20]. However, the relationship between DlRhV and *D. longicaudata* is not obligate, and the involvement of DlRhV during wasp parasitism is still unknown [21]. DlEPV virions, in contrast, have been observed within and budding from the blood cells (hemocytes) of fly hosts throughout parasitism [18]. *D. longicaudata* parasitism was shown to cause adverse alterations in host hemocyte morphology, and the melanization process performed by hemocytes in response to an immune challenge was inhibited [22]. DlEPV was therefore claimed to suppress host immunity through the infection and disruption of host hemocyte function, thereby promoting the parasitism success of *D. longicaudata* [22]. Furthermore, characterization of DlEPV virion morphology and later sequencing of several DlEPV genes confirmed its classification as a poxvirus, representing the only poxvirus to be identified as a symbiont of parasitoid wasps to date

[23–26]. Poxviruses are divergent from the known ancestors of other parasitoid viruses or EVEs, and so a broader knowledge of this system has the potential to transform our conception of these associations.

Previous studies of DlEPV came to several conclusions regarding the replication and virulence strategies of this virus [18,22]. However, these findings were based almost entirely on qualitative data. Many aspects of the biology and evolution of this system therefore remain unknown, such as the transmission and replication dynamics of DlEPV, the pathogenic capability of DlEPV in fly hosts, and the consequences of DlEPV on wasp fitness. Here, we characterize these elements of the DlEPV system to address whether features displayed by PDVs and other parasitoid EVEs are also shared by DlEPV. Our collective results provide strong evidence that convergent evolution has occurred between DlEPV and other heritable parasitoid viruses, resulting in a novel case of beneficial virus evolution from a divergent pathogenic ancestor.

2.4 RESULTS

DlEPV is vertically transmitted within wasp eggs and consumed by wasp larvae while feeding from fly tissue. Approximately 24 to 48 h postparasitism (hpp) of an *A. suspensa* host, a *D. longicaudata* egg hatches and subsequently undergoes 3 larval instar stages while feeding on host tissue. The fly host begins its transition from larva to pupa during *D. longicaudata* parasitism, although fly pupal development is short-lived. Once the wasp has progressed to the third larval instar (144 to 168 hpp), all fly tissue has been consumed with the exception of the fly pupal casing (puparium). Afterwards, the wasp enters a transitory prepupal stage characterized by cessation of movement and compound eye pigmentation that marks the beginning of pupation [27]. We used

quantitative PCR (qPCR) estimation of viral abundance to first investigate the modes of DlEPV transmission and replication within *D. longicaudata* wasps.

The presence of viral DNAs within wasp eggs was used as an indication of vertical transmission. Wasp eggs were dissected from fly hosts after oviposition and washed to minimize the number of virions on egg surfaces in order to determine whether eggs contain viral DNAs. A substantial amount of DlEPV (approximately 1×10^4 genome copies) was detected from each laid wasp egg using this approach, providing suggestive evidence for vertical transmission of DlEPV from wasp mother to offspring (Figure 2.1A). Next, we measured viral copy number throughout wasp development to explore any changes that were indicative of virus replication. Significant differences in mean viral copy number for individual effects and interaction effects were tested using analysis of variance (ANOVA) with F test results indicated as follows ($F_{\text{between group df, within group df}}$ = F ratio). DlEPV abundance rose significantly in wasps during early larval development, peaked during the second instar stage, and significantly dropped by the prepupal stage ($F_{4,25}$ = 43.27, $P < 0.0001$) (Figure 2.1A). While this initial increase in viral copy number could be attributed to virus replication within internal wasp tissues, it could alternatively represent the mere accumulation of virus-infected fly tissue within the wasp gut. We hypothesized that the latter scenario best explained the observed pattern, given (i) previous observations showing DlEPV infection of fly tissue during parasitism [18], (ii) the continual ingestion of fly tissue by the developing wasp larva [27], and (iii) the large proportion of the wasp larva occupied by the gut [28]. DlEPV quantification in second-instar, third-instar, and prepupal wasp gut tissues revealed a significant interaction between tissue and developmental stage effects ($F_{4,45} = 27.71$, $P < 0.0001$) (Figure 2.1B). More than 99% of second- and third-instar DlEPV genome copies were localized to the gut contents, and relatively little virus was detected in the gut epithelial tissue or elsewhere

in the wasp (second-instar tissue effect: $F_{2,15} = 318.28$, $P < 0.0001$; third-instar tissue effect: $F_{2,15} = 108.63$, $P < 0.0001$) (Figure 2.1B). This suggests that the inflation of DlEPV abundance in wasp larvae is the result of virus ingestion rather than virus replication within wasp tissue. Prepupae also displayed a significant difference in viral abundance among gut tissues, but this difference was much less extreme than in second- and third-instar larvae (prepupa tissue effect: $F_{2,15} = 8.46$, $P = 0.0035$) (Figure 2.1B). Furthermore, there was a relative lack of DlEPV in prepupal gut contents compared to that in second- and third-instar larvae, suggesting that when the larva has finished consuming the fly host and is preparing to pupate, virions in the gut are degraded as the fly tissue is digested (Figure 2.1B).

DlEPV replicates in female wasps prior to eclosion but is discontinued during adulthood. After a *D. longicaudata* wasp pupates, processes such as eye pigmentation and cuticle sclerotization characterize the progression of pupal development. Therefore, we denoted each wasp with red eyes (incomplete eye pigmentation) and a white body (no sclerotization) as an early pupa, and each wasp with black eyes and an orange sclerotized body as a late pupa. We could also differentiate male and female wasps beginning in the pupal stage due to the long ovipositor characteristic of female *D. longicaudata*. Once pupal development has concluded, each wasp undergoes a final molt into an adult. However, the adult wasp will remain in the host puparium for 2 to 3 days before it emerges [27]. Here, we refer to this stage as "unemerged" adult.

There was a significant interaction between wasp sex and developmental stage effects when analyzing the amount of DlEPV in wasp pupae and adults ($F_{4,50} = 70.45$, $P < 0.0001$) (Figure 2.1C). We detected a small amount of virus within male and female wasps throughout early and late pupal stages (Figure 2.1C). DlEPV copy number rapidly increased by >5 orders of magnitude in female wasps beginning in the unemerged adult stage, and high viral abundance persisted after

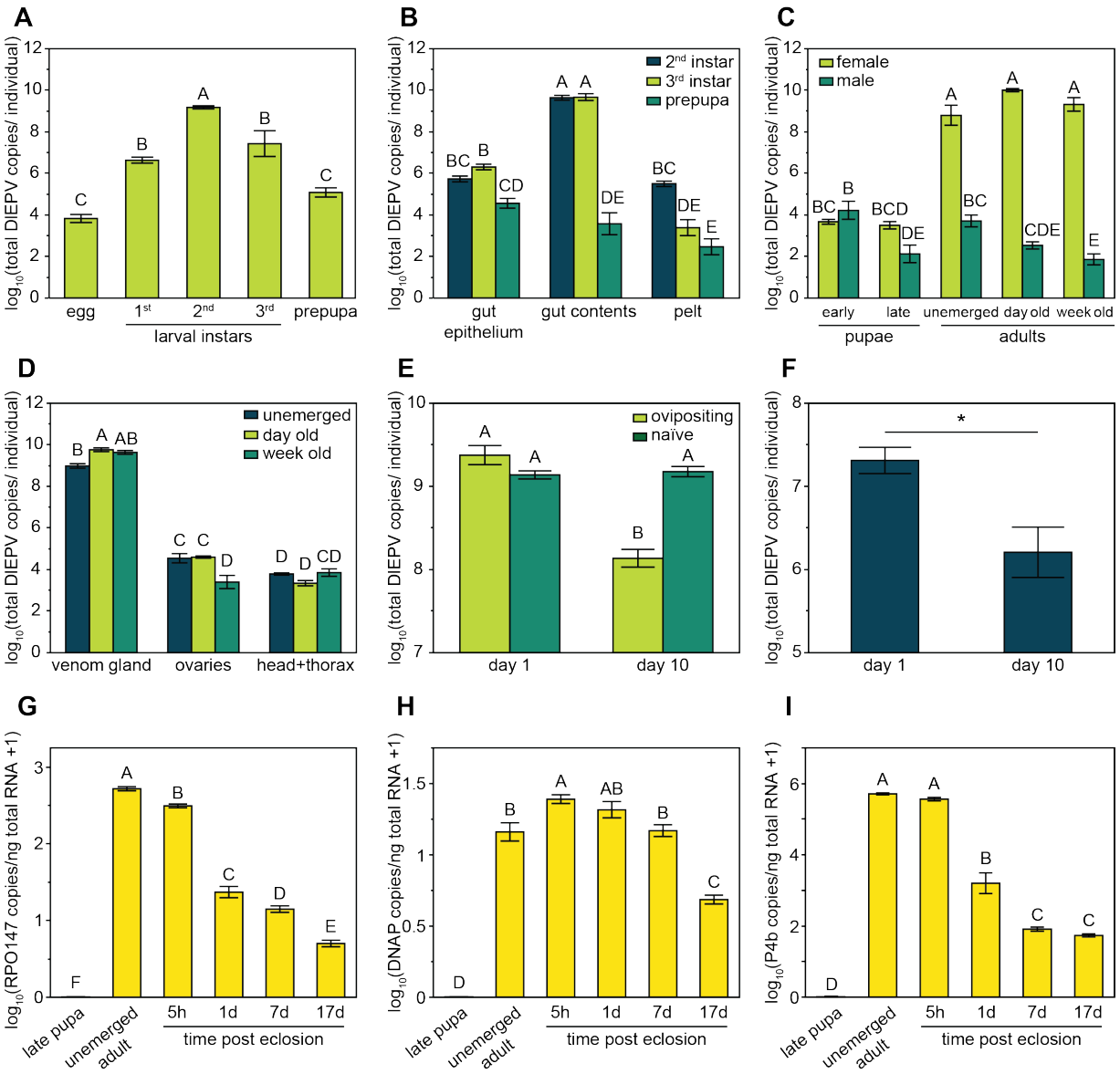

Figure 2.1. DlEPV replication and transcriptional activity within *D. longicaudata* wasps. DlEPV abundance was estimated via qPCR in egg and larval wasp stages (A), larval wasp gut tissues (B), male and female pupal through adult wasp stages (C), and adult female reproductive tissues (D). Eggs, first-instar, and second-instar larvae required the pooling of 10, 6, and 3 specimens per biological replicate, respectively. Venom glands and ovaries from adult females were also pooled in triplicates for each biological replicate. One-week-old female wasps were given either daily oviposition opportunities for 10 consecutive days (ovipositing) or no oviposition opportunities (naive). (E) DlEPV copy number per wasp venom gland (replicates pooled in triplicates) at day 1 and day 10. (F) DlEPV introduced per fly larva by an ovipositing wasp at day 1 and day 10. For graphs in panels A to F, each bar represents the $\log_{10}$-transformed absolute DlEPV genome copy number per individual averaged from 6 biological replicates. Expression of DlEPV genes

measured with RT-qPCR in female wasp venom glands from late pupa to 17-day-old adult. Profiled DlEPV genes include the 147-kDa RNA polymerase subunit RPO147 (G), the DNA polymerase DNAP (H), and the structural protein P4b (I). Each mean copy number bar in panels G to I represents the $\log_{10}$-transformed mean cDNA copy number per nanogram total RNA averaged from 6 biological replicates. Six venom glands from late pupa, 7-day-old, and 17-day-old wasps were pooled for each replicate in panels G to I, while 3 venom glands were pooled for unemerged, 5-h-old, and 1-day-old replicates. Error bars in all graphs represent one standard error above and below the mean. The uppercase letter(s) above each bar indicates statistically distinct mean values from Tukey's HSD tests, and each bar in a graph that includes multiple main effects (B to E) was analyzed independently of either effect. Statistical significance of the *t* test in panel F is indicated: *, $P < 0.01$.

emergence (eclosion) and into adulthood (female life stage effect: $F_{4,25} = 131.47$, $P < 0.0001$) (Figure 2.1C). Male wasps also showed a significant fluctuation in viral copy number throughout pupal development and adulthood (male life stage effect: $F_{4,25} = 9.67$, $P < 0.0001$), although the severity of this effect (<3 orders of magnitude difference) was lower than that of female wasps, and the biological significance of this variation is unclear (Figure 2.1C). To identify where DlEPV is localized when most abundant in female wasps, we measured the amount of DlEPV within reproductive tissues, as well as head plus thorax tissues of female adults. The interaction effect of life stage and tissue type was significant ($F_{4,45} = 12.53$, $P < 0.0001$) (Figure 2.1D). The majority of DlEPV copies in unemerged, newly emerged (day-old), and week-old female wasps was located within the venom gland (unemerged tissue effect: $F_{2,15} = 381.94$, $P < 0.0001$; day-old tissue effect: $F_{2,15} = 1184.59$, $P < 0.0001$; week-old tissue effect: $F_{2,15} = 263.12$, $P < 0.0001$), which is consistent with previous qualitative reports [18], while a small amount of virus was also observed in the ovaries and head plus thorax (Figure 2.1D).

As adults, female *D. longicaudata* require approximately 1 week to reach reproductive maturity and mate before they begin the process of oviposition [29]. Patterns of DlEPV abundance

associated with female wasp oviposition behavior were examined by comparing the viral copy number in wasps that were allowed to oviposit (ovipositing wasps) to that in wasps that were given no oviposition opportunities (naive wasps). Starting 1 week after eclosion, we gave ovipositing wasps daily opportunities for oviposition with *A. suspensa* larvae for 10 consecutive days and measured starting and ending venom gland viral loads. We observed a significant interaction between day and treatment effects ($F_{1,20} = 53.33$, $P < 0.0001$) (Figure 2.1E). DlEPV genome copy number dropped by 94.6% within the venom glands of ovipositing wasps from day 1 to day 10 (ovipositing day effect: $F_{1,10} = 62.59$, $P < 0.0001$), while the amount of virus in the venom glands of naive wasps showed no significant change over the same time frame (naive day effect: $F_{1,10} = 0.26$, $P = 0.62$) (Figure 2.1E). Furthermore, the average amount of DlEPV injected into a fly larva by an ovipositing female decreased from day 1 to day 10 in parallel with the drop in viral load (Figure 2.1F). These results signify that female wasps become depleted of DlEPV after repeated oviposition and suggest that DlEPV replication either cannot match the rate of depletion or may not persist into adulthood.

We next estimated the expression of 3 DlEPV genes in the venom glands of female wasps using reverse transcription-qPCR (RT-qPCR) to corroborate the virus replication pattern indicated by our DlEPV abundance measurements. We chose the 147-kDa RNA polymerase subunit (RPO147), the DNA polymerase (DNAP), and the virion structural component P4b genes, because they are representative of the transcription, replication, and morphogenesis functions of poxviruses, respectively [30]. Viral gene expression was barely detected in the venom glands of late pupal stage wasps but was initiated rapidly after the final molt, including an average 3.19-fold change from late pupa to the unemerged adult stage (Figure 2.1G to I). After female wasp eclosion, peak levels of the detected viral mRNAs were reduced an average 94.3% by 17 days posteclosion

(RPO147 $F_{5,30}$ = 637.17, P < 0.0001; DNAP $F_{5,30}$ = 150.67, P < 0.0001; P4b $F_{5,30}$ = 329.93, P < 0.0001) (Figure 2.1G to I). These expression data confirm that high-level viral replication is not continuous and demonstrate that female wasps have a finite amount of DlEPV to utilize throughout their lifetime.

Virus replication also occurs in parasitized hosts and primarily infects host hemocytes. DlEPV abundance was measured within *A. suspensa* flies that had been parasitized by *D. longicaudata* to determine whether virus replication also occurs in host insects. During oviposition by a naive female wasp, approximately 1×10^7 DlEPV genome copies were injected into each fly larva (Figure 2.2A). DlEPV genome copy number began to rise in whole flies soon after oviposition and steadily grew to $>1 \times 10^{10}$ copies throughout parasitism by the wasp ($F_{4,25}$ = 31.58, P < 0.0001) (Figure 2.2A). We then dissected fly larvae 4 hpp to investigate which tissue(s) DlEPV virions initially infect within the host. We found that >98% of total DlEPV genome copies were localized to host hemocytes at 4 hpp, while the fat body accounted for only 1.15% of viral copies, and <1% was observed within the gut, brain, and salivary gland tissues combined ($F_{4,25}$ = 102.25, P < 0.0001) (Figure 2.2B). These data are consistent with the localization of many pathogenic entomopoxviruses (EPVs) within the hemocytes of infected hosts [31] and demonstrate that DlEPV introduced during oviposition contains the ability to replicate its DNA within parasitized flies. In this regard, DlEPV represents a major exception to the characteristic absence of virus replication in hosts that is observed for parasitoid EVEs.

Microinjection of venom gland-derived DlEPV inhibits fly eclosion. Although DlEPV has been shown to infect *A. suspensa* hemocytes [18,22] (Figure 2.2B), the antagonistic impact of this virus within fly hosts has not yet been directly demonstrated. We therefore investigated the effects

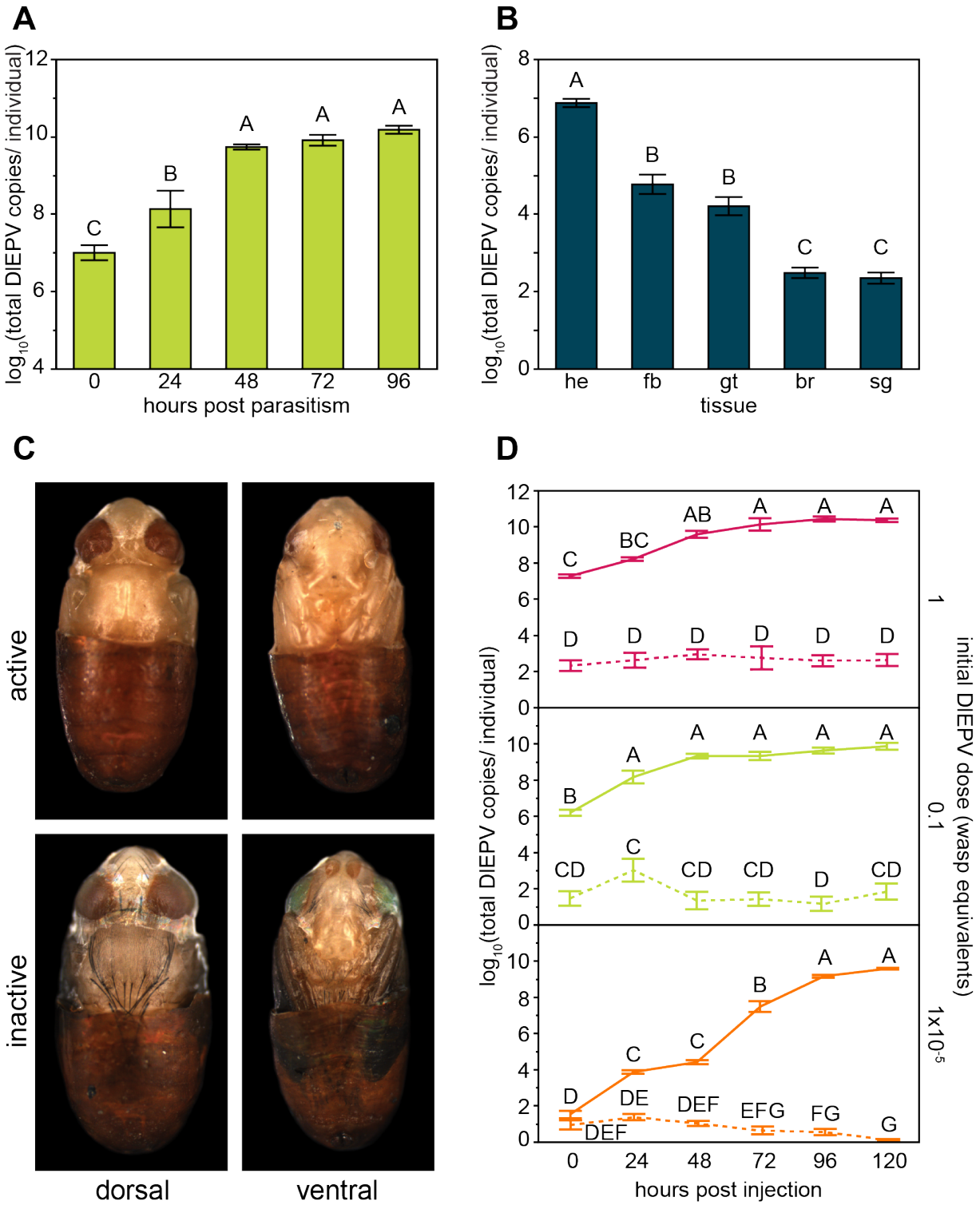

Figure 2.2. DlEPV replication and phenotypic effects within *A. suspensa* flies. DlEPV abundance was estimated with qPCR in parasitized fly tissue after oviposition by 1-week-old naive wasps. Absolute DlEPV copy number within whole flies throughout parasitism (A) and larval fly tissues at 4 hpp (B), including hemocytes (he), fat body (fb), gut (gt), brain (br), and salivary gland (sg).

Brain and salivary gland tissues were pooled in triplicates per biological replicate. (C) Images showing the cessation of fly pupal development at 12 days postinjection (dpi) in flies injected with 1 oviposition equivalent of active DlEPV compared to the normal progression of development observed in flies injected with the same dose of UV-inactivated DlEPV. Background debris from dissections in images was removed using Adobe Photoshop CC 2019. (D) qPCR estimation of DlEPV abundance in flies injected with one of three doses of either active or UV-inactivated DlEPV: (top) 1 oviposition equivalent, (middle) 0.1 oviposition equivalents, (bottom) 1×10^{-5} oviposition equivalents. Solid lines in each graph of panel D indicate viral copy number in flies injected with active DlEPV, while dashed lines indicate viral copy number in flies injected with UV-inactivated DlEPV. Mean copy numbers, error bars, and statistical significance are indicated as defined in the legend for Figure 2.1.

of DlEPV infection on *A. suspensa* by injecting several doses of purified venom gland-derived virus into nonparasitized third-instar fly larvae. The effect on flies when injected with 1 naive wasp's oviposition equivalent (1×10^7 viral genome copies) of unaltered (active) DlEPV was an absolute failure to emerge as adults (0/126 adult flies). Additionally, injection of 0.1 oviposition equivalents (1×10^6 copies) also resulted in 0% adult fly emergence (0/100 flies), and injection of 1×10^{-5} oviposition equivalents (1×10^2 copies) resulted in only 2% emergence (4/208 flies). In contrast, flies injected with UV-inactivated (inactive) virus at the same 3 initial doses had an average of 91% emergence (1 oviposition equivalent, 105/112 adult flies; 0.1 oviposition equivalents, 68/80 flies; 1×10^{-5} oviposition equivalents, 130/136 flies). Flies injected with either active or inactive DlEPV successfully completed pupation and remained alive throughout the pupal developmental period, but dissection of puparia at the end of the pupal stage revealed that flies injected with active DlEPV failed to complete development (Figure 2.2C). These pupae showed a lack of several adult morphological features compared to their control counterparts, such as complete eye pigmentation, darkened wing coloration, and dense setae on the head and thorax (Figure 2.2C).

We next measured the amount of virus within virus-injected flies to examine how virus replication corresponds to these emergence data. At all 3 doses, we observed a significant interaction between treatment and time effects (1 oviposition equivalent interaction effect: $F_{5,60}$ = 7.52, $P < 0.0001$; 0.1 oviposition equivalent interaction effect: $F_{5,60} = 10.41$, $P < 0.0001$; 1×10^{-5} oviposition equivalent interaction effect: $F_{5,60} = 222.02$, $P < 0.0001$) (Figure 2.2D). Whereas limited DlEPV qPCR amplification and no virus replication occurred within flies injected with inactive virus due to viral DNA cross-linking, all doses of active DlEPV resulted in rapid virus amplification by 120 h postinjection (hpi) (1 oviposition equivalent active time effect: $F_{5,30}$ = 50.86, $P < 0.0001$; 0.1 oviposition equivalents active time effect: $F_{5,30} = 40.18$, $P < 0.0001$; 1×10^{-5} oviposition equivalents active time effect: $F_{5,30} = 62.50$, $P < 0.0001$) (Figure 2.2D). Furthermore, the average viral load of flies injected with the lowest initial dose of 1×10^{-5} oviposition equivalents was within 1 order of magnitude of those injected with higher initial doses by 120 hpi (Figure 2.2D). Our collective findings therefore demonstrate that DlEPV infection and replication are responsible for high mortality of *A. suspensa* flies associated with arrested development.

RNAi successfully knocks down DlEPV gene expression and diminishes viral abundance in wasps. Given the high virulence of DlEPV observed within flies, we sought to empirically test whether DlEPV provides a fitness benefit to *D. longicaudata* wasps by comparing the parasitism success of wasps with virus to those that are virus deprived. We used RNA interference (RNAi) to target the same 3 DlEPV genes used for RT-qPCR simultaneously in order to clear female wasps of virus. Injection of this DlEPV-specific double-stranded RNA (dsRNA) cocktail into early female wasp pupae successfully knocked down target viral gene expression in the adult venom gland by an average 2.9 orders of magnitude compared to that in control wasps injected with

dsRNA targeting the unrelated *egfp* gene (Figure 2.3A to C). Furthermore, DlEPV genome copy number was reduced by >99.99% in DlEPV dsRNA-treated wasps, indicating that the knockdown of RPO147, DNAP, and P4b gene expression deprives wasps of the vast majority of virus that is normally present (Figure 2.3D and E).

We then allowed dsRNA-treated wasps to oviposit within fly larvae in order to quantify whether any remaining virus within DlEPV-deprived wasps was transferred to fly hosts and, if so, how much virus replication occurred throughout parasitism by the progeny of dsRNA-treated wasps. A significant interaction between time and treatment effects was observed ($F_{5,60}$ = 13.01, P < 0.0001) (Figure 2.3F). Approximately 1×10^2 copies were introduced during oviposition by DlEPV-deprived wasps, although viral copy number fell to <10 copies on average by 24 hpp. Some amount of virus replication then proceeded and peaked at 96 hpp, with a maximum viral abundance of approximately 1×10^3 copies (ds-viral mix time effect: $F_{5,30}$ = 9.99, P < 0.0001) (Figure 2.3F). While this level of virus replication was largely reduced in comparison to that provided by control wasps (ds-*egfp* time effect: $F_{5,30}$= 40.56, P < 0.0001), our data on DlEPV virulence described above argue that a modest amount of viral activity could be sufficient for the potential benefit that is provided by the virus to the developing wasp. These dsRNA-treated wasps would therefore be inappropriate for use in fitness comparisons between virus-free and control wasps.

Parental RNAi effect further deprives second-generation female wasps of DlEPV. As an alternative, we examined the daughters of DlEPV-deprived wasps for a possible parental RNAi effect. Viral gene expression in the venom gland of second-generation DlEPV-deprived adult female wasps was effectively absent (Figure 2.4A to C) and, therefore, suppressed to a greater extent than the parental generation that directly received the ds-viral mix treatment. Additionally, viral copy number in second-generation DlEPV-deprived wasps was approximately 2 orders of

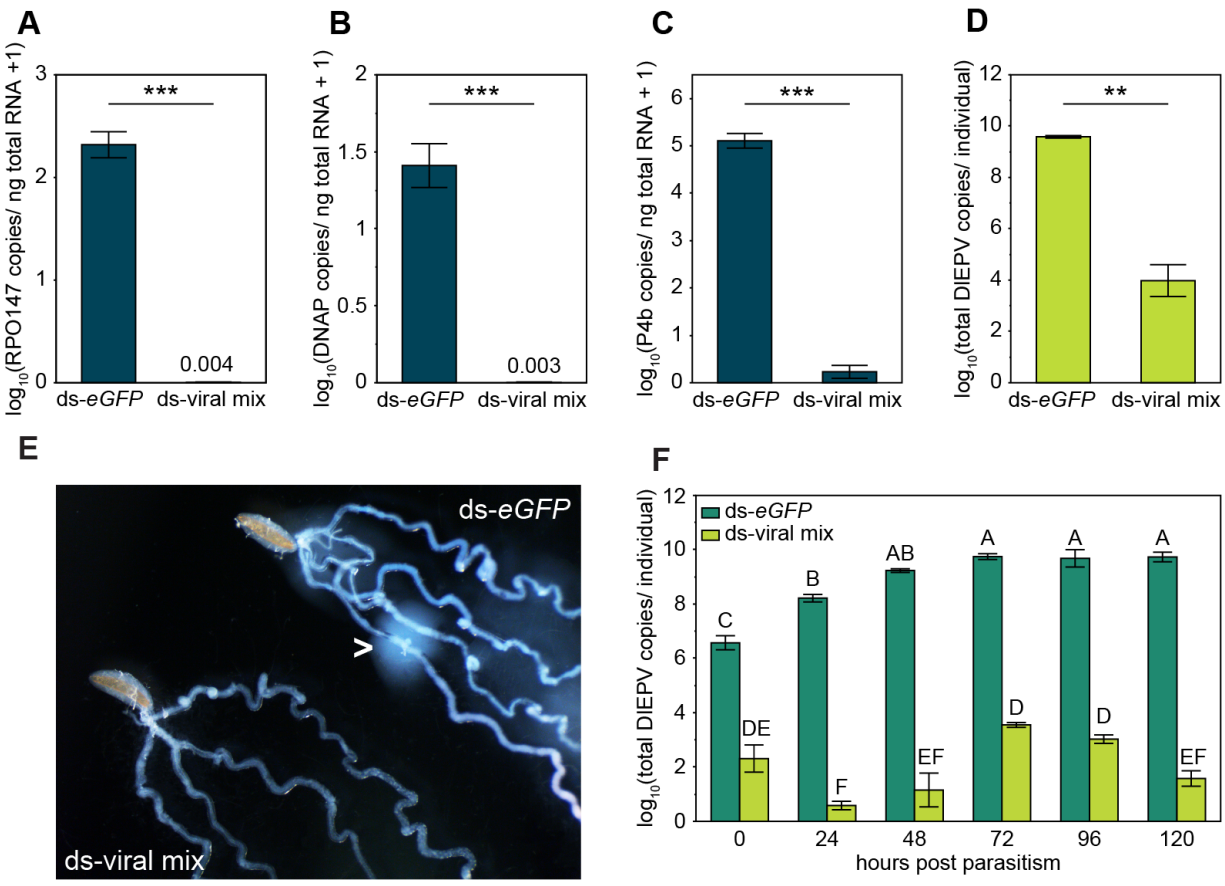

Figure 2.3. RNAi knockdown of three DlEPV genes reduces viral abundance within both *D. longicaudata* wasps and in *A. suspensa* fly hosts during parasitism. RT-qPCR estimation of viral gene expression after early female wasp pupae were injected with a double-stranded RNA (dsRNA) cocktail specific for RPO147 (A), DNAP (B), and P4b (C) genes (ds-viral mix). Control pupae were injected with ds-*egfp*. Venom glands were dissected from newly emerged adult wasps for total RNA isolation. Venom glands were combined in triplicates for each ds-*egfp* biological replicate, and 6 venom glands were combined for each ds-viral mix biological replicate. Each bar in graphs in panels A to C represents the $\log_{10}$-transformed mean copy number for the target gene per nanogram total RNA calculated from 6 biological replicates. Numerical labels above bars in panels A and B represent minute mean copy number values. (D) qPCR-estimated viral abundance of dsRNA-treated wasps (3 venom glands were pooled per ds-*egfp* replicate, 6 venom glands per ds-viral mix replicate). (E) Light microscope image showing the natural blue fluorescence caused by DlEPV particles in the venom gland of a control (ds-*egfp*) wasp compared to a lack of blue fluorescence in the venom gland of a virus-deprived (ds-viral mix) wasp. Arrowhead indicates DlEPV-containing venom that has leaked out of a ruptured accessory tubule. Background dissection debris in the image was removed using Adobe Photoshop CC 2019. (F) qPCR-estimated viral abundance in flies following oviposition by dsRNA-treated wasps. Mean copy numbers in graphs in panels D and F are as defined in the legend for Figure 2.1. Statistical significance of *t* tests in panels A to D is indicated: **, $P < 0.001$; ***, $P < 0.0001$. Statistical significance for the

uppercase letters above bars in panel F are as defined in the legend for Figure 2.1. Error bars in each graph represent one standard error above and below the mean.

magnitude further reduced compared to that in the parental generation, with each venom gland containing an average of 100 viral genome copies (Figure 2.4D). When these second-generation DlEPV-deprived females were allowed to oviposit within fly larvae, a significant interaction between time and treatment effects was again observed ($F_{5,60} = 11.64$, $P < 0.0001$) (Figure 2.4E). Almost no DlEPV was introduced and no viral replication occurred in flies during parasitism (second-generation ds-viral mix time effect: $F_{5,30} = 0.85$, $P = 0.53$) (Figure 2.4E). These data indicate that transgenerational effects of parental RNAi have resulted in the effective elimination of DlEPV from female *D. longicaudata* wasps and that second-generation dsRNA-treated wasps can be utilized to determine the symbiotic role of DlEPV in this system.

Second-generation DlEPV-deprived females have significantly reduced parasitism success. The fitness of second-generation dsRNA-treated wasps was estimated through parasitism success assays, in which the percentage of wasp progeny that survived to adulthood for DlEPV-deprived and control treatments was measured. We first offered third-instar fly larvae to second-generation wasps for these assays, which was the larval stage used for oviposition in other analyses of viral activity within flies. A significant reduction of wasp emergence was observed for DlEPV-deprived wasp progeny compared to those of control wasps, which demonstrates that DlEPV provides a fitness benefit to wasps (Figure 2.5A). In addition, fly emergence was rare when parasitized by control wasps but significantly improved when parasitized by DlEPV-deprived wasps, further supporting the virulence role of the virus within flies (Figure 2.5B). Surprisingly, high proportions (>60%) of “no emergence” were observed in both treatments of these assays

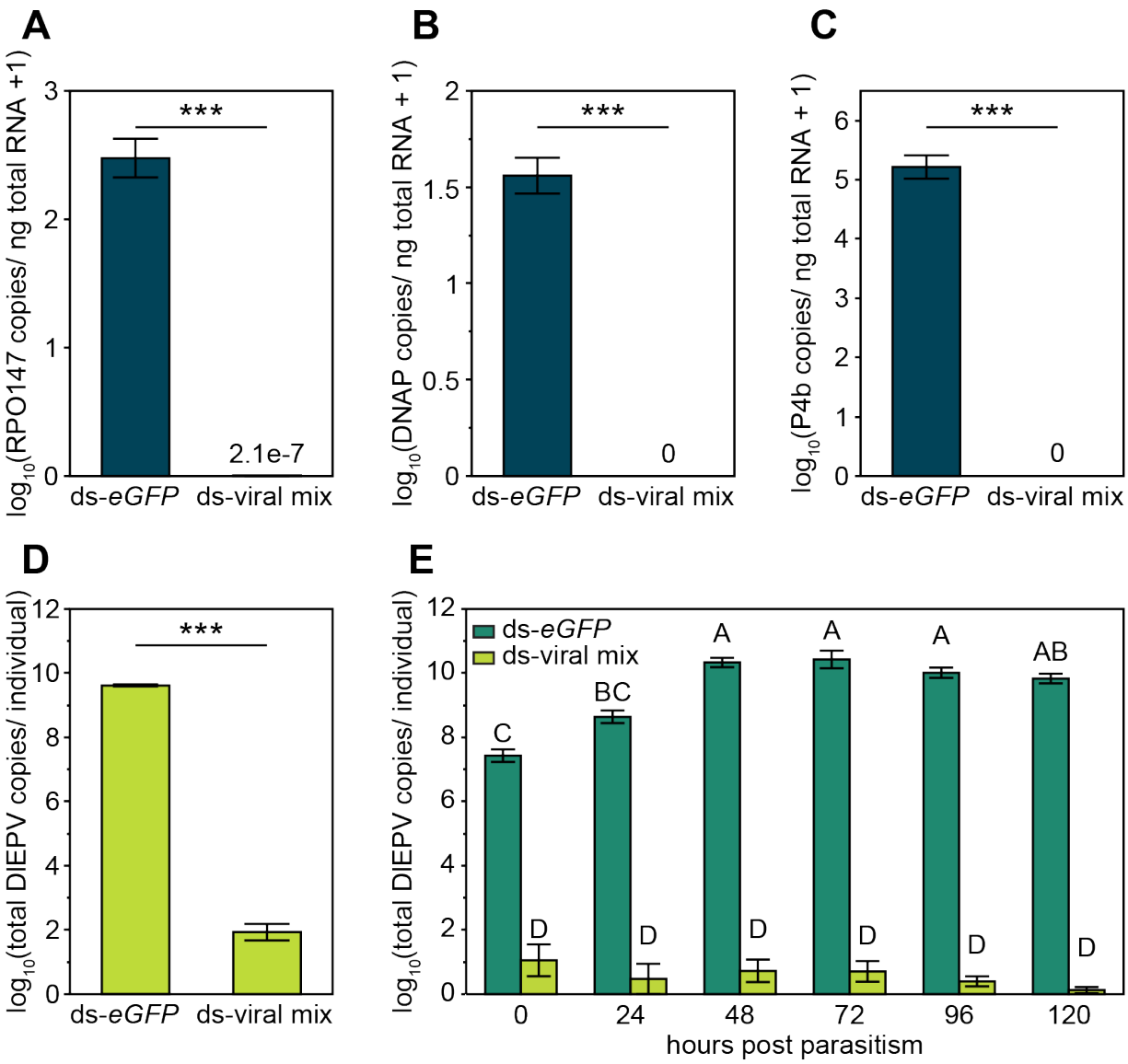

Figure 2.4. RNAi knockdown phenotype persists in second-generation wasps. (A to C) RT-qPCR estimation of viral gene expression in female progeny of dsRNA-treated wasps. Venom glands were sampled from newly emerged daughters of control (ds-*egfp*) and virus-deprived (ds-viral mix) wasps as described in the legend for Figure 2.3. Numerical labels above bars in panels A to C represent minute or nonexistent mean copy number values. (D) qPCR estimation of DlEPV abundance in second-generation female wasps. Samples were prepared as described in the legend for Figure 2.3 (E) Viral abundance in flies after oviposition by second-generation dsRNA-treated wasps. Mean copy numbers, error bars, and statistical significance for all graphs are as indicated in the legend for Figure 2.3.

(Figure 2.5C), often due to dual mortality of both developing wasp and fly. Although our laboratory colony of *D. longicaudata* is maintained by exposing third-instar flies to wasps for oviposition, this late stage of larval fly development does not appear to be ideal for downstream wasp survival in these assays. However, *D. longicaudata* is known to oviposit within both second- and third-instar stages of *A. suspensa* [32].

We therefore conducted additional emergence assays that allowed wasps to oviposit within younger, second-instar fly larvae (Figure 2.5D to F). In these modified assays, an average 63% of wasp progeny emerged as adults from flies parasitized by *egfp* second-generation female wasps, while only 2% of wasp progeny survived to adulthood from flies parasitized by DlEPV-deprived second-generation wasps (Figure 2.5D). These data expand upon the wasp emergence pattern from the third-instar fly assays by showing a severe fitness cost associated with wasps that lack DlEPV. Fly emergence rates in both treatments were similar to those observed in the first set of assays (Figure 2.5E), suggesting that fly life stage at the time of oviposition does not affect fly emergence patterns in the presence and absence of DlEPV. However, "no emergence" rates were significantly lower in control wasp assays than in DlEPV-deprived assays (Figure 2.5F), which differs from the overall high "no emergence" rates in assays using older fly larvae. This increased dual mortality in DlEPV-deprived assays is therefore likely due to increased rates of wasp death in the absence of DlEPV. Collectively, these emergence assay results provide strong evidence that virus activity is beneficial to wasp survival and that DlEPV is thus a mutualist of *D. longicaudata* wasps.

2.5 DISCUSSION

Parasitoid wasps are one of the few taxonomic groups for which numerous instances of heritable virus associations have been observed [33], offering a unique opportunity to build a

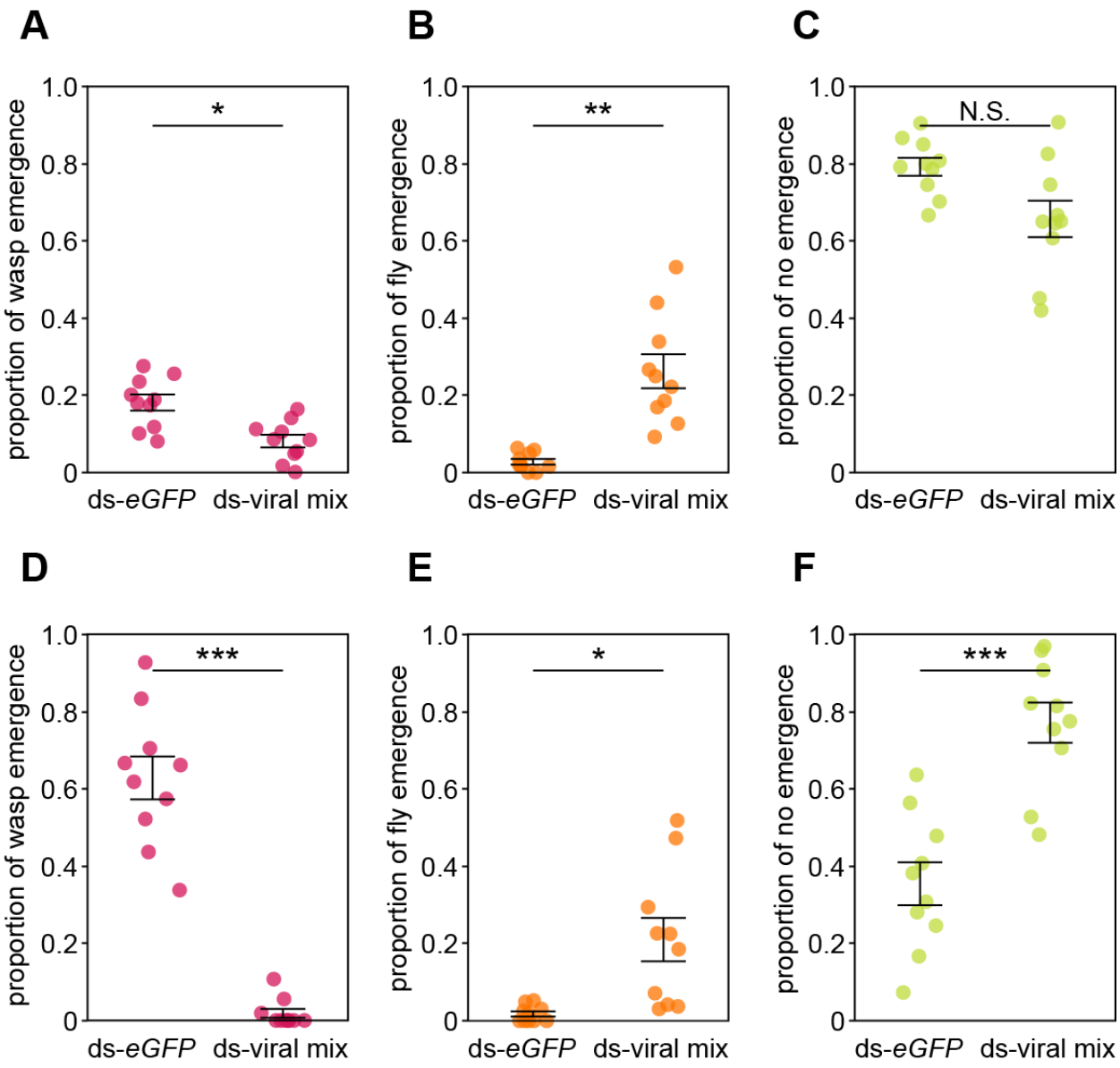

Figure 2.5. Progeny of second-generation dsRNA-treated wasps without accompanying virus show heavily reduced parasitism success. Proportional emergence rates of wasps (A and D), flies (B and E), or no emergence (C and F) after oviposition by second-generation dsRNA-treated wasps. Flies were offered to wasps for oviposition as either third-instar larvae (A to C) or second-instar larvae (D to F). Each dot represents a single trial, in which second-generation wasps were allowed to oviposit within fly larvae, and the emergence fate of singly parasitized flies was recorded. A total of 10 replicate trials were conducted per treatment for all assays. An average of 53 singly parasitized flies were recorded for each trial in panels A to C and an average of 57 flies were recorded per trial in panels D to F. Trials in which <40 singly parasitized flies were found were omitted from analysis. Statistical significance of *t* tests is as indicated in the legend for Figure 2.3.

comparative framework for understanding the evolution of beneficial viruses. However, characterization of these systems has been primarily focused on PDVs, and in turn, the present consensus on parasitoid viruses or EVEs is heavily skewed toward insights from PDV associations [12]. The lack of comparative data from lineages of diverse viral ancestry led us to investigate DlEPV in order to gain insight on alternative mechanisms through which mutualistic viruses may arise. The aim of this work was to establish a foundation for the DlEPV system that would allow us to draw thorough comparisons to PDVs and other parasitoid EVEs in order to identify both common characteristics and key differences between these systems. Our combined results have uncovered several important features of the DlEPV system that unite this virus with parasitoid EVEs in serving an analogous role during parasitism. We also observed distinctions with DlEPV that pose new questions regarding the mechanisms that maintain its relationship with *D. longicaudata*.

DlEPV is maternally inherited via transovarial transmission. Strict vertical transmission is a feature of many endosymbiotic relationships between insects and microbes, and the corresponding alignment of fitness experienced by both partners in these mergers helps to maintain symbiotic stability [34,35]. All currently identified EVEs in parasitoid wasps display stable vertical transmission from wasp mother to eggs due to independent ancestral acquisition events, in which a viral genome integrated into the germ line of a wasp [3]. Present day EVEs are therefore permanently incorporated within wasp genomes. In particular, PDVs represent ancient associations that have achieved extreme stability within parasitoid lineages, due in part to the faithful transmission of these EVEs through the wasp germ line [16]. qPCR detection of DlEPV genome copies within laid *D. longicaudata* eggs and adult wasp ovaries supports a transovarial

form of vertical transmission. While these results suggest DlEPV shares a similar transmission strategy with parasitoid EVEs, we have not yet ruled out other possible routes of vertical and/or horizontal transmission utilized by DlEPV. A second mode of vertical transmission among wasps could involve the transfer of virions injected into a host by a wasp mother to her progeny via the ingestion and sequestration of virions by the developing wasp larva. This form of oral transmission, in which offspring consume microbial symbionts that the mother has deposited nearby, has been observed in the obligate bacterial symbiont of the tsetse fly, *Wigglesworthia glossinidia*. These bacteria are not transmitted through the germ line but are secreted within the fly mother's milk, ingested by the intrauterine larva, and migrate through the gut epithelium to the symbiont-housing organ (bacteriome) and milk gland of the fly progeny [36,37]. Therefore, DlEPV virions may similarly migrate through the wasp gut following ingestion of fly tissue and colonize the venom gland before eclosion. Horizontal transmission of DlEPV between *D. longicaudata* individuals may also be possible using the above-described route. Superparasitism, or parasitism of one host by multiple wasps of the same species, is common in both laboratory and field populations of *D. longicaudata* [38,39]. A fly host that is superparasitized by *D. longicaudata* could potentially be infected with a mixture of DlEPV strains that could then be exchanged by unrelated wasps feeding within the same host. Additional modes of transmission such as these have not been assessed in this system, but our results here suggest that transovarial transmission is a major, if not the sole, mode of DlEPV transmission within *D. longicaudata* wasps. The prevalence of transovarial transmission among parasitoid viruses or EVEs, regardless of viral origin, further signifies maternal inheritance as a major stabilizing force within persistent parasitoid-virus associations.

DlEPV successfully replicates in both wasps and flies. PDV gene expression and, consequently, virus replication begin during late pupal wasp development, occur specifically within the calyx region of the ovaries, and are continuous throughout adulthood [40]. Moreover, the stage and tissue specificity of VcENV and FaENV viral gene expression and VLP production strongly resembles that of PDVs [10,11]. Initial work on the DlEPV system demonstrated the presence of virions in both wasp and parasitized fly tissues but did not adequately investigate whether the virus could replicate in either insect species [18]. Our results thus provide unprecedented resolution into DlEPV replication strategies. In wasps, our data show that DlEPV replication initiates at the end of pupal development in the female venom gland but tapers off soon after eclosion, at which point wasps appear to have a finite amount of virus to deposit into hosts at oviposition. This is supported by the rapid rise in viral genome copy number in adult female wasps that is largely concentrated in venom gland tissue as well as the depletion of venom gland viral load after repeated oviposition opportunities. Our RT-qPCR data of DlEPV expression in wasp venom glands also support this interpretation by demonstrating a surge of viral gene expression that begins in unemerged adult wasps and diminishes drastically after eclosion. Taken together, our data reveal broad similarities in the developmental coordination of DlEPV and EVE replication within wasps, including replication initiation during the pupal stage and virus localization within female reproduction-associated tissues. Subtle distinctions regarding the tissue specificity and discontinuity of high-level virus replication, however, indicate that DlEPV is likely regulated through different mechanisms than parasitoid EVEs while within wasp tissue. Nevertheless, the restriction of virion or VLP production within all of these examples, including DlEPV, illustrates the importance of controlled viral activity in these long-term relationships.

Due to viral gene losses and rearrangements within wasp genomes, virus replication genes are not included within encapsidated PDV genomes, and PDVs therefore cannot replicate within the caterpillar hosts of their associated wasps [41]. Instead, PDV virions infect host hemocytes and express virulence genes that allow wasps to mitigate host immune defenses and feed from host tissue [9]. The replication deficiency of PDV virions maintains their dependency on wasps for transmission and replication, creating a reliable coexistence between wasp and virus [42]. The similar genomic architectures of other parasitoid EVEs produce VLPs that contain virulence proteins but no viral genome, and so replication in host tissue is also not possible in these cases [10,11]. This recurrent adaptation in parasitoid EVE systems implies that restriction of virus replication to wasp tissue is important for the persistence of these associations. Therefore, the most striking difference between DlEPV and parasitoid EVEs uncovered by our qPCR analysis was that DlEPV replicates in fly tissue throughout parasitism in addition to its replication in wasps. The detection of the highest levels of DlEPV DNA in host hemocytes at 4 hpp is congruent with the hemocytic localization of other EPVs, as well as PDVs. However, the steady rise in DlEPV genome copy number within whole fly tissue from 0 to 96 hpp is not consistent with PDV biology, in which no virus replication occurs inside host tissue [41]. This finding was recapitulated by our qPCR data of microinjected DlEPV within nonparasitized fly hosts, in which a similar trajectory of viral genome copy number increase from 0 to 120 hpi was observed. In contrast to the dependency of EVEs on associated wasps for survival, the ability of DlEPV to amplify its DNA within fly hosts suggests that it is not dependent on *D. longicaudata* wasps for virus replication. Although the infectivity of fly-propagated DlEPV virions remains untested, our results here suggest that viruses do not necessarily have to be “domesticated” by parasitoid wasps to serve a valuable function.

DlEPV is highly virulent within fly hosts. Suppression of host immunity and manipulation of host developmental processes are two conserved strategies of the products that parasitoids introduce into hosts, which serve to bolster wasp survival and maximize host nutrient availability, respectively [43]. PDVs have been shown to cause both immunosuppression and developmental arrest in the hosts of their associated wasps [44]. For example, injection of PDVs isolated from various parasitoid species into nonparasitized caterpillar hosts has repeatedly resulted in arrested host development with symptoms that include prolonged larval development, failed pupation, and eventual mortality [8]. Knowledge on the effects of DlEPV within *A. suspensa* hosts has been limited to previous work that described the presence of virions within host hemocytes and the detrimental effects of *D. longicaudata* parasitism, in general, on host hemocyte function [18,22]. However, multiple factors introduced into the host insect during parasitism, such as maternally derived venom proteins or secretions from the developing wasp, could affect host physiology and do so in other parasitoid systems [43]. Our results thus directly establish that DlEPV is virulent within fly hosts by showing that microinjection of purified virus caused an overwhelming failure of flies to emerge as adults. Additionally, the mortality of DlEPV-infected flies was associated with phenotypic alterations that were indicative of developmental arrest during the pupal stage. Parasitism by *D. longicaudata* was previously shown to cause an elevation in juvenile hormone (JH) titer in *A. suspensa* flies, a hormone that is normally depleted at the end of larval development to initiate the larval-pupal molt [45]. In addition, EPV infection often elevates JH and slows development in host insects [46–48]. Furthermore, the PDVs of *Microplitis demolitor* wasps are responsible for raised JH titers, causing stunted growth and development of caterpillar hosts [49,50]. These findings combined with our data indicate that DlEPV infection may contribute to high JH titers as a mode of action that prevents parasitized flies from completing normal

development. The disruption of *A. suspensa* hemocyte function shown by Lawrence [22] and the disruption of *A. suspensa* development shown here suggest that the two conserved strategies of parasitoid products mentioned above are also employed by *D. longicaudata* and could be credited, in part, to the gene products of DlEPV.

DlEPV is beneficial to *D. longicaudata* wasps. PDVs were shown to be obligate entities for associated wasps through experiments that measured wasp survival when eggs were dissected from parasitized hosts, washed of any external virions, and injected into nonparasitized hosts with or without purified virus [51]. All developing wasps that were not accompanied by PDV particles failed to survive to adulthood, which demonstrated that the virus is critical for wasp development [51]. While our data strongly support the virulent nature of DlEPV within *A. suspensa* flies, a persistent DlEPV infection does not appear to be detrimental to *D. longicaudata* wasps throughout their life cycle. However, previous studies have failed to provide direct evidence that the virus is advantageous for wasp fitness. We therefore explored methods to rid female *D. longicaudata* wasps of their viral load to test whether DlEPV is important for the survival of this wasp species. RNAi technology was previously used to successfully knockdown PDV gene expression in *M. demolitor* wasps [52]. We utilized similar methods here but targeted 3 DlEPV genes at once to achieve an all-encompassing suppression of viral gene expression with the goal of completely obstructing virus activity.

Our results in this study represent a novel use of RNAi to effectively eliminate an insect's microbial symbiont population. We achieved a remarkable knockdown efficiency that rendered DlEPV largely incapable of replication within the wasp venom gland. Furthermore, the knockdown effect was augmented in the next generation of female wasps. When second-generation dsRNA-treated wasps were allowed to oviposit within fly larvae, we observed a

significant drop in parasitism success for wasps that were devoid of virus. This decrease was modest when third-instar fly larvae were offered to wasps, likely due to the high overall mortality rate that was present in these assays. We reasoned that assays involving third-instar flies are less likely to reveal a clear pattern of parasitism success between wasps with and without DlEPV, because flies at this stage are preparing for the next phase of development that entails major tissue remodeling. In addition, third-instar fly larvae normally crawl out of the diet substrate to pupate in the soil [53], and so we would not necessarily expect to find fly larvae at this late stage in the fruit that *D. longicaudata* seek out for oviposition in nature. Previous work has also indicated that *D. longicaudata* survive at highest rates when oviposited within *A. suspensa* hosts well before the end of larval development [32]. When we presented wasps with younger fly larvae, we noticed a more drastic drop in parasitism success associated with a lack of DlEPV. These cumulative parasitism assay results directly demonstrate that DlEPV is beneficial to *D. longicaudata* survival. DlEPV therefore shares with PDVs this fundamental role in successful parasitism.

Summary. This investigation has revealed several features shared by DlEPV and parasitoid EVEs that are likely due to the similar overall contributions of these viruses throughout the wasp life cycle. These commonalities include (i) vertical transmission of virus to wasp offspring, (ii) confinement of virus replication within wasps to a specific sex, developmental stage, and tissue, and (iii) reliance on virus activity for successful wasp development within the host. However, we also found evidence that suggests DlEPV represents a more autonomous entity than EVEs, including the ability of DlEPV to replicate within host flies. This and other differences described here may be products of the contrasting viral ancestry between DlEPV and parasitoid EVEs. For example, nudiviruses and poxviruses have fundamentally different replication cycles: a nudivirus must invade the nucleus of an infected cell for successful replication, while a poxvirus replicates

within the cytoplasm of infected cells and does not require nuclear localization or genomic integration for virus propagation [54,55]. Intrinsic differences in biology such as this may affect how these viruses are acquired and maintained by parasitoid wasp lineages. Despite its unique origin, it is clear that DlEPV exhibits strong convergent evolution with parasitoid EVEs, and further study of this system will provide important insights into the evolutionary transition of viruses from pathogens into mutualists.

2.6 MATERIALS AND METHODS

Insect tissue collection. *D. longicaudata* wasps and *A. suspensa* flies were reared as previously described [21]. For the collection of developing wasps, we allowed adult wasps to oviposit within late-third-instar fly larvae and followed the wasp developmental stages as delineated by Paladino *et al.* [27]. Wasps were then dissected from within fly puparia in 1× phosphate-buffered saline (PBS) and subsequently washed in three successive PBS droplets. We controlled for initial quantities of DlEPV introduced during oviposition by using strictly 7-day-old, naive adult female wasps for parasitisms and by collecting wasp specimens from flies that had only been parasitized once (i.e., fly puparia that exhibited only one parasitism scar). As an exception, wasp eggs were oftentimes dissected from flies with multiple parasitism scars, with the reasoning that if eggs were washed in PBS thoroughly after dissection, superparasitism would not affect the amount of DlEPV present within unhatched eggs. Gut tissue dissections of second- and third-instar wasp larvae as well as prepupae entailed removal of the gut from the wasp larva, followed by tearing the gut epithelium open and washing in PBS to separate the gut contents from the epithelial tissue. Adult wasps were individually surface sterilized prior to collection by vortex mixing in 1 ml 3% bleach for 1 min, followed by three rounds of 1-min vortex mixing in 1 ml

water. For female adult reproductive tissue dissections, the head and thorax were first cut and removed from the abdomen. The venom gland and ovaries were then dissected from the abdomen and separately washed in PBS.

Investigation of DlEPV abundance associated with repeated oviposition by adult female wasps involved cages of mixed same-age male and female wasps that were designated either "ovipositing" or "naive." Beginning 7 days after eclosion, ovipositing cages were offered third-instar fly larvae for 2 h of collective oviposition every day, while females in naive cages were not given any oviposition opportunities. Female wasp venom glands were sampled from both ovipositing and naive cages at days 1 and 10 of this routine following that day's oviposition opportunity. Fly larvae that bore one parasitism scar following the 2-h oviposition period were also collected to determine the amount of virus injected by ovipositing wasps of both ages.

DlEPV replication in host flies during natural parasitism was measured using a similar parasitism protocol to that used for developing wasp tissue collection. Singly scarred third-instar flies resulting from oviposition by 7-day-old naive wasps were collected at 0 to 96 hpp. Flies that were not collected immediately following parasitism were kept under standard rearing conditions until the specified time point. The developing wasp was removed from each larval or pupal fly sample prior to collection via dissection in PBS. Acquisition of larval fly tissues at 4 hpp included collection of fly hemocytes, followed by dissection of the fat body, gut, brain, and salivary tissues. Hemocytes were obtained by creating a longitudinal cut spanning the entire dorsal side of the larva and washing the pelt in PBS to remove the hemolymph. Centrifugation of the hemolymph at 1,000 × g for 5 min was then performed to pellet and isolate the hemocytes from other hemolymph components. After removal of the hemolymph, the remaining tissues were dissected from the larval pelt in fresh PBS.

DNA isolation and qPCR estimation of viral abundance. DNA was isolated from each tissue sample above with a phenol-chloroform extraction method. Briefly, each sample was homogenized in 500 µl PBS, followed by viral lysis using 250 µg of proteinase K (Roche) and 2% Sarkosyl. Following 1 h of incubation at 62°C, each sample was treated with 4 µl RNase A (6.6 µg/µl) for 4 min and then subjected to phenol-chloroform extraction. DNA was precipitated with 0.3 M sodium acetate (pH 5.2), 25 µg glycogen, and 100% isopropanol. The DNA pellet was washed with 70% ethanol and then eluted in 30 µl water. qPCR primers were designed with Primer3 [56] (Table 1.1). We estimated DlEPV genome copy number using the DlEPV putative

Table 1.1. Primer sequences used in this study.

Primer Set	Forward Sequence (5' – 3')	Reverse Sequence (5' – 3')
PolyAPol qPCR	GCTCCAGTAAAACCGTTTCC	GGCTTTGGATCGTAAAACCA
RPO147 RT-qPCR	AACGATGCGTTGGTGATTTT	CAAGATGCCCAAAGATGGAC
DNAP RT-qPCR	AAAATTGGAATCGGGTGGAT	TTGCGAAAGTTGGTTGTGAG
P4b RT-qPCR	CGTGGGGAAACTGATATGCT	GGATTCCCCTCCAGTTTGTT
RPO147 RNAi	TAATACGACTCACTATAGGGTGG TGTTCACAAAGGCAAAA	TAATACGACTCACTATAGGGTGAGT GATCCAGCGTTACCA
DNAP RNAi	TAATACGACTCACTATAGGGGCC ACTGGTGCCAAAACTAT	TAATACGACTCACTATAGGGCCAAG CATTTCTCCGATTTC
P4b RNAi	TAATACGACTCACTATAGGGCCA CACTTTTGGCTCGTACA	TAATACGACTCACTATAGGGATATT GGCTTCTGCGGTTTG

poly(A) polymerase regulatory small subunit gene (polyAPol; accession number AY598432). We generated an absolute standard curve for polyAPol through PCR amplification using wasp venom gland DNA and specific primers, followed by cloning of the PCR product into the pSC-A-amp/kan

vector with the StrataClone PCR Cloning kit (Agilent), isolation of the plasmid with the GeneJET Plasmid Miniprep kit (Thermo Scientific), and confirmation of the cloned sequence with Sanger sequencing. Afterwards, the threshold cycle (CT) values for serial dilutions of 10^2 to 10^7 plasmid copies were used to produce the standard curve for absolute copy number quantification. Creation of the standard, as well as experimental qPCR and melting curve analyses, was conducted with a Rotor-Gene Q machine using the Rotor-Gene SYBR green PCR kit (Qiagen) with 1 μM primers and 1 μl of DNA per 10-μl reaction mixture. After 5 min of denaturation at 95°C, a two-step amplification cycle with 95°C for 5 s of denaturation and 60°C for 20 s of annealing and extension was used for 45 cycles. Each sample was internally replicated with 4 separate qPCRs, and the mean copy number was calculated from these 4 technical replicates for each biological replicate. Total copy number was calculated by multiplying the mean copy number by the DNA dilution factor and total DNA elution volume and then dividing by the number of specimens that comprised each sample. All comparisons of DlEPV abundance across life stages, tissue types, or experimental treatments were conducted using absolute DlEPV copy number per individual.

DlEPV gene sequence acquisition. A preliminary transcriptome generated from *D. longicaudata* venom gland tissue was used to obtain DlEPV gene sequences for estimation of viral gene expression and RNAi targeting. Total RNA was extracted from the pooled venom glands of 3 unemerged adult wasps using the RNeasy kit (Qiagen) and subjected to 75-bp paired-end sequencing on an Illumina NextSeq machine at the Georgia Genomics and Bioinformatics Core (GGBC). The resulting 20.8 million reads were quality filtered using fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and only reads that contained >90% bases with a Phred score of at least 90 were retained. The remaining 13 million quality reads were assembled de novo with Trinity v2.8.4 [57]. BLASTX was then used with default parameters and a 0.01 E value cutoff

to query the venom gland transcriptome against a custom protein sequence database composed of all NCBI entomopoxvirus genes (taxid 10284), as well as genes from *Diachasma alloeum*, the most closely related parasitoid genome sequence to *D. longicaudata* that was available (taxid 454923). From these results, we could distinguish between wasp and DlEPV transcripts and identified open reading frame (ORF) sequences for DlEPV homologs of DNAP, RPO147, and P4b genes.

RNA isolation and quantification of DlEPV gene expression. Venom gland samples were stored in a guanidine hydrochloride lysis buffer consisting of 4.9 M guanidine hydrochloride, 2% Sarkosyl, 50 mM Tris-Cl (pH 7.6), and 10 mM EDTA. Total RNA was isolated using the phenol-chloroform extraction method described above (excluding RNase treatment), followed by DNase treatment with the Turbo DNA-free kit (Ambion) and elution in 30 µl water. First-strand cDNA was synthesized for each sample with 400 ng RNA according to the Superscript III reverse transcriptase protocol (Invitrogen) using oligo(dT) primers. qPCR standards were generated as described above for specific primers representing the DNAP, RPO147, and P4b genes (Table 1.1). qPCRs were run with 1 µl cDNA for each primer set to measure expression of the 3 viral genes in each venom gland sample. cDNA copy number per nanogram total RNA was calculated for each biological replicate by multiplying the mean copy number (across 4 technical replicates) by the total cDNA volume and dividing by the amount of RNA used for cDNA synthesis.

Purification and microinjection of DlEPV. Venom gland-derived DlEPV was obtained from the dissection of 40 venom glands from naive female wasps in 500 µl PBS. Venom glands were homogenized with a pestle and centrifuged at 1,000 × g for 3 min to pellet tissue debris. The supernatant was passed through a 0.45-µm filter, and the filtrate was centrifuged for 30 min at 20,000 × g and 4°C to pellet DlEPV virions. The supernatant was removed, and after washing the

pellet with 500 µl PBS and undergoing a second round of centrifugation, the pelleted virus was resuspended in 400 µl PBS. Half of the resulting DlEPV suspension was subjected to UV inactivation of viral DNA through exposure to 120,000 µJ using a UV Crosslinker (Stratalinker), and both active and inactive DlEPV stocks were stored at −80°C. The concentration of undiluted virus stock was equal to 1 naive wasp's oviposition equivalent per µl. Serial dilutions of the virus stock were made to obtain lower doses for initial virus infection of flies. Late-third-instar fly larvae were each injected with 1 µl of either active or inactive DlEPV stock at various doses and then transferred to moist vermiculite to pupate. Flies were collected at 0 to 120 hpi to quantify viral abundance with qPCR as described above or were left undisturbed under standard rearing conditions to measure adult emergence rates.

RNAi assays. dsRNA targeting RPO147, DNAP, and P4b was synthesized with the MEGAscript T7 High Yield Transcription kit (Invitrogen) using gene-specific primers with added T7 promoter adaptors (Table 1.1) and venom gland DNA as the template. The three resulting types of DlEPV-specific dsRNA were then mixed together in equal concentrations of 333.3 ng/µl to form the "viral mix" dsRNA cocktail. Control dsRNA targeting the egfp gene was also synthesized in this manner using *egfp*-specific primers and *egfp* plasmid DNA as the template. *D. longicaudata* female wasps of the early pupal stage were each microinjected in the abdomen with 500 ng of either viral mix or *egfp* dsRNA (0.5 µl at 1,000 ng/µl) and were left under standard rearing conditions until the wasps emerged as adults. Daughters of dsRNA-treated wasps were obtained by allowing 1-week-old dsRNA-treated wasps to collectively oviposit within third-instar fly larvae for up to 16 h, followed by storage of parasitized flies under standard rearing conditions until wasp eclosion. Venom glands from dsRNA-treated wasps and their daughters were collected <24 h upon eclosion for quantification of viral gene expression or viral abundance.

Parasitism assays. Second-generation wasps to be used for parasitism assays were placed in either a "viral mix" or "*egfp*" cage upon eclosion with same-age male wasps and were left undisturbed for at least 7 days. For parasitism assays using third-instar fly larvae, subgroups of 3 female wasps in the same treatment group were transferred to an empty cage and presented with at least 200 fly larvae for oviposition, which lasted approximately 16 h. Wasps were allowed to oviposit in groups rather than individually due to the increased rate of oviposition exhibited by groups of *D. longicaudata* females compared to that of single females [58]. The cuticle of each fly was then examined, and approximately 50 flies that contained one laid wasp egg (e.g., one oviposition scar) were kept for observation under standard rearing conditions. After 4 weeks, the number of adult wasps and adult flies that had emerged from the singly scarred fly puparia were counted, along with the number of puparia from which nothing emerged. The parasitism success rate for each wasp triplicate was calculated as the number of wasp progeny that emerged as adults divided by the total number of singly scarred puparia. Second-instar fly larvae for modified assays were extracted from within the larval diet approximately 2 days prior to the appearance of third-instar flies. Subgroups of 6 wasps were used for each parasitism assay and were allowed to oviposit within second-instar flies for 4 h. Flies were then transferred back into fresh larval diet to complete larval development. Singly scarred flies were sorted and kept for observation once the fly larvae had crawled from the larval diet and pupated. Parasitism success was calculated as described for the third-instar assays.

Statistical analyses. JMP v13 was used for statistical analysis of all qPCR data. One-way ANOVA or *t* test assuming equal variances was used to test for differences in means from biological replicates, and Tukey's honestly significant difference (HSD) was used for multiple-comparison tests. For A × B factorial data sets, we used two-way ANOVA to test for differences

in means between levels of either effect as well as the interaction between the two effects. Total copy numbers were $\log^{10}$ transformed prior to analysis to obtain a normal distribution of residuals.

Data availability. Sequencing reads from the venom gland transcriptome can be found in the NCBI Gene Expression Omnibus under accession number GSE144541.

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2.8 REFERENCES

1. Strand MR, Pech LL. Immunological basis for compatibility in parasitoid host relationships. Annu Rev Entomol. 1995;40: 31–56.

2. Asgari S, Rivers DB. Venom proteins from endoparasitoid wasps and their role in host-parasite interactions. Annu Rev Entomol. 2011;56: 313–335.

3. Drezen J-M, Leobold M, Bézier A, Huguet E, Volkoff A-N, Herniou EA. Endogenous viruses of parasitic wasps: variations on a common theme. Curr Opin Virol. 2017;25: 41–48.

4. Burke GR, Strand MR. Polydnaviruses of parasitic wasps: domestication of viruses to act as gene delivery vectors. Insects. 2012;3: 91–119.

5. Bézier A, Annaheim M, Herbinière J, Wetterwald C, Gyapay G, Bernard-Samain S, et al. Polydnaviruses of braconid wasps derive from an ancestral nudivirus. Science. 2009;323: 926–930.

6. Volkoff A-N, Jouan V, Urbach S, Samain S, Bergoin M, Wincker P, et al. Analysis of virion structural components reveals vestiges of the ancestral ichnovirus genome. PLoS Pathog. 2010;6: e1000923.

7. Herniou EA, Huguet E, Thézé J, Bézier A, Periquet G, Drezen J-M. When parasitic wasps hijacked viruses: genomic and functional evolution of polydnaviruses. Philos Trans R Soc Lond B Biol Sci. 2013;368: 20130051.

8. Beckage NE. Polydnaviruses as endocrine regulators. In: Beckage NE, Drezen J-M, editors. Parasitoid Viruses. San Diego, CA: Academic Press; 2012. pp. 163–168.

9. Strand MR. Polydnavirus gene products that interact with the host immune system. In: Beckage NE, Drezen J-M, editors. Parasitoid Viruses. San Diego, CA: Academic Press; 2012. pp. 149–161.

10. Pichon A, Bézier A, Urbach S, Aury JM, Jouan V, Ravallec M, et al. Recurrent DNA virus domestication leading to different parasite virulence strategies. Sci Adv. 2015;1: e1501150.

11. Burke GR, Simmonds TJ, Sharanowski BJ, Geib SM. Rapid viral symbiogenesis via changes in parasitoid wasp genome architecture. Mol Biol Evol. 2018;35: 2463–2474.

12. Lawrence PO. Non-poly-DNA viruses, their parasitic wasps, and hosts. J Insect Physiol. 2005;51: 99–101.

13. Burke GR. Common themes in three independently derived endogenous nudivirus elements in parasitoid wasps. Curr Opin Insect Sci. 2019;32: 28–35.

14. Béliveau C, Cohen A, Stewart D, Periquet G, Djoumad A, Kuhn L, et al. Genomic and proteomic analyses indicate that banchine and campoplegine polydnaviruses have similar, if not identical, viral ancestors. J Virol. 2015;89: 8909–8921.

15. Ovruski S, Aluja M, Sivinski J, Wharton R. Hymenopteran parasitoids on fruit-infesting Tephritidae (Diptera) in Latin America and the Southern United States: diversity, distribution, taxonomic status and their use in fruit fly biological control. Integr Pest Manage Rev. 2000;5: 81–107.

16. Strand MR, Burke GR. Polydnaviruses: nature's genetic engineers. Annu Rev Virol. 2014;1: 333–354.

17. Edson KM, Barlin MR, Vinson SB. Venom apparatus of braconid wasps: comparative ultrastructure of reservoirs and gland filaments. Toxicon. 1982;20: 553–562.

18. Lawrence PO, Akin D. Virus-like particles from the poison glands of the parasitic wasp *Biosteres longicaudatus* (Hymenoptera: Braconidae). Can J Zool. 1990;68: 539–546.

19. Luo L, Zeng L. A new rod-shaped virus from parasitic wasp *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae). J Invertebr Pathol. 2010;103: 165–169.

20. Lawrence PO, Matos LF. Transmission of the Diachasmimorpha longicaudata rhabdovirus (DlRhV) to wasp offspring: an ultrastructural analysis. J Insect Physiol. 2005;51: 235–241.

21. Simmonds TJ, Carrillo D, Burke GR. Characterization of a venom gland-associated rhabdovirus in the parasitoid wasp *Diachasmimorpha longicaudata*. J Insect Physiol. 2016;91-92: 48–55.

22. Lawrence PO. Morphogenesis and cytopathic effects of the Diachasmimorpha longicaudata entomopoxvirus in host haemocytes. J Insect Physiol. 2005;51: 221–233.

23. Hashimoto Y, Lawrence PO. Comparative analysis of selected genes from Diachasmimorpha longicaudata entomopoxvirus and other poxviruses. J Insect Physiol. 2005;51: 207–220.

24. Lawrence PO. Purification and partial characterization of an entomopoxvirus (DlEPV) from a parasitic wasp of tephritid fruit flies. J Insect Sci. 2002;2: 10.

25. Lawrence PO, Dillard BE. A homolog of the vaccinia virus D13L rifampicin resistance gene is in the entomopoxvirus of the parasitic wasp, *Diachasmimorpha longicaudata*. J Insect Sci. 2008;8: 8.

26. Mwaengo DM, Lawrence PO. A putative DNA helicase and novel oligoribonuclease in the Diachasmimorpha longicaudata entomopoxvirus (DlEPV). Arch Virol. 2003;148: 1431–1444.

27. Paladino LZC, Papeschi AG, Cladera JL. Immature stages of development in the parasitoid wasp, *Diachasmimorpha longicaudata*. J Insect Sci. 2010;10: 56.

28. Quicke DLJ. Parasitic wasps. London, UK: Chapman & Hall; 1997.

29. Lawrence PO. Host vibration - a cue to host location by the parasite, *Biosteres longicaudatus*. Oecologia. 1981;48: 249–251.

30. Upton C, Slack S, Hunter AL, Ehlers A, Roper RL. Poxvirus orthologous clusters: toward defining the minimum essential poxvirus genome. J Virol. 2003;77: 7590–7600.

31. Perera S, Li Z, Pavlik L, Arif B. Entomopoxviruses. In: Asgari S, Johnson KN, editors. Insect Virology. Norfolk, UK: Caister Academic Press; 2010. pp. 83–102.

32. Lawrence PO, Baranowski RM, Greany PD. Effect of host age on development of *Biosteres* (= *Opius*) *longicaudatus*, a parasitoid of the Caribbean fruit fly, *Anastrepha suspensa*. Fla Entomol. 1976;59: 33–39.

33. Roossinck MJ. The good viruses: viral mutualistic symbioses. Nat Rev Microbiol. 2011;9: 99–108.

34. Moran NA, McCutcheon JP, Nakabachi A. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet. 2008;42: 165–190.

35. Douglas AE. The symbiotic habit. Princeton University Press; 2010.

36. Attardo GM, Lohs C, Heddi A, Alam UH, Yildirim S, Aksoy S. Analysis of milk gland structure and function in *Glossina morsitans*: milk protein production, symbiont populations and fecundity. J Insect Physiol. 2008;54: 1236–1242.

37. Balmand S, Lohs C, Aksoy S, Heddi A. Tissue distribution and transmission routes for the tsetse fly endosymbionts. J Invertebr Pathol. 2013;112: S116–S122.

38. Gonzalez PI, Montoya P, Perez-Lachaud G, Cancino J, Liedo P. Superparasitism in mass reared *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae), a parasitoid of fruit flies (Diptera: Tephritidae). Biol Control. 2007;40: 320–326.

39. Montoya P, Ruiz L, Perez-Lachaud G, Cancino J, Liedo P. Field superparasitism by *Diachasmimorpha longicaudata* attacking *Anastrepha* spp. larvae on mango fruits. Biol Control. 2013;64: 160–165.

40. Strand MR. Polydnavirus gene expression profiling: what we know now. In: Beckage NE, Drezen J-M, editors. Parasitoid Viruses. San Diego, CA: Academic Press; 2012. pp. 139–147.

41. Dupuy C, Gundersen-Rindal DE, Cusson M. Genomics and replication of polydnaviruses. In: Beckage NE, Drezen J-M, editors. Parasitoid Viruses. San Diego, CA: Academic Press; 2012. pp. 47–61.

42. Strand MR, Burke GR. Polydnaviruses as symbionts and gene delivery systems. PLoS Pathog. 2012;8: e1002757.

43. Pennacchio F, Strand MR. Evolution of developmental strategies in parasitic Hymenoptera. Annu Rev Entomol. 2006;51: 233–258.

44. Strand MR. Polydnaviruses. In: Asgari S, Johnson KN, editors. Insect Virology. Norfolk, UK: Caister Academic Press; 2010. pp. 171–197.

45. Lawrence PO, Baker FC, Tsai LW, Miller CA, Schooley DA, Geddes LG. JH-III levels in larvae and pharate pupae of *Anastrepha suspensa* (Diptera: Tephritidae) and in larvae of the parasitic wasp *Biosteres longicaudatus* (Hymenoptera: Braconidae). Arch Insect Biochem Physiol. 1990;13: 53–62.

46. Palli SR, Ladd TR, Tomkins WL, Shu S, Ramaswamy SB, Tanaka Y, et al. Choristoneura fumiferana entomopoxvirus prevents metamorphosis and modulates juvenile hormone and ecdysteroid titers. Insect Biochem Mol Biol. 2000;30: 869–876.

47. Nakai M, Shiotsuki T, Kunimi Y. An entomopoxvirus and a granulovirus use different mechanisms to prevent pupation of *Adoxophyes honmai*. Virus Res. 2004;101: 185–191.

48. Nakai M, Kinjo H, Takatsuka J, Shiotsuki T, Kamita SG, Kunimi Y. Entomopoxvirus infection induces changes in both juvenile hormone and ecdysteroid levels in larval *Mythimna separata*. J Gen Virol. 2016;97: 225–232.

49. Balgopal MM, Dover BA, Goodman WG, Strand MR. Parasitism by *Microplitis demolitor* induces alterations in the juvenile hormone titers and juvenile hormone esterase activity of its host, *Pseudoplusia includens*. J Insect Physiol. 1996;42: 337–345.

50. Strand MR, Dover BA. Developmental disruption of *Pseudoplusia includens* and *Heliothis virescens* larvae by the calyx fluid and venom of *Microplitis demolitor*. Arch Insect Biochem Physiol. 1991;18: 131–145.

51. Edson KM, Vinson SB, Stoltz DB, Summers MD. Virus in a parasitoid wasp: suppression of the cellular immune response in the parasitoid's host. Science. 1981;211: 582–583.

52. Burke GR, Thomas SA, Eum JH, Strand MR. Mutualistic polydnaviruses share essential replication gene functions with pathogenic ancestors. PLoS Pathog. 2013;9: e1003348.

53. Lawrence PO. Immature stages of the Caribbean fruit fly, *Anastrepha suspensa*. Fla Entomol. 1979;62: 214–219.

54. Moss B. Poxviridae: the viruses and their replication. 5th ed. In: Knipe DM, Howley PM, editors. Fields Virology. 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2007. pp. 2905–2945.

55. Jehle JA. Nudiviruses: their Biology and Genetics. In: Asgari S, Johnson KN, editors. Insect Virology. Norfolk, UK: Caister Academic Press; 2010. pp. 153–170.

56. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3-new capabilities and interfaces. Nucleic Acids Res. 2012;40: e115.

57. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011;29: 644–652.

58. Montoya P, Liedo P, Benrey B, Barrera JF, Cancino J, Aluja M. Functional response and superparasitism by *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae), a parasitoid of fruit flies (Diptera: Tephritidae). Ann Entomol Soc Am. 2000;93: 47–54.

CHAPTER 3

Genomic Analysis Reveals an Exogenous Viral Symbiont with Dual Functionality in Parasitoid Wasps and their Hosts[2]

[2] Coffman KA, Burke GR. Accepted by *PLoS Pathogens*, 11/08/2020.
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3.1 ABSTRACT

Insects are known to host a wide variety of beneficial microbes that are fundamental to many aspects of their biology and have substantially shaped their evolution. Notably, parasitoid wasps have repeatedly evolved beneficial associations with viruses that enable developing wasps to survive as parasites that feed from other insects. Ongoing genomic sequencing efforts have revealed that most of these virus-derived entities are fully integrated into the genomes of parasitoid wasp lineages, representing endogenous viral elements (EVEs) that retain the ability to produce virus or virus-like particles within wasp reproductive tissues. All documented parasitoid EVEs have undergone similar genomic rearrangements compared to their viral ancestors characterized by viral genes scattered across wasp genomes and specific viral gene losses. The recurrent presence of viral endogenization and genomic reorganization in beneficial virus systems identified to date suggest that these features are crucial to forming heritable alliances between parasitoid wasps and viruses. Here, our genomic characterization of a mutualistic poxvirus associated with the wasp *Diachasmimorpha longicaudata*, known as Diachasmimorpha longicaudata entomopoxvirus (DlEPV), has uncovered the first instance of beneficial virus evolution that does not conform to the genomic architecture shared by parasitoid EVEs with which it displays evolutionary convergence. Rather, DlEPV retains the exogenous viral genome of its poxvirus ancestor and the majority of conserved poxvirus core genes. Additional comparative analyses indicate that DlEPV is related to a fly pathogen and contains a novel gene expansion that may be adaptive to its symbiotic role. Finally, differential expression analysis during virus replication in wasps and fly hosts demonstrates a unique mechanism of functional partitioning that allows DlEPV to persist within and provide benefit to its parasitoid wasp host.

3.2 AUTHOR SUMMARY

Viruses have repeatedly formed long-term associations with insects called parasitoid wasps, which grow as parasites within other insect hosts. While these viruses were once pathogenic, they have since been co-opted by parasitoid wasps to benefit the survival of wasp offspring during parasitism. The genomes of most identified beneficial viruses are fully integrated into the genomes of the parasitoid wasps that produce them. Because these virus-derived entities have lost the ability to exist apart from their associated wasps, they are considered endogenous viral elements (EVEs) of the wasps rather than mutualistic symbionts. We sequenced the genome of the beneficial parasitoid virus Diachasmimorpha longicaudata entomopoxvirus (DlEPV) and found that its genome is not integrated into the genome of *Diachasmimorpha longicaudata* wasps and has largely retained the genomic structure of its pathogenic ancestor. Given the importance of viral genome integration in the overall stability of parasitoid wasp-EVE systems, we identified a novel strategy used by DlEPV to maintain its relationship with *D. longicaudata* despite its lack of endogenization. Our findings in this study demonstrate the first instance of a mutualistic viral symbiont in insects and provide new insight into the means through which beneficial viruses can arise.

Keywords: *Diachasmimorpha longicaudata*, symbiosis, endogenous viral elements, poxvirus, parasitism, parasitoid wasp, genome evolution

3.3 INTRODUCTION

Microbial symbionts have been increasingly identified as major drivers of animal evolution due to the novel capabilities microbes provide to their hosts and the speed at which symbiosis can

cause adaptive change in animal lineages [1,2]. Insects, in particular, have repeatedly formed symbiotic alliances with microbes that highly vary with respect to taxonomic classification, localization on or within the insect, transmission strategies, and phenotypic traits provided to the insect [3]. Bacterial symbionts have been the primary focus of study for many insect groups, such as plant sap-feeders, blood-feeders, and social insects [4–6]. However, parasitoid wasps, whose young are obligate parasites of other arthropods, are better known for their numerous associations with viruses [7–9]. Parasitoid wasp lineages have repeatedly acquired heritable viruses in conjunction with evolutionary arms race dynamics between wasps and their hosts [10–12]. Many of these associations are extraordinary examples of endogenous viral elements (EVEs) within wasp genomes, in which components of viral machinery are retained from their pathogenic ancestors to produce virus or virus-like particles within wasp ovaries [13,14]. The resulting virus-derived particles accompany wasp eggs when delivered into host insects and can function to protect parasitoid eggs from attack by the host immune system and/or actively disrupt host developmental and immunological pathways [10,15,16].

Rather than existing in a wasp genome as a contiguous region of proviral DNA, parasitoid EVEs share an unconventional genomic architecture characterized by the dispersal of viral genes to separate regions of the wasp genome [17–22]. Key virus replication genes have also been lost in all cases for which the viral ancestor is known, which implies that wasp genes are instead needed to complete virus particle production [23]. These genomic anomalies have three major consequences that are thought to be important adaptations in parasitoid-EVE associations. First, permanent integration of the viral genome into the wasp genome ensures viral transmission to future wasp generations. Second, viral gene dispersal and gene loss forfeits EVE autonomy over their own propagation, allowing for strict regulation of virus replication by the wasp. Third, virus

particles produced in wasp tissue do not contain the necessary genes for further replication outside of the wasp. The functional outcome of these genomic features is most clearly understood in the polydnaviruses (PDVs), a group of ancient EVEs formed from multiple, independent viral acquisition events [17,18]. Due to their unusual genome organization, PDVs contain a dual functionality that is effectively split between two insects: PDV replication occurs exclusively in wasp tissue, while PDV virulence is confined to parasitized host tissue [24,25]. This separation of virus function promotes stability within these associations, because it minimizes wasp-virus conflict and establishes an interdependency for survival, in which wasp offspring depend on PDV virulence within the host, and PDVs depend on wasps for transmission and amplification [26,27]. Furthermore, the recurrent observation of this distinctive genomic architecture in more recently acquired EVEs supports the notion that viral genome integration and reorganization is fundamental to the persistence of wasp-virus mergers [21,22].

However, additional examples of heritable viruses have been identified in parasitoid lineages that are of unique viral origin and may deviate from this pattern. An ascovirus carried by the wasp *Diadromus pulchellus*, named Diadromus pulchellus toursvirus (DpTV), contains a circular, episomal DNA genome present in the nuclei of wasp cells, and when DpTV virions infect the caterpillar hosts of *D. pulchellus*, the virus inhibits the host melanization response during an immune challenge [28–30]. Additionally, a heritable iflavirus discovered within *Dinocampus coccinellae* parasitoid wasps, known as Dinocampus coccinellae paralysis virus (DcPV), contains an exogenous RNA viral genome that replicates within the neural tissue of coccinellid beetle hosts of the wasps during parasitism. This viral activity is thought to cause a behavioral manipulation within the host, in which parasitized beetles will guard the parasitoid pupa against predation [31]. While these examples provide suggestive evidence that the virus in each case is beneficial for the

parasitoid that transmits it, neither example has been experimentally shown to provide a direct fitness benefit for its associated wasp. An effective method to determine whether heritable viruses are truly mutualistic for wasps is to remove the virus population from wasps and compare the success of "cured" wasps to those with a normal viral load. Lower survivorship of wasp progeny when not accompanied by virus is strong evidence that the virus provides a net benefit to wasp fitness.

This has been recently demonstrated for *Diachasmimorpha longicaudata* parasitoid wasps and the heritable poxvirus female wasps maintain within their venom gland, known as Diachasmimorpha longicaudata entomopoxvirus (DlEPV) [32,33]. We showed that DlEPV is vertically transmitted to each wasp generation within oviposited wasp eggs and provides a considerable boost to wasp survival during parasitism within Anastrepha suspensa fruit fly hosts, because wasps reared without DlEPV survive at a drastically reduced rate compared to wasps with a typical viral load [34]. DlEPV is currently the only mutualistic poxvirus to be identified in parasitoid wasps, and unlike EVEs, DlEPV can replicate within both wasps and fruit fly hosts of the wasps [34]. Therefore, DlEPV appears to have replicative autonomy within both insects, suggesting that this virus retains more features from its pathogenic ancestor than other parasitoid viral elements. Despite these ancestral characteristics, we have also demonstrated that DlEPV replication is highly virulent within host fly tissue, while replication within wasp tissue has no observable pathogenic effects [34]. These results imply that DlEPV utilizes a similar strategy of functional partitioning to that observed in parasitoid EVEs. DlEPV is therefore unique in that it maintains features of both an autonomous viral pathogen and a beneficial viral symbiont. In this study, we sequenced the complete DlEPV genome to ascertain whether DlEPV shares the genomic architecture of parasitoid EVEs and to determine how DlEPV has evolved in comparison to other

poxviruses, including the identification of its closest known relative. Using the results from our comparative analyses, we then performed a functional genomic investigation to elucidate the novel means through which DlEPV may achieve its beneficial relationship with *D. longicaudata*.

3.4 RESULTS

Sequencing and Assembly of the DlEPV Genome

The DlEPV genome is non-endogenous. Poxviruses are large DNA viruses that infect vertebrates (chordopoxviruses, or CPVs), as well as insects (entomopoxviruses, or EPVs) [35]. The study of poxviruses has historically focused on CPVs and the prototype CPV, known as vaccinia virus (VACV), due to the societal impact of smallpox [36]. EPVs have been comparatively neglected but function similarly to CPVs in many ways, while exhibiting differences that can often be attributed to the biology of their insect hosts [37]. Both CPV and EPV genomes exist as linear, double-stranded DNA (dsDNA) molecules that contain a hairpin loop at each terminus. The two extreme ends of the genome consist of sequence repeats that are inversions of one another, known as inverted terminal repeats (ITRs), while the genome interior contains the majority of viral genes [38]. The DlEPV genome sequence, obtained from high-throughput sequencing of *D. longicaudata* venom gland DNA, was assembled into a single 253 kilobase (kb) contiguous sequence, including two 17 kb ITR regions and 193 open reading frames (ORFs) (Figure 3.1, Supplemental Table 3.1). The contiguity and lack of flanking wasp genes in our assembly implies that the DlEPV genome is not endogenous within the wasp genome. Normalized quantitative PCR (qPCR) data of viral abundance in wasp tissue also support this finding by showing that the number of DlEPV genome copies is less than the number of wasp genome copies for several tissues, developmental stages, and all male wasps (Supplemental Figure 3.1).

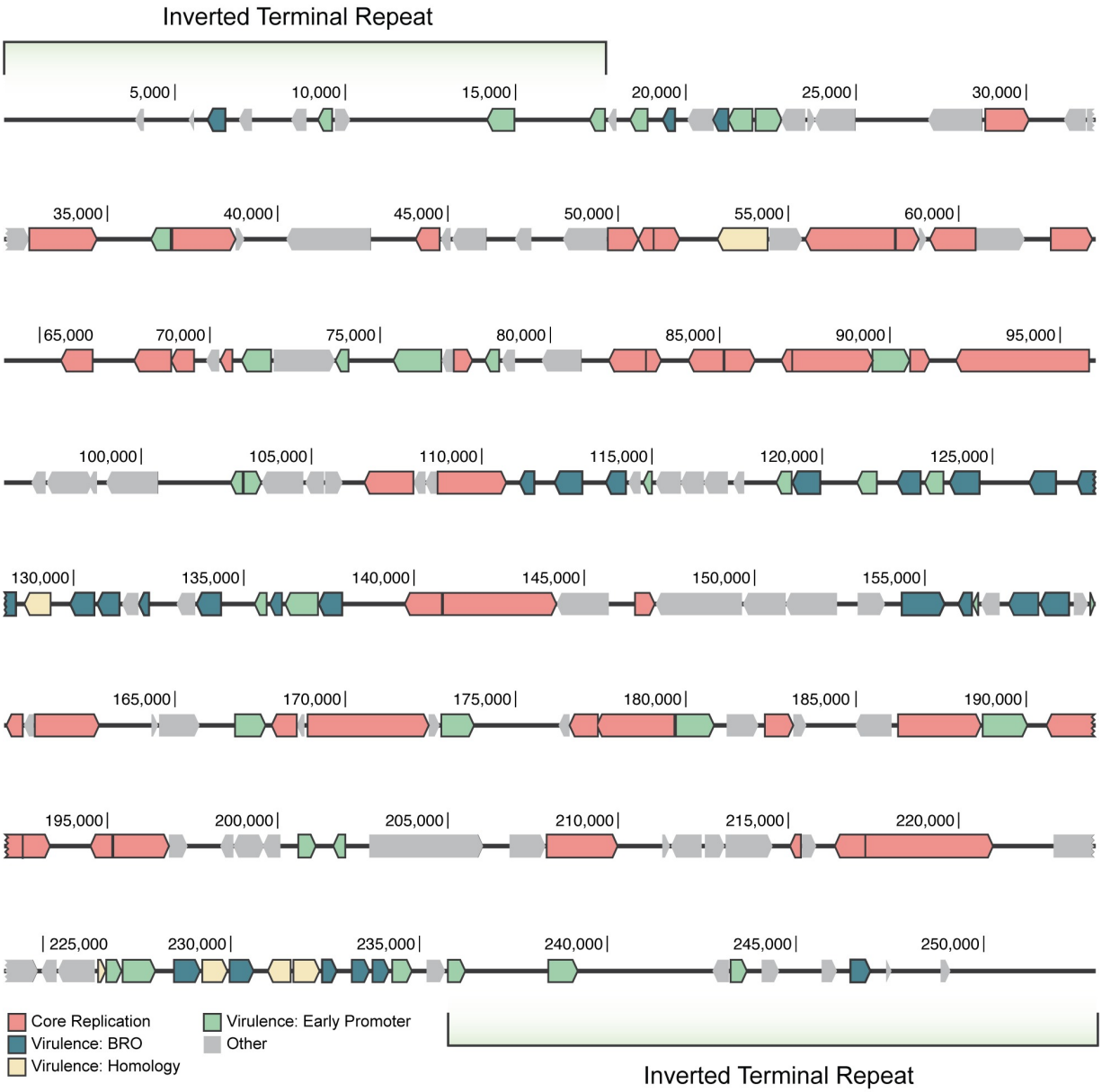

Figure 3.1. Linear map of the DlEPV genome. Each arrow indicates the genomic position of a DlEPV ORF, and the direction of the arrow corresponds to its strand orientation. Arrows are colored based on the putative functional category of each ORF as defined in the legend at the bottom of the map. Core Replication refers to the 45 poxvirus core genes identified in the DlEPV genome. Virulence: BRO refers to the 27 DlEPV BRO genes, Virulence: Homology indicates the 6 ORFs with similarity to known virulence genes, and Virulence: Early Promoter are the additional 34 putative virulence genes based on the presence of the conserved EPV early promoter sequence and no other assigned function.

The DlEPV genome has abnormally low coding density and high GC content. The majority of publicly available EPV genomes are from lepidopteran (moth and butterfly) poxviruses: Amsacta moorei entomopoxvirus (AMEV), Adoxophyes honmai entomopoxvirus (AHEV), Choristoneura biennis entomopoxvirus (CBEV), Choristoneura rosaceana entomopoxvirus (CREV), and Mythimna separata entomopoxvirus (MySEV) [39,40]. Orthopteran (grasshopper) and coleopteran (beetle) poxvirus genomes contain single representatives: Melanoplus sanguinipes entomopoxvirus (MSEV) and Anomala cuprea entomopoxvirus (ACEV), respectively [41,42]. Recently, two additional EPV sequences have been reported. A partial poxvirus genome sequence identified within the argentine ant, named Linepithema humile entomopoxvirus 1 (LHEV), represents the first hymenopteran (ant, wasp, and bee) poxvirus to be sequenced [43]. In addition, a complete poxvirus genome obtained from *Drosophila melanogaster*, known as Yalta virus, represents the first sequenced dipteran (fly) poxvirus [44].

DlEPV, in comparison to these other EPVs, has a similar overall genome length, ITR length, and ORF number (Supplemental Table 3.2). However, the DlEPV genome is peculiar with respect to its coding capacity and GC content. DlEPV is extremely gene-sparse relative to its genome size and contains a heavily reduced coding density of 65.1% compared to the 89.9 ± 3.0% coding density of other EPV genomes (Supplemental Table 3.2). This reduced gene density is an exception to poxvirus genomes, in general, which are highly compact with a dense array of non-overlapping genes [38]. The nucleotide composition of the DlEPV genome also varies compared to other EPV genomes, which consistently exhibit the most severe AT-bias found in the poxvirus family [45]. The GC content of the DlEPV genome at 30.1% is substantially higher than the average 20.5 ± 2.4% of its EPV relatives (Supplemental Table 3.2). Since viral GC content can be correlated to the GC of the host genome [46], we also estimated *D. longicaudata* and *A. suspensa* genome nucleotide composition using transcriptomes produced for a subsequent differential

expression analysis (see Functional Genomic Analysis of DlEPV). Assembled *D. longicaudata* transcripts had 40.7% GC overall, and *A. suspensa* fly hosts contained transcripts with a GC content of 39.7%.

DlEPV Genome Annotation

DlEPV contains most poxvirus core genes. We next annotated the DlEPV genome to assess its completeness compared to other poxviruses. The central region of the linear poxvirus genome generally contains genes that are required for virus replication, including the 49 core genes conserved among all sequenced poxviruses [45,47,48]. We were able to identify the majority of poxvirus core genes in the DlEPV genome, with the exception of the following four genes: the heparin binding surface protein (VACV core gene H3L), a virion core protein (E6R), the NlpC/P60 superfamily protein (G6R), and a RNA polymerase subunit (A29L) [49] (Supplemental Table 3.1). VACV core genes H3L and E6R are both required for the correct assemblage of mature virions, a process known as morphogenesis [50–54]. G6R is unique among the poxvirus core gene set, as its protein product is not required for VACV replication *in vitro* but instead is involved in virulence [55]. A29L encodes the 35 kDa RNA polymerase subunit (RPO35), one of five conserved subunits of the poxvirus RNA polymerase holoenzyme responsible for viral gene transcription [56]. We utilized our previously reported transcriptome of the *D. longicaudata* venom gland [34] to determine whether these four genes had been transferred from the DlEPV genome to the *D. longicaudata* genome. Endogenized PDV replication genes were first identified in PDV-producing wasps using transcriptome sequencing of wasp ovary tissue collected during PDV replication [17,18]. We therefore hypothesized that DlEPV transcripts with sequence similarity to the undetected genes would be present during virus replication in the venom gland if these genes were endogenous. However, our transcriptome searches yielded no hits to the aforementioned genes.

DlEPV is most closely related to a *Drosophila* poxvirus. Due to the exogenous state of the DlEPV genome and its relatively complete set of core genes, DlEPV appears to be more biologically similar to its viral progenitor than has been observed of parasitoid EVEs. This level of genomic preservation led us to investigate the origin of DlEPV among other poxviruses through phylogenetic reconstruction and identification of its closest relative. Because DlEPV replicates in both a parasitoid wasp and the wasp's fruit fly hosts, it is likely that DlEPV originated as either a parasitoid pathogen or as a fly pathogen. The two most recently published EPV genomes, LHEV and Yalta virus, could therefore give more context on the origin of DlEPV.

We generated a maximum likelihood (ML) phylogeny using 16 concatenated poxvirus core genes from all sequenced EPVs and the following CPVs to test the two hypotheses: VACV, orf virus (ORFV), molluscum contagiosum virus (MOCV), fowlpox virus (FWPV), crocodilepox virus (CRV), and salmon gill poxvirus (SGPV) (Figure 3.2; Supplemental Table 3.3). The placement of the 7 originally sequenced EPVs (MSEV, AMEV, AHEV, MySEV, CREV, CBEV, and ACEV) on the tree shows concordance with the higher phylogenetic relationships of their insect hosts, which is consistent with previous EPV phylogenetic analyses [37,40]. In contrast, LHEV and Yalta virus show a clear divergence from other EPVs [44]. The inclusion of DlEPV in our phylogeny revealed that it shares a more recent common ancestor with Yalta virus than LHEV, suggesting that DlEPV is more likely derived from a fly pathogen rather than a parasitoid pathogen (Figure 3.2). The shared common ancestry between DlEPV and Yalta virus was maintained in an expanded 44 core gene phylogeny that excluded the partial LHEV genome, and the overall tree topology was robust to Bayesian inference analysis (Supplemental Figure 3.2). While the position of the DlEPV/Yalta virus clade appears to bridge the gap between EPVs and CPVs in the unrooted phylogeny, the inclusion of members of the sister group to poxviruses, known as Asfarviridae [57],

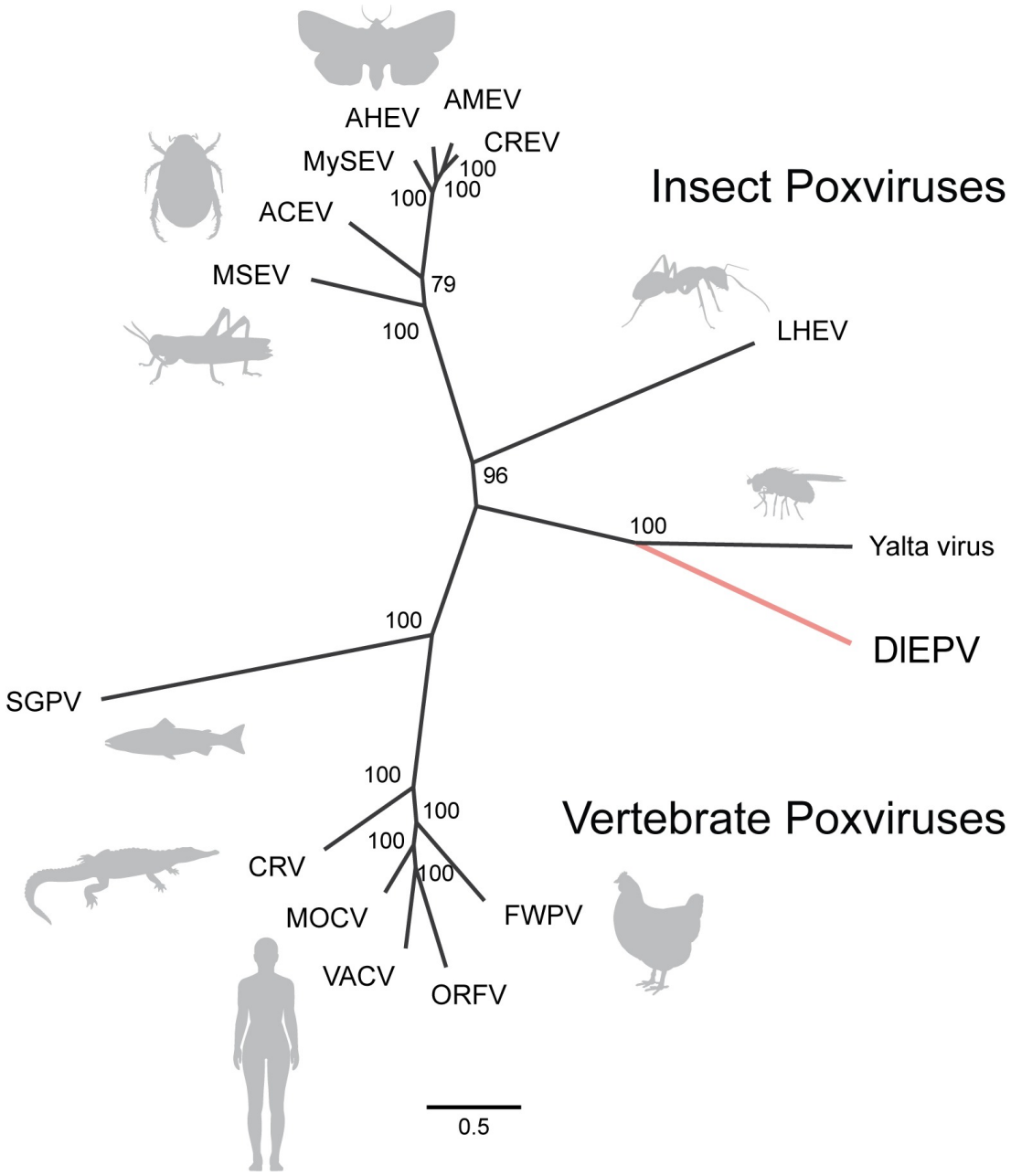

Figure 3.2. Poxvirus core gene phylogeny demonstrates that the fly-infecting Yalta virus is the closest known relative of DlEPV. Phylogenetic tree constructed from a maximum likelihood analysis using the concatenated amino acid multiple sequence alignment from 16 conserved poxvirus core genes. Node support (%) was inferred with 1,000 bootstrap iterations. Insect and vertebrate poxvirus orthologs used to build the phylogeny are indicated in Supplemental Table 3.3. Genome abbreviations are as defined in the Results section, and accessions are included in the Materials and Methods section.

as an outgroup in a 10 poxvirus core gene phylogeny confirmed that DlEPV and Yalta virus are, in fact, more closely related to EPVs than to CPVs (Supplemental Figure 3.2).

We further investigated similarities between the DlEPV and Yalta virus genomes by searching for additional orthologous genes shared among them. We determined that 44 of the 49 poxvirus core genes are shared between the DlEPV and Yalta virus genomes (Supplemental Table 3.3). Interestingly, 2 of the 3 "missing" genes in the Yalta virus genome were also not detected in the DlEPV genome [44]. This suggests that the absence, or more likely, extreme sequence divergence of these genes is lineage-specific to fly poxviruses, rather than due to genome incompleteness. In addition to the 44 core genes shared between the two genomes, we found 24 single-copy orthologs and 3 orthologs that had undergone duplication in either genome (Supplemental Table 3.1). Most of these orthologous groups are of unknown function, while some have putative functions that are not found in other EPVs and therefore, may be unique to fly poxviruses. These include a ribonucleotide reductase large subunit (DLEV028/Yalta121), an alpha/beta fold hydrolase (DLEV038/Yalta165), and a type II topoisomerase (DLEV158/Yalta014).

While many CPV genomes have a highly conserved gene order [58], this colinear pattern does not hold true for EPVs, which display little synteny with CPVs or EPVs from separate host genera [39,40,42]. We investigated genome synteny between DlEPV and Yalta virus by generating two-dimensional dot plots comparing the genomic positions of their shared 44 core genes (Figure 3.3). The DlEPV-Yalta virus dot plot revealed a moderate amount of synteny between the two viral genomes. In particular, a large syntenic region of approximately 50 kb was identified, as indicated by the negative linear arrangement of orthologs in the lower-right quadrant of the plot (Figure 3.3A). This partial synteny further supports a closer relationship between DlEPV and Yalta virus, because both genomes have relatively low synteny when compared to the next closest relative MSEV (Figure 3.3B to C).

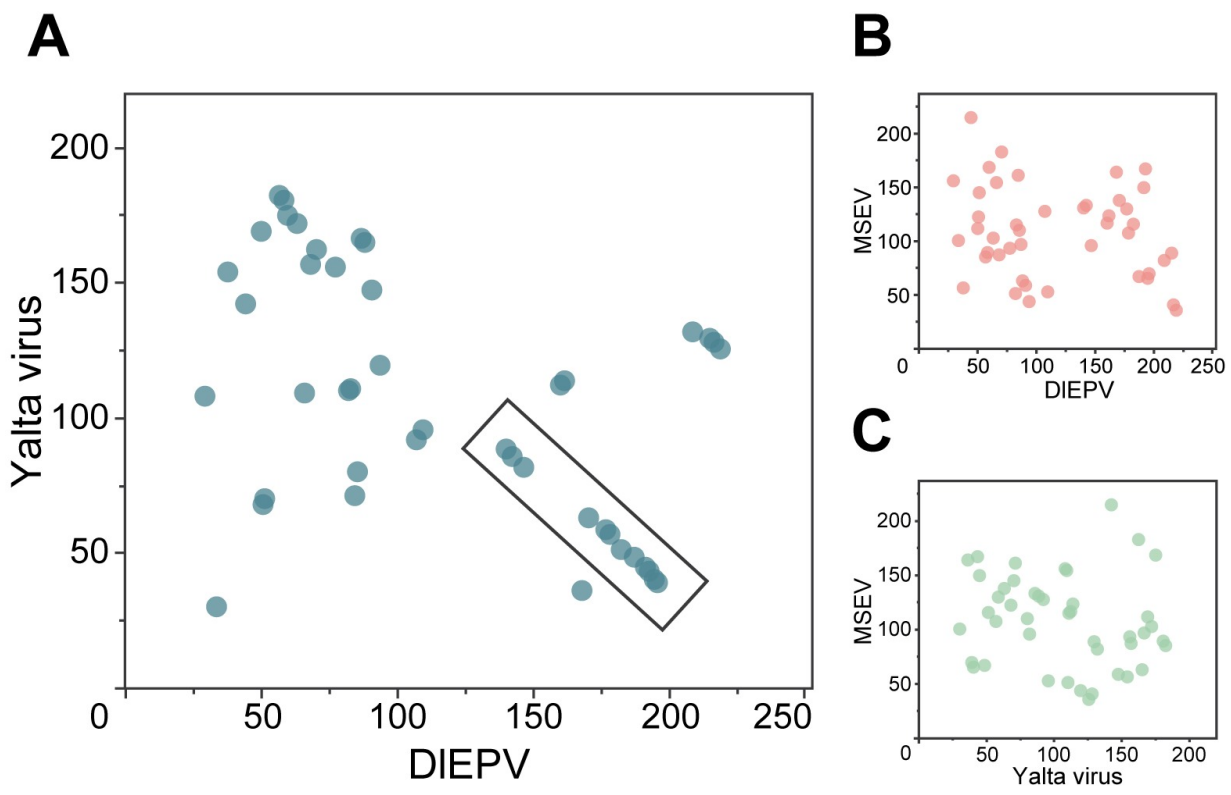

Figure 3.3. Core gene synteny further supports the close relationship between DlEPV and Yalta virus. Dot plots show the relative genomic location of the 44 poxvirus core genes shared between the DlEPV and Yalta virus genomes when compared to (A) one another, or (B-C) when either is compared to MSEV. Each dot represents a homologous core gene, and axes indicate the genomic position in kilobases. Box in panel A highlights a highly syntenic region between DlEPV and Yalta virus core genes.

The DlEPV genome contains a novel BRO gene expansion. In contrast to the more conserved center of the typical poxvirus genome, the exterior regions contain a variable assortment of virulence genes, which are involved in host interactions [45,59]. Multigene families are common components of large DNA viral genomes that likely represent lineage-specific adaptations resulting from coevolution between the virus and its host [60]. One such family, known as baculovirus repeated ORF (BRO) proteins, are found in many insect DNA viruses, including

baculoviruses, iridoviruses, ascoviruses, and EPVs [61]. The function of BRO proteins remains unclear, although they localize to the nuclei of insect cells infected with the baculovirus Bombyx mori nucleopolyhedrovirus [62], and the characteristic N-terminal BRO domain can bind DNA [63]. Therefore, it has been proposed that BRO proteins may play a role in virulence through the regulation of host DNA transcription and/or replication [63]. BRO genes are consistently found in the terminal regions of EPV genomes amongst other virulence genes [39–42]. Strikingly, the DlEPV genome contains 27 BRO genes, which is >3 times the average quantity found in other EPVs (7.6 ± 4.8 gene copies) (Supplemental Table 3.4). Furthermore, 14 of the DlEPV BRO genes are clustered together in the most central 30 kb of the genome, while 4 additional BRO genes form a secondary cluster within 17 kb of the primary cluster (Figure 3.1). The uniform strand orientation and relative size of BRO genes in the primary cluster suggests that this region represents a large gene family expansion via tandem duplication events.

Few other homologous virulence genes were identified. Three DlEPV virulence genes could be identified by sequence similarity to virulence genes in other viruses, including a thymidylate kinase (DLEV176), a thymidine kinase (DLEV178), and a F-box protein (DLEV179) (Supplemental Table 3.1). Thymidine and thymidylate kinases are ubiquitous genes involved in DNA biosynthesis of both eukaryotes and prokaryotes, in which they sequentially phosphorylate nucleosides before incorporation into a growing DNA strand [64]. Although poxvirus thymidine and thymidylate kinases similarly function in viral DNA replication, they are not highly conserved and are not required for replication of all poxviruses [58,65]. Furthermore, thymidine kinase has a proposed virulence role in VACV due to the drop in pathogenicity associated with thymidine kinase-negative viral recombinants [66]. F-box proteins are commonly found in eukaryotic genomes and contribute to the cellular ubiquitination system for protein degradation [67–69]. F-box-like proteins are also highly abundant virulence genes in CPV genomes, where they interact

with host cell ubiquitin-proteasome components and lead to the degradation of important immunity-related proteins, such as nuclear factor kappa B (NF-kB) transcription factors [70–74]. Only one F-box domain-containing gene has been previously reported in an EPV, which is AMV254, a tryptophan repeat gene family protein in the AMEV genome [39]. The DlEPV putative F-box gene shows no similarity to AMV254 and differs from CPV F-box-like genes in that it does not contain the characteristic ankyrin repeats of poxvirus F-box-like genes [71]. In addition, the F-box domain of DLEV179 is located at the N-terminus, which is more similar to eukaryotic F-box proteins [75], rather than the C-terminal location common to viral F-box-like domains [71].

The DlEPV genome also contains three genes with protein domains not found in other viruses that may also be involved in virulence, such as a gamma-glutamyl transpeptidase domain (DLEV037), a type IV secretion system domain (DLEV099), and a thermostable hemolysin domain (DLEV172). The gamma-glutamyl transpeptidase (GGT) gene was first identified in DlEPV by Hashimoto and Lawrence [76]. A GGT has also been identified in the venom of *Aphidius ervi* parasitoid wasps, where it was shown to cause ovarian cell apoptosis in the aphid hosts of the wasp [77]. The DlEPV genes that encode the type IV secretion system (T4SS) and hemolysin domains could also be involved in host cell death, as these domains are used by many bacterial pathogens for cell membrane pore formation. T4SSs are used broadly by bacteria for the transfer of macromolecules, like DNA or proteins, to bacterial or eukaryotic cells [78,79]. Furthermore, hemolysins are toxins specifically used by bacterial pathogens, such as *Vibrio* species, to rupture blood cells of the infected host [80].

Additional putative virulence genes characterize the DlEPV genome. Due to the overall lack of poxvirus virulence gene conservation, we conducted a promoter sequence analysis to identify additional putative virulence genes in the DlEPV genome. Transcription of poxvirus genes is temporally regulated during infection, based on promoter sequence recognition by viral

transcription factors that are specific to different stages of the replication cycle [56]. Viral genes transcribed soon after infection are known as early genes and include the virulence genes, which are expressed quickly to combat host defenses against infection [56,81,82]. CPV early genes contain a conserved upstream promoter sequence that is recognized by the early transcription factor packaged within virions [83–85]. Similarly, the promoter sequence TGAAAXXXXA is conserved among EPV early genes [39,41], so we searched the 100 bp upstream region of DlEPV ORFs without an assigned putative function for the EPV early promoter motif. We identified 34 additional putative virulence genes from this approach (Figure 3.1, Supplemental Table 3.1). When combined with the 27 BRO genes and 6 virulence genes described above, a total of 67 putative virulence genes were identified in the DlEPV genome.

Functional Genomic Analysis of DlEPV

We next used our annotation of the DlEPV genome to investigate the differential functionality of this virus in its two insect hosts. We have previously shown that DlEPV replicates in both wasp and fly tissue, but only flies are susceptible to the virulent effects of the virus [34]. In order to maintain a stable symbiosis, we hypothesized that DlEPV activity is altered within the wasp venom gland, such that replication is maximized and virulence is minimized. Conversely, we predicted that DlEPV replication and virulence activity follows a more standard poxvirus trajectory within the fly host. Because DlEPV shows no evidence of endogenization within the *D. longicaudata* genome, this variation in replication and virulence functions within the wasp would have to be achieved through a different mechanism than the genomic integration and reorganization feature of parasitoid EVEs. We therefore utilized RNA sequencing (RNA-seq) to examine whether differential viral gene expression is associated with the selective virulence demonstrated by DlEPV.

Identification of peak DlEPV expression for transcriptome sequencing. Stages of peak viral gene expression were first determined in both wasp and fly tissues to select for transcriptome sequencing. Our previously reported DlEPV expression profiles from wasp venom gland tissue indicated that viral gene expression peaked in female wasps at the time of the final molt into adulthood [34] (Figure 3.4A to C). Here, we generated similar reverse transcription qPCR (RT-qPCR) profiles of whole flies throughout parasitism using the same 3 genes that were analyzed in venom gland tissue: the 147 kDa RNA polymerase subunit RPO147 (DLEV067), the DNA polymerase DNAP (DLEV168), and the P4b structural component (DLEV147). DlEPV gene expression rose steadily in flies 4-24 hours post parasitism (hpp) and plateaued at 48-96 hpp (RPO147 $F_{5,30} = 12.29$, $P < 0.0001$; DNAP $F_{5,30} = 9.82$, $P < 0.0001$; P4b $F_{5,30} = 111.54$, $P < 0.0001$) (Figure 3.4D to F), which is congruent with prior qPCR quantification of viral genome copy growth in parasitized flies [34]. Using these data, we determined that the unemerged adult female wasp and the 72 hpp fly were comparable stages of maximum DlEPV expression activity.

Differential viral gene expression supports two DlEPV functional roles. Total RNA was isolated from venom gland tissue of unemerged female wasps, as well as whole fly body tissue at 72 hpp (with wasp larva removed), including 6 biological replicates of each treatment. Because we had previously generated a transcriptome from unemerged wasp venom gland tissue [34], only 5 additional replicates were sequenced for this treatment. Paired-end sequencing followed by read quality filtering yielded an average 11.8 million read pairs per wasp sample and 8.9 million read pairs per fly sample. An average 38.9% of venom gland reads and 7.8% of parasitized fly reads aligned to the DlEPV genome. 91.2% (176 of 193) of DlEPV genes showed significant differential expression (FDR, $q < 0.05$) during virus replication in wasps and flies (Supplemental Table 3.5). Hierarchical clustering yielded two main groups of differentially expressed DlEPV genes: 86 genes were significantly upregulated, and 90 genes were significantly downregulated during virus

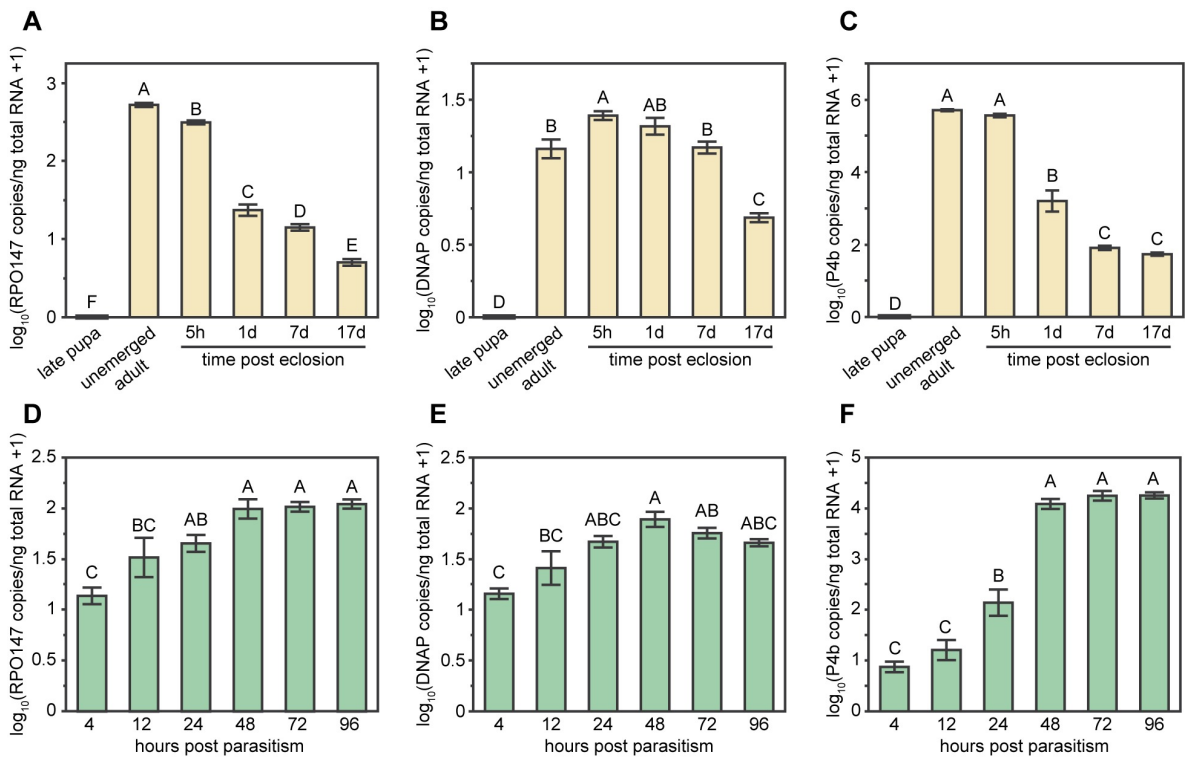

Figure 3.4. DlEPV gene expression profiles for selection of RNA-seq timepoints. Expression of DlEPV genes measured with RT-qPCR in (A-C) female wasp venom glands from late pupa to 17-day-old adult and (D-F) parasitized flies from 4-96 hours post parasitism (hpp). Profiled DlEPV genes encode (A,D) the 147kDa RNA polymerase subunit RPO147, (B,E) the DNA polymerase DNAP, and (C,F) the structural protein P4b. Each bar represents the mean $\log_{10}$ transformed cDNA copy number per ng total RNA averaged from 6 biological replicates. Error bars represent one standard error above and below the mean, and the letter(s) above each bar indicates statistically distinct mean values from Tukey's HSD tests. Data in (A-C) were modified from Coffman *et al.* 2020 [34].

replication in the wasp venom gland compared to the parasitized fly (Figure 3.5). Genes upregulated in wasp tissue displayed an average $\log_2$ fold change of 2.3, which is nearly a 5x greater level of expression in wasps compared to flies. Even more drastic were genes downregulated in wasp tissue, which had an average $\log_2$ fold change of 3.4, or >10x lower expression in wasps compared to flies (Supplemental Table 3.5). We then looked for differential

expression patterns associated with the two main functional gene groups identified in the DLEPV genome: core replication genes and virulence genes. Remarkably, 82.2% (37 of 45) DlEPV core replication genes fell within the former cluster of genes upregulated in wasp tissue. The latter cluster of DlEPV genes downregulated in wasp tissue contained 79.1% (53 of 67) of DlEPV putative virulence genes, including 22 of the 27 BRO genes, 5 of the 6 virulence genes identified by sequence similarity, as well as 26 of the 34 additional virulence genes identified by their early promoter motif.

3.5 DISCUSSION

The genomic architecture of PDVs underpins many aspects of their associations with parasitoid wasps, including Mendelian inheritance of the proviral genome, the shift of virus replication control to the wasp, and restriction of replication to wasps and virulence to hosts [27]. We have previously shown that DlEPV broadly shares these features with PDVs as products of convergent evolution [34]. However, our work in this study has shown that DlEPV lacks the fundamental integration event that facilitated the evolution of these characteristics in PDV and other EVE systems. Our findings therefore raise intriguing questions regarding how features like vertical transmission, controlled virus replication, and selective virulence arose and are maintained in the DlEPV system. Furthermore, functional data presented here demonstrate that one of these features, partitioning of viral activity, is accomplished by DlEPV through a method not before observed for a beneficial virus.

DlEPV represents an exogenous parasitoid virus. The presence of wasp genes surrounding multiple viral gene clusters is repeatedly observed with parasitoid EVE genome sequences [17–22]. Conversely, our assembly of the DlEPV genome into a single contig without bordering wasp

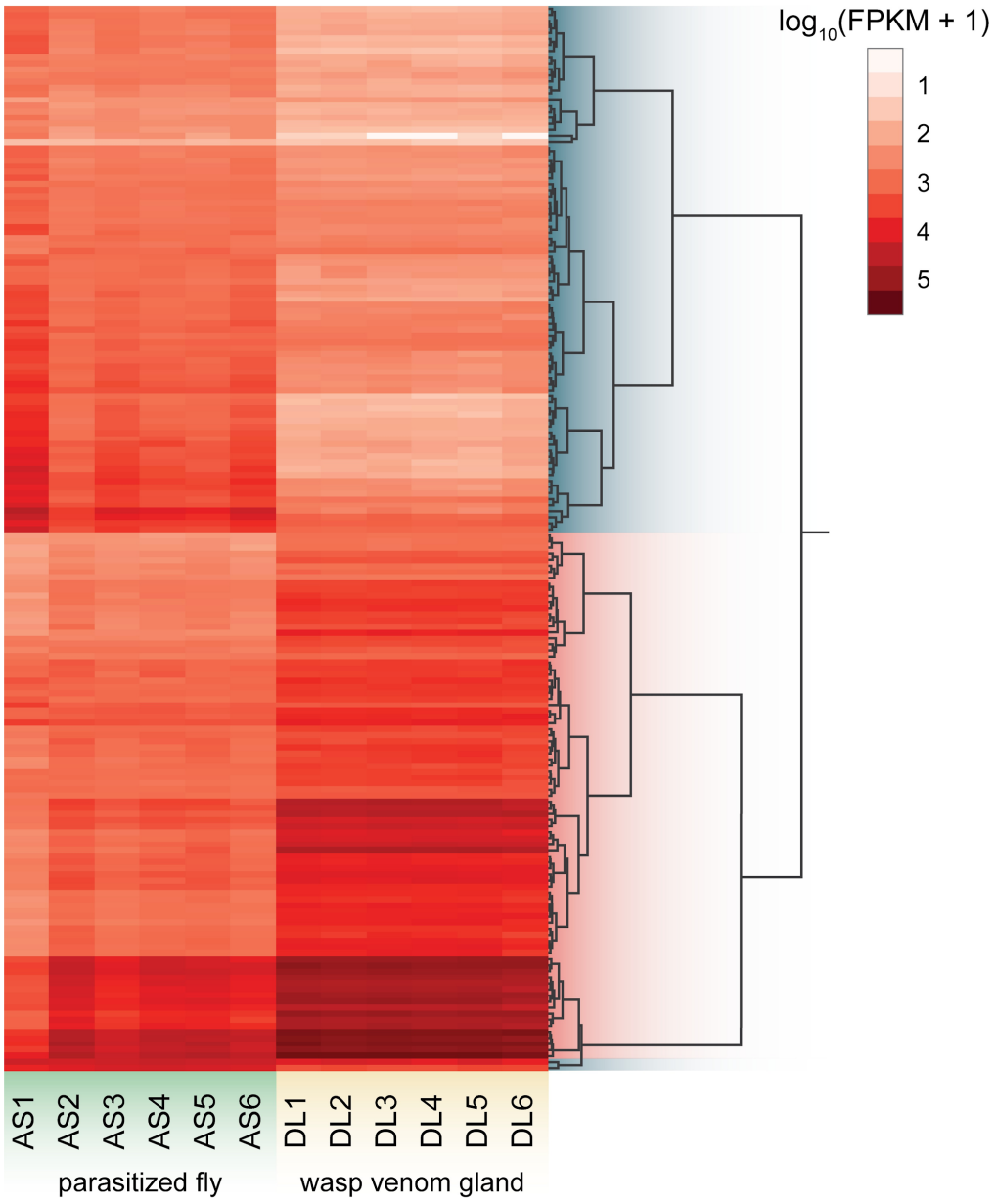

Figure 3.5. DlEPV shows widespread differential expression during replication in *D. longicaudata* wasps compared to *A. suspensa* flies. Heatmap showing significantly (FDR $q < 0.05$) differentially expressed DlEPV genes in wasp and fly tissue. Each row represents a DlEPV gene, and each column represents that gene's expression in each of the 12 RNA samples. Expression is depicted as the $\log_{10}$ transformed FPKM value. Columns AS1-6 correspond to the 6 parasitized fly RNA replicates, and DL1-6 correspond to the 6 wasp venom gland replicates. Rows were clustered using the Ward method based on similarity in gene expression pattern across the 12 samples: DlEPV genes that were significantly downregulated in wasp tissue are highlighted in blue, and genes that were significantly upregulated in wasp tissue are highlighted in pink.

DNA provides evidence that DlEPV is not integrated into the *D. longicaudata* genome and thus, is not an EVE. qPCR measurements of DlEPV genome copy number normalized by *D. longicaudata* genome copy number provide further support for the non-endogenous status of this virus, because they reveal that DlEPV is not consistently present in all wasp cells, which we would expect if DlEPV was an endogenous provirus. The notion that DlEPV is not integrated into the *D. longicaudata* genome is perhaps not surprising given the atypical replication strategy of other poxviruses. The family *Poxviridae* is unique among most other DNA viruses, as the poxvirus replication cycle is completed entirely within the cytoplasm of infected cells and does not require localization to the nucleus or integration of viral DNA into the host genome [38]. Nevertheless, the exogenous nature of the DlEPV genome contrasts starkly with the integrated and dispersed viral genome architecture of PDVs and other parasitoid EVEs [14,22].

Our DlEPV genome assembly is similar in total length to other poxvirus genomes, and our annotation of the DlEPV genome yielded the majority of conserved poxvirus core genes, indicating that we have successfully obtained the entire viral genome sequence. However, 4 of the 49 poxvirus core genes were not identified through sequence similarity searches. Given the absence of these genes in the *D. longicaudata* venom gland transcriptome, it is unlikely that these genes have integrated into the wasp genome. In addition, the high sequence divergence of identifiable DlEPV core genes displayed in our phylogeny suggests that the missing core genes still reside within the DlEPV genome but have diverged in sequence past the point of detection by our search methods. Furthermore, the similar level of sequence divergence in Yalta virus core genes, combined with the mutual absence of 2 core genes between the DlEPV and Yalta virus genomes support a lineage-specific divergence of these core genes. We can not rule out, however, that these genes may have instead been lost entirely from this poxvirus lineage and are not required for successful infection and replication within their respective hosts.

DlEPV may have originated as a fly pathogen. Several EPVs with dipteran hosts have been described, but most representatives have been isolated from chironomid midges or mosquitoes and lack genetic sequence data [35]. Yalta virus is the first dipteran poxvirus isolated from the higher flies (suborder Brachycera), which also contains the tephritid fruit flies that serve as hosts for *D. longicaudata* wasps. The close relationship found between DlEPV and Yalta virus in this investigation supports the hypothesis that DlEPV arose from a fly pathogen. However, more taxonomic sampling of fly poxvirus genomes would be required to rule out the possibility that Yalta virus is instead a remnant parasitoid virus within a drosophilid host. How the DlEPV progenitor was acquired by the *D. longicaudata* lineage is unknown but could have occurred through a variety of events, since parasitoid wasps can come into contact with the pathogens of their hosts during development, as well as adulthood. For example, ascoviruses are pathogenic insect DNA viruses exclusively vectored to new lepidopteran hosts via contamination of adult parasitoid wasp ovipositors that are used to lay eggs within them [86]. The precise origin of most other parasitoid viruses and EVEs remains somewhat obscure due to limited taxonomic sampling of closely related insect DNA viruses, but many are suspected to be derived from pathogens of the parasitoids' host insects [87]. The recent discoveries of hymenopteran and *Drosophila* poxviruses have allowed us to conduct a closer examination that suggests DlEPV originated as a pathogen from a host fly of the *D. longicaudata* ancestor. DlEPV thus provides more evidence for how viruses can be acquired by parasitoid wasps and lead to symbiogenesis events.

The genomic differences of DlEPV and Yalta virus compared to other sequenced EPVs suggest that insect poxviruses are more diverse than originally understood. The proximity of DlEPV and Yalta virus to CPVs in our unrooted phylogeny (Figure 3.2) makes the once clear divide between the EPV and CPV subfamilies appear more ambiguous. Other genomic features shared by DlEPV and Yalta virus differ from EPVs, such as their nucleotide base composition and

gene content. Both DlEPV and Yalta virus contain higher than average GC content compared to other EPVs and therefore are more similar to CPVs, which can range widely in GC content from 25-65% [45]. The approximate 40% GC estimated for the *D. longicaudata* genome could also contribute to the elevated GC composition of DlEPV, in particular, due to the vertical transmission of this virus within an insect host to which other EPVs are not subjected. Additionally, apart from the core genes shared by all poxviruses, DlEPV and Yalta virus contain very few of the additional 50 genes shared by all EPVs [40,42]. One notably absent EPV-specific core gene from both DlEPV and Yalta virus genomes is that which encodes the protein spheroidin. This protein is not found in CPVs but is the main component of the characteristic EPV spheroid occlusion bodies, which are thought to protect EPV virions from environmental inactivation agents, such as UV light [88]. While the spheroidin gene may be divergent to a point beyond detection, a second scenario is that spheroidin is truly absent and not required for successful transmission of these viruses.

Transcriptomic data support DlEPV functional dichotomy and genomic adaptations. The ability of DlEPV to replicate within both wasps and flies but only cause pathogenic effects during fly replication implies that DlEPV virulence is mitigated during virus replication in wasps. This strategy would promote a more stable association between virus and wasp, but as DlEPV is not endogenous, the viral genome integration and dispersion observed in other parasitoid viruses fails to explain how DlEPV completes nonpathogenic replication within wasp tissue. We looked at differential viral gene expression during replication in wasps and flies as an alternative mechanism that might corroborate the selective virulence phenotype of DlEPV. Our findings demonstrate that DlEPV transcriptional activity varies largely during replication in wasps compared to flies, supporting a promotion of virus replication and inhibition of virulence in wasp tissue. These distinct expression patterns are correlated to the different predicted roles of the virus in its two hosts: maximum virus replication in wasp tissue produces an abundance of virions for injection

into hosts during oviposition, and restriction of virulence to fly tissue manipulates the host physiology for successful parasitism by the developing wasp. Of note, putative virulence genes, such as the BRO genes, were expressed at extremely low levels compared to other DlEPV genes during virus replication in the venom gland, and they represented many of the most differentially expressed viral genes in wasps compared to flies. We hypothesize that regulatory mechanisms exist within *D. longicaudata* that suppress BRO and other virulence gene expression during virus replication in the venom gland to deter viral pathogenicity. Possible measures of DlEPV control such as this warrant future study, because they differ from what is observed in PDV systems. The differential DlEPV gene expression reported here thus represents convergent evolution with endogenous parasitoid viruses to maintain a separation of viral function that aligns with parasitoid wasp survival and fitness.

Results from our transcriptomes also hint that DlEPV genomic features, such as the BRO gene expansion, are adaptive to symbiotic life. The DlEPV BRO genes are far more extensive in copy number than observed in other EPVs, likely due to a large tandem duplication in the DlEPV genome center. In addition, the majority of DlEPV BRO genes demonstrated upregulation in fly tissue, supporting their involvement in virulence within the fly hosts of *D. longicaudata* wasps. Poxviruses have experimentally been shown to undergo rapid, tandem virulence gene duplications as a means of adaptation [89,90]. Therefore, tandem duplication of the BRO genes may be adaptive to the success of DlEPV, or by extension, the success of the developing wasp that is also fighting for survival within the fly host. Similar to DlEPV, PDV genomes contain large gene families with members that function as host virulence factors, such as the protein tyrosine phosphatases (PTPs) [91]. Several PTP members arose by tandem duplication events, and some also show evidence for positive selection [92]. The gene duplications in DlEPV and PDV genomes may therefore

represent similar adaptations to respective hosts due to the shared selective pressures accompanied by their associations with parasitoid wasps.

DlEPV is a true mutualistic viral symbiont of parasitoid wasps. Heritable associations between insects and beneficial microbes are often highly complex, due to the unconventional evolutionary forces that act on host-associated microbes [93]. Vertically transmitted bacterial symbionts that are completely restricted to live within insect hosts often exhibit extreme genome degradation compared to their free-living relatives [94,95]. Even though this genomic decay causes the bacteria to become dependent on their host for survival, the symbionts still maintain an exogenous genome that replicates and evolves separately from the host genome [94,95]. PDVs are similar to many of these bacteria in that they provide essential functions for their parasitoid wasp hosts. However, PDVs are less commonly considered to be true symbionts, because they do not contain a replicative genome external to the wasp genome [96]. Until now, genetic characterization of heritable parasitoid viruses has challenged the very notion of a 'viral symbiont' given the shared endogenous nature of those currently described. Furthermore, known examples of heritable viruses that are not endogenous, such as DpTV and DcPV, remain to be definitively demonstrated as mutualistic. DlEPV is thus an unprecedented example of a virus that fully meets the requirements of a heritable mutualistic symbiont, including an exogenous genome and a beneficial function within *D. longicaudata* wasps. As the first genuine mutualistic viral symbiont of parasitoid wasps to be characterized, DlEPV shows promise as a tractable system from which to gain valuable knowledge on the viral side of microbial mutualism in insects.

3.6 MATERIALS AND METHODS

Viral genome sequencing and assembly. *D. longicaudata* wasps and *A. suspensa* flies were reared as previously described [97]. Dissected venom glands from six *D. longicaudata* adult female wasps were pooled into one sample to enrich for DlEPV DNA, followed by phenol:chloroform DNA extraction as reported previously [34]. The resulting DNA was subjected to both Pacific BioSciences (PacBio) and Illumina technologies to sequence the DlEPV genome. 7.5 μg of venom gland DNA was used to make a 10 kb insert size library using PacBio standard SMRT library construction chemistry. The PacBio library was sequenced using a 120 min movie on one SMRT cell. PacBio data were analyzed using the smrtanalysis-2.2.0 Amazon Machine Image hosted on Amazon Web Services. 150,283 PacBio reads were filtered to retain 78,011 reads with a minimum read score of 0.8 and length of 100 bp. 615 long reads (>6 kb in length) were pre-assembled and error-corrected by aligning short reads (>500 bp) to the longer reads and taking the consensus with HGAP v3.0 [98]. Following this, 522 long error-corrected reads with an N50 of 9,071 bp were assembled into 19 unitigs to form a draft assembly using the Celera Assembler [99]. All reads were aligned to the assembly to give coverage reports and perform polishing with Quiver from SMRT Analysis v2.2. Each unitig was split into 3 kb pieces and analyzed with blastx against the NCBI non-redundant (nr) protein sequence database (downloaded in September 2014). 7 unitigs of putative EPV origin were selected based upon BLAST results and unitig depth of sequence coverage. These unitigs were compared to each other using blastn, which revealed sets that were almost identical and completely nested within each other, and may have been split apart during assembly due to differing numbers of short repeat sequences in each assembled unitig. The nested unitigs were excluded to retain the longest version of each sequence, resulting in three final

unitigs. These unitigs contained areas of overlap ranging from 5-10 kb in length, which were assembled manually to form the full DlEPV genome.

Illumina-compatible library construction was performed using 1 μg of venom gland DNA and the standard protocol with Kapa Biosystems DNA library preparation chemistry. The library was sequenced with 8.6 million 75 bp paired-end reads on an Illumina MiSeq instrument at the Georgia Genomics and Bioinformatics Core (GGBC). Reads were filtered with the fastx toolkit (hannonlab.cshl.edu/fastx_toolkit/) to retain reads with >90% of bases with a PHRED score of 30 or higher for both reads in a pair. 6,243,436 read pairs were mapped against the DlEPV genome assembly with bowtie2 v2.2.4 [100] to correct any potential errors that arose from PacBio sequencing. Variant SNPs, insertions, and deletions present in the Illumina short read alignment were identified with SAMtools v1.0 [101], as well as through manual inspection. 140 total corrections were made to the reference genome.

DlEPV genome annotation. ORFs with a methionine start codon and a length of at least 50 amino acids were predicted using a combination of Prokka v1.6 [102] and Artemis v16 [103]. ORFs with highly repetitive amino acid sequences were manually discarded as illegitimate proteins. The remaining ORFs were subjected to blastp protein searches against the nr database (downloaded in September 2019), as well as custom BLAST databases composed of poxvirus proteins (taxid: 10240) or strictly EPV proteins (taxid: 10284). Conserved protein domain searches were also conducted against the Pfam database using hmmsearch from HMMER v3.1b1 [104]. An *e*-value cutoff of 0.001 for blastp searches and 0.01 for Pfam searches were used for the bulk of viral gene annotations. These combined searches provided putative functions for 88 of the 193 identified DlEPV ORFs, including 44 of the 49 poxvirus core genes.

To find distant homologs to the poxvirus core genes missed by initial blastp and Pfam searches, we looked for possible matches to the core genes that were beyond our original *e*-value cutoffs but

found no additional hits despite these relaxed blastp and Pfam search parameters. We then scoured the DlEPV gene set using core gene hidden Markov model (HMM) searches. HMMs were constructed for each poxvirus core gene by aligning amino acid sequences from available EPV and VACV orthologs with MAFFT v7.215 using the --auto alignment setting [105] and hmmbuild from HMMER. Each HMM was then queried against all DlEPV protein sequences with hmmscan. Only one additional core gene, the L5R entry/fusion membrane protein (DLEV060), was identified from this approach. We also queried our HMMs against intergenic regions of the DlEPV genome to identify core genes that may have been pseudogenized but found no hits from this approach.

A *de novo* transcriptome assembly was generated from previously published venom gland RNA-seq reads (accession GSE144541) to check if the four remaining core genes had integrated into the *D. longicaudata* genome. First, bowtie2 v2.2.4 was used to map quality filtered reads from the venom gland transcriptome to the DlEPV genome. Reads that failed to map to the reference genome were collected and fed as input for *de novo* transcript assembly using Trinity v2.0.6 [106]. A BLAST nucleotide database was created from the resulting assembly, and the missing core genes were queried against it with tblastn. We then queried the HMMs of the missing genes against the translated transcriptome assembly with HMMER but found no significant hits from either approach.

Comparative genomic analyses. We used publicly available annotations to calculate genome metrics for the majority of poxvirus genomes featured in this study: ACEV (accession NC_023426), AHEV (NC_021247), AMEV (NC_002520), CBEV (NC_021248), CREV (NC_021249), MSEV (NC_001993), MySEV (NC_021246), Yalta virus (MT364305), VACV (NC_006998), ORFV (NC_005336), MOCV (NC_001731), FWPV (NC_002188), CRV (NC_008030), and SGPV (NC_027707). However, the partial LHEV genome (NC_040577) required re-annotation for use in our analyses. We used Prokka v1.13 to call ORFs within the 46

kb LHEV genome segment and identified 53 total ORFs (Supplemental Table 3.6). BLAST searches against poxvirus and EPV protein databases yielded 18 LHEV ORFs that showed similarity to poxvirus core genes. There were two instances in which two adjacent ORFs had sequence similarity to opposite ends of the same core gene. In both cases, the two ORFs had the same strand orientation and were separated by a single frame shift. Therefore, we assumed the original core gene was incorrectly split into two ORFs due to a single nucleotide sequence error. A total of 16 unique core genes were thus identified from the partial LHEV genome.

Nucleotide composition (% GC) was estimated for the *D. longicaudata* and *A. suspensa* genomes using RNA-seq transcriptomes that were generated as described below. For each species, Trinity was used to construct a *de novo* assembly from RNA reads that failed to map to the DlEPV genome combined for all 6 RNA replicate samples per insect. GC content was then calculated from the resulting assemblies.

To generate the poxvirus core gene phylogeny, amino acid sequences for EPV and CPV orthologs of the 16 core genes found in LHEV (Supplemental Table 3.3) were aligned with MAFFT --auto, concatenated using Geneious Prime 2019.2.3 (https://www.geneious.com), and trimmed of alignment positions in which >50% of taxa contained a gap using trimAl v1.4.1 [107]. The ML phylogenetic tree was generated using RAxML v8.2.11 [108], in which the Gamma model of rate heterogeneity and the LG amino acid substitution matrix were utilized.

RNA isolation and RT-qPCR estimation of viral gene expression. DlEPV gene expression in host flies during parasitism was measured by offering third instar fly larvae to 7-day-old adult wasps that had no prior oviposition experience for 2 h. Resulting flies containing a single laid wasp egg (i.e. those with one oviposition scar) were collected at 4-96 hpp. Flies were kept in standard rearing conditions until each sampling time point. Whole fly samples were collected in a guanidine hydrochloride lysis buffer consisting of 4.9M guanidine hydrochloride, 2% sarkosyl, 50 mM Tris-

Cl (pH 7.6), and 10 mM EDTA. Total RNA was isolated using phenol:chloroform, followed by DNase treatment with the TURBO DNA-*free* Kit (Ambion), and elution in 30 μL water. First-strand cDNA was synthesized with 1,000 ng fly RNA according to the Superscript III reverse transcriptase protocol (Invitrogen) using oligo(dT) primers. qPCR reactions were performed as described previously for wasp venom gland DlEPV expression profiling [34]. JMP Pro 14 was used for statistical analysis of RT-qPCR data. One-way ANOVA was used to test for differences in means of biological replicates, and Tukey's HSD was used for multiple comparison tests. Copy numbers were $\log_{10}$ transformed prior to statistical analysis.

Transcriptome sequencing and analysis. Unemerged wasp venom glands were collected in triplicate as was done for our initial venom gland transcriptome to obtain 5 additional venom gland samples for a total of 6 biological replicates. Singly-scarred fly pupae were collected 72 hpp by first removing the developing wasp larva by dissection in PBS. A total of 6 flies were collected in this manner with each specimen representing one biological replicate. Total RNA for sequencing was extracted using the RNeasy Mini Kit (QIAGEN) with on-column DNase digestion, followed by a secondary DNase treatment using the TURBO DNA-*free* Kit (Ambion) after RNA isolation. Illumina-compatible stranded RNA libraries for the 12 samples were constructed at the GGBC with the Kapa Biosystems RNA library preparation chemistry using 3 μg RNA from each fly sample and 1μg RNA from each venom gland sample. Libraries were sequenced using an Illumina NextSeq instrument at the GGBC, which generated an average of 18.5 million and 14.2 million 75 bp paired-end reads for each wasp and fly sample, respectively. Reads were quality filtered using the fastx toolkit as described before with the Illumina DNA sequencing. Quality reads for each sample were separately mapped to the DlEPV reference genome using bowtie2 v2.2.4. Cuffquant from Cufflinks v2.2.1 was used to calculate the average fragments per kilobase of transcript per million mapped reads (FPKM) values for each DlEPV ORF in both wasp and fly tissues, and

Cuffdiff was used to test for differential expression between the two treatments [109]. Differential expression for a gene was considered significant for FDR-adjusted q-values < 0.05 [110]. Hierarchical clustering of the significantly differentially expressed DlEPV genes was performed with JMP Pro 14.

Data Availability. The authors confirm that all data underlying the findings are fully available without restriction. The GenBank accession number for the complete, annotated DlEPV genome is KR095315 (https://www.ncbi.nlm.nih.gov/nuccore/KR095315). Transcriptomic data were deposited in the Gene Expression Omnibus (GEO) under accession number GSE122240 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE122240).

3.7 ACKNOWLEDGMENTS

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3.8 REFERENCES

1. Moran NA. Symbiosis as an adaptive process and source of phenotypic complexity. Proc Natl Acad Sci U S A. 2007;104: 8627–8633.

2. McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Lošo T, Douglas AE, et al. Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci. 2013;110: 3229–3236.

3. Douglas AE. Multiorganismal insects: diversity and function of resident microorganisms. Annu Rev Entomol. 2015;60: 17–34.

4. Baumann P. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microbiol. 2005;59: 155–189.

5. Rio RVM, Attardo GM, Weiss BL. Grandeur alliances: symbiont metabolic integration and obligate arthropod hematophagy. Trends Parasitol. 2016;32: 739–749.

6. Engel P, Moran NA. The gut microbiota of insects – diversity in structure and function. FEMS Microbiol Rev. 2013;37: 699–735.

7. Roossinck MJ. The good viruses: viral mutualistic symbioses. Nat Rev Microbiol. 2011;9: 99–108.

8. Gauthier J, Drezen J-M, Herniou EA. The recurrent domestication of viruses: major evolutionary transitions in parasitic wasps. Parasitology. 2018;145: 713–723.

9. Dicke M, Cusumano A, Poelman EH. Microbial symbionts of parasitoids. Annu Rev Entomol. 2020;65: 171–190.

10. Strand MR, Pech LL. Immunological basis for compatibility in parasitoid host relationships. Annu Rev Entomol. 1995;40: 31–56.

11. Pennacchio F, Strand MR. Evolution of developmental strategies in parasitic Hymenoptera. Annu Rev Entomol. 2006;51: 233–258.

12. Asgari S, Rivers DB. Venom proteins from endoparasitoid wasps and their role in host-parasite interactions. Annu Rev Entomol. 2011;56: 313–335.

13. Strand MR, Burke GR. Polydnavirus-wasp associations: evolution, genome organization, and function. Curr Opin Virol. 2013;3: 587–594.

14. Drezen J-M, Leobold M, Bézier A, Huguet E, Volkoff A-N, Herniou EA. Endogenous viruses of parasitic wasps: variations on a common theme. Curr Opin Virol. 2017;25: 41–48.

15. Beckage NE. Polydnaviruses as endocrine regulators. In: Beckage NE, Drezen J-M, editors. Parasitoid Viruses. San Diego, CA: Academic Press; 2012. pp. 163–168.

16. Strand MR. Polydnavirus gene products that interact with the host immune system. In: Beckage NE, Drezen J-M, editors. Parasitoid Viruses. San Diego, CA: Academic Press; 2012. pp. 149–161.

17. Bézier A, Annaheim M, Herbinière J, Wetterwald C, Gyapay G, Bernard-Samain S, et al. Polydnaviruses of braconid wasps derive from an ancestral nudivirus. Science. 2009;323: 926–930.

18. Volkoff A-N, Jouan V, Urbach S, Samain S, Bergoin M, Wincker P, et al. Analysis of virion structural components reveals vestiges of the ancestral ichnovirus genome. PLoS Pathog. 2010;6: e1000923.

19. Burke GR, Walden KKO, Whitfield JB, Robertson HM, Strand MR. Widespread genome reorganization of an obligate virus mutualist. PLoS Genet. 2014;10: e1004660.

20. Béliveau C, Cohen A, Stewart D, Periquet G, Djoumad A, Kuhn L, et al. Genomic and proteomic analyses indicate that banchine and campoplegine polydnaviruses have similar, if not identical, viral ancestors. J Virol. 2015;89: 8909–8921.

21. Pichon A, Bézier A, Urbach S, Aury JM, Jouan V, Ravallec M, et al. Recurrent DNA virus domestication leading to different parasite virulence strategies. Sci Adv. 2015;1: e1501150.

22. Burke GR, Simmonds TJ, Sharanowski BJ, Geib SM. Rapid viral symbiogenesis via changes in parasitoid wasp genome architecture. Mol Biol Evol. 2018;35: 2463–2474.

23. Burke GR. Common themes in three independently derived endogenous nudivirus elements in parasitoid wasps. Curr Opin Insect Sci. 2019;32: 28–35.

24. Strand MR. Polydnaviruses. In: Asgari S, Johnson KN, editors. Insect Virology. Norfolk, UK: Caister Academic Press; 2010. pp. 171–197.

25. Herniou EA, Huguet E, Thézé J, Bézier A, Periquet G, Drezen J-M. When parasitic wasps hijacked viruses: genomic and functional evolution of polydnaviruses. Philos Trans R Soc Lond B Biol Sci. 2013;368: 20130051.

26. Strand MR, Burke GR. Polydnaviruses as symbionts and gene delivery systems. PLoS Pathog. 2012;8: e1002757.

27. Strand MR, Burke GR. Polydnaviruses: nature's genetic engineers. Annu Rev Virol. 2014;1: 333–354.

28. Bigot Y, Rabouille A, Doury G, Sizaret PY, Delbost F, Hamelin MH, et al. Biological and molecular features of the relationships between Diadromus pulchellus ascovirus, a parasitoid hymenopteran wasp (*Diadromus pulchellus*) and its lepidopteran host, *Acrolepiopsis assectella*. J Gen Virol. 1997;78: 1149–1163.

29. Renault S, Petit A, Benedet F, Bigot S, Bigot Y. Effects of the Diadromus pulchellus ascovirus, DpAV-4, on the hemocytic encapsulation response and capsule melanization of the leek-moth pupa, *Acrolepiopsis assectella*. J Insect Physiol. 2002;48: 297–302.

30. Bigot Y, Renault S, Nicolas J, Moundras C, Demattei MV, Samain S, et al. Symbiotic virus at the evolutionary intersection of three types of large DNA viruses; iridoviruses, ascoviruses, and ichnoviruses. PLoS One. 2009;4: e6397.

31. Dheilly NM, Maure F, Ravallec M, Galinier R, Doyon J, Duval D, et al. Who is the puppet master? Replication of a parasitic wasp-associated virus correlates with host behaviour manipulation. Proc R Soc Lond B Biol Sci. 2015;282: 20142773.

32. Edson KM, Barlin MR, Vinson SB. Venom apparatus of braconid wasps: comparative ultrastructure of reservoirs and gland filaments. Toxicon. 1982;20: 553–562.

33. Lawrence PO, Akin D. Virus-like particles from the poison glands of the parasitic wasp *Biosteres longicaudatus* (Hymenoptera: Braconidae). Can J Zool. 1990;68: 539–546.

34. Coffman KA, Harrell TC, Burke GR. A mutualistic poxvirus exhibits convergent evolution with other heritable viruses in parasitoid wasps. J Virol. 2020;94: e02059–19.

35. Skinner MA, Buller RM, Damon IK, Lefkowitz EJ, McFadden G, McInnes CJ, et al. Poxviridae. In: King AMQ, Lefkowitz E, Adams MJ, Carstens EB, editors. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier; 2012. pp. 291–309.

36. Smith GL. Genus *Orthopoxvirus*: vaccinia virus. In: Mercer AA, Schmidt A, Weber O, editors. Poxviruses. Basel, CH: Birkhäuser Verlag; 2007. pp. 1–45.

37. Perera S, Li Z, Pavlik L, Arif B. Entomopoxviruses. In: Asgari S, Johnson KN, editors. Insect Virology. Norfolk, UK: Caister Academic Press; 2010. pp. 83–102.

38. Moss B. Poxviridae: the viruses and their replication. 5th ed. In: Knipe DM, Howley PM, editors. Fields Virology. 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2007. pp. 2905–2945.

39. Bawden AL, Glassberg KJ, Diggans J, Shaw R, Farmerie W, Moyer RW. Complete genomic sequence of the Amsacta moorei entomopoxvirus: analysis and comparison with other poxviruses. Virology. 2000;274: 120–139.

40. Thézé J, Takatsuka J, Li Z, Gallais J, Doucet D, Arif B, et al. New insights into the evolution of *Entomopoxvirinae* from the complete genome sequences of four entomopoxviruses infecting *Adoxophyes honmai*, *Choristoneura biennis*, *Choristoneura rosaceana*, and *Mythimna separata*. J Virol. 2013;87: 7992–8003.

41. Afonso CL, Tulman ER, Lu Z, Oma E, Kutish GF, Rock DL. The genome of Melanoplus sanguinipes entomopoxvirus. J Virol. 1999;73: 533–552.

42. Mitsuhashi W, Miyamoto K, Wada S. The complete genome sequence of the *Alphaentomopoxvirus* Anomala cuprea entomopoxvirus, including its terminal hairpin loop sequences, suggests a potentially unique mode of apoptosis inhibition and mode of DNA replication. Virology. 2014;452: 95–116.

43. Viljakainen L, Holmberg I, Abril S, Jurvansuu J. Viruses of invasive Argentine ants from the European main supercolony: characterization, interactions and evolution. J Gen Virol. 2018;99: 1129–1140.

44. Wallace MA, Coffman KA, Gilbert C, Ravindran S, Albery GF, Abbott J, et al. The discovery, distribution and diversity of DNA viruses associated with *Drosophila melanogaster* in Europe. bioRxiv [Preprint]. 2020; 2020.10.16.342956.

45. Lefkowitz EJ, Wang C, Upton C. Poxviruses: past, present and future. Virus Res. 2006;117: 105–118.

46. Shackelton LA, Parrish CR, Holmes EC. Evolutionary basis of codon usage and nucleotide composition bias in vertebrate DNA viruses. J Mol Evol. 2006;62: 551–563.

47. Upton C, Slack S, Hunter AL, Ehlers A, Roper RL. Poxvirus orthologous clusters: toward defining the minimum essential poxvirus genome. J Virol. 2003;77: 7590–7600.

48. Gubser C, Hué S, Kellam P, Smith GL. Poxvirus genomes: a phylogenetic analysis. J Gen Virol. 2004;85: 105–117.

49. Goebel SJ, Johnson GP, Perkus ME, Davis SW, Winslow JP, Paoletti E. The complete DNA sequence of vaccinia virus. Virology. 1990;179: 247–266.

50. Lin CL, Chung CS, Heine HG, Chang W. Vaccinia virus envelope H3L protein binds to cell surface heparan sulfate and is important for intracellular mature virion morphogenesis and virus infection in vitro and in vivo. J Virol. 2000;74: 3353–3365.

51. da Fonseca FG, Wolffe EJ, Weisberg A, Moss B. Characterization of the vaccinia virus H3L envelope protein: topology and posttranslational membrane insertion via the C-terminal hydrophobic tail. J Virol. 2000;74: 7508–7517.

52. da Fonseca FG, Wolffe EJ, Weisberg A, Moss B. Effects of deletion or stringent repression of the H3L envelope gene on vaccinia virus replication. J Virol. 2000;74: 7518–7528.

53. Boyd O, Turner PC, Moyer RW, Condit RC, Moussatche N. The E6 protein from vaccinia virus is required for the formation of immature virions. Virology. 2010;399: 201–211.

54. Condit RC, Moussatche N. The vaccinia virus E6 protein influences virion protein localization during virus assembly. Virology. 2015;482: 147–156.

55. Senkevich TG, Wyatt LS, Weisberg AS, Koonin EV, Moss B. A conserved poxvirus NlpC/P60 superfamily protein contributes to vaccinia virus virulence in mice but not to replication in cell culture. Virology. 2008;374: 506–514.

56. Broyles SS. Vaccinia virus transcription. J Gen Virol. 2003;84: 2293–2303.

57. Koonin EV, Yutin N. Evolution of the Large Nucleocytoplasmic DNA Viruses of Eukaryotes and Convergent Origins of Viral Gigantism. In: Kielian M, Mettenleiter TC, Roossinck MJ, editors. Advances in Virus Research. 2019. pp. 167–202.

58. McLysaght A, Baldi PF, Gaut BS. Extensive gene gain associated with adaptive evolution of poxviruses. Proc Natl Acad Sci. 2003;100: 15655–15660.

59. Nazarian SH, McFadden G. Immunomodulation by poxviruses. In: Mercer AA, Schmidt A, Weber O, editors. Poxviruses. Basel, CH: Birkhäuser Verlag; 2007. pp. 273–296.

60. Iyer LA, Balaji S, Koonin EV, Aravind L. Evolutionary genomics of nucleo-cytoplasmic large DNA viruses. Virus Res. 2006;117: 156–184.

61. Bideshi DK, Renault S, Stasiak K, Federici BA, Bigot Y. Phylogenetic analysis and possible function of bro-like genes, a multigene family widespread among large double-stranded DNA viruses of invertebrates and bacteria. J Gen Virol. 2003;84: 2531–2544.

62. Kang WY, Suzuki M, Zemskov E, Okano K, Maeda S. Characterization of baculovirus repeated open reading frames (bro) in Bombyx mori nucleopolyhedrovirus. J Virol. 1999;73: 10339–10345.

63. Zemskov EA, Kang WK, Maeda S. Evidence for nucleic acid binding ability and nucleosome association of Bombyx mori nucleopolyhedrovirus BRO proteins. J Virol. 2000;74: 6784–6789.

64. Anderson EP. Nucleoside and nucleotide kinases. In: Boyer PD, editor. The Enzymes. Academic Press; 1973. pp. 49–96.

65. Gentry GA. Viral thymidine kinases and their relatives. Pharmacol Ther. 1992;54: 319–355.

66. Buller RML, Smith GL, Cremer K, Notkins AL, Moss B. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature. 1985;317: 813–815.

67. Bai C, Sen P, Hofmann K, Ma L, Goebl M, Harper JW, et al. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell. 1996;86: 263–274.

68. Skowyra D, Craig KL, Tyers M, Elledge SJ, Harper JW. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell. 1997;91: 209–219.

69. Kipreos ET, Pagano M. The F-box protein family. Genome Biol. 2000;1: reviews3002.

70. Camus-Bouclainville C, Fiette L, Bouchiha S, Pignolet A, Counor D, Filipe U, et al. A virulence factor of myxoma virus colocalizes with NF-kappa B in the nucleus and interferes with inflammation. J Virol. 2004;78: 2510–2516.

71. Mercer AA, Fleming SB, Ueda N. F-box-like domains are present in most poxvirus ankyrin repeat proteins. Virus Genes. 2005;31: 127–133.

72. Hsiao JC, Chao CC, Young MJ, Chang YT, Cho EC, Chang W. A poxvirus host range protein, CP77, binds to a cellular protein, HMG20A, and regulates its dissociation from the vaccinia virus genome in CHO-K1 cells. J Virol. 2006;80: 7714–7728.

73. Sonnberg S, Seet BT, Pawson T, Fleming SB, Mercer AA. Poxvirus ankyrin repeat proteins are a unique class of F-box proteins that associate with cellular SCF1 ubiquitin ligase complexes. Proc Natl Acad Sci. 2008;105: 10955–10960.

74. Mohamed MR, Rahman MM, Lanchbury JS, Shattuck D, Neff C, Dufford M, et al. Proteomic screening of variola virus reveals a unique NF-kappa B inhibitor that is highly conserved among pathogenic orthopoxviruses. Proc Natl Acad Sci. 2009;106: 9045–9050.

75. Jin JP, Cardozo T, Lovering RC, Elledge SJ, Pagano M, Harper JW. Systematic analysis and nomenclature of mammalian F-box proteins. Genes Dev. 2004;18: 2573–2580.

76. Hashimoto Y, Lawrence PO. Comparative analysis of selected genes from Diachasmimorpha longicaudata entomopoxvirus and other poxviruses. J Insect Physiol. 2005;51: 207–220.

77. Falabella P, Riviello L, Caccialupi P, Rossodivita T, Valente MT, De Stradis ML, et al. A gamma-glutamyl transpeptidase of *Aphidius ervi* venom induces apoptosis in the ovaries of host aphids. Insect Biochem Mol Biol. 2007;37: 453–465.

78. Voth DE, Broederdorf LJ, Graham JG. Bacterial Type IV secretion systems: versatile virulence machines. Future Microbiol. 2012;7: 241–257.

79. Grohmann E, Christie PJ, Waksman G, Backert S. Type IV secretion in Gram-negative and Gram-positive bacteria. Mol Microbiol. 2018;107: 455–471.

80. Zhang X-H, Austin B. Haemolysins in *Vibrio* species. J Appl Microbiol. 2005;98: 1011–1019.

81. Yang ZL, Bruno DP, Martens CA, Porcella SF, Moss B. Simultaneous high-resolution analysis of vaccinia virus and host cell transcriptomes by deep RNA sequencing. Proc Natl Acad Sci. 2010;107: 11513–11518.

82. Yang ZL, Cao S, Martens CA, Porcella SF, Xie Z, Ma M, et al. Deciphering poxvirus gene expression by RNA sequencing and ribosome profiling. J Virol. 2015;89: 6874–6886.

83. Broyles SS, Yuen L, Shuman S, Moss B. Purification of a factor required for transcription of vaccinia virus early genes. J Biol Chem. 1988;263: 10754–10760.

84. Broyles SS, Li J, Moss B. Promoter DNA contacts made by the vaccinia virus early transcription factor. J Biol Chem. 1991;266: 15539–15544.

85. Davison AJ, Moss B. Structure of vaccinia virus early promoters. J Mol Biol. 1989;210: 749–769.

86. Bideshi DK, Bigot Y, Federici BA, Spears T. Ascoviruses. In: Asgari S, Johnson KN, editors. Insect Virology. Caister Academic Press; 2010. pp. 3–34.

87. Whitfield JB, Asgari S. Virus or not? Phylogenetics of polydnaviruses and their wasp carriers. J Insect Physiol. 2003;49: 397–405.

88. Becker MN, Moyer RW. Subfamily *Entomopoxvirinae*. In: Mercer AA, Schmidt A, Weber O, editors. Poxviruses. Basel, CH: Birkhäuser Verlag; 2007. pp. 253–271.

89. Elde NC, Child SJ, Eickbush MT, Kitzman JO, Rogers KS, Shendure J, et al. Poxviruses deploy genomic accordions to adapt rapidly against host antiviral defenses. Cell. 2012;150: 831–841.

90. Cone KR, Kronenberg ZN, Yandell M, Elde NC. Emergence of a viral RNA polymerase variant during gene copy number amplification promotes rapid evolution of vaccinia virus. J Virol. 2017;91: e01428–16.

91. Burke GR, Strand MR. Polydnaviruses of parasitic wasps: domestication of viruses to act as gene delivery vectors. Insects. 2012;3: 91–119.

92. Serbielle C, Dupas S, Perdereau E, Héricourt F, Dupuy C, Huguet E, et al. Evolutionary mechanisms driving the evolution of a large polydnavirus gene family coding for protein tyrosine phosphatases. BMC Evol Biol. 2012;12: 253.

93. Moran NA, McCutcheon JP, Nakabachi A. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet. 2008;42: 165–190.

94. McCutcheon JP, Moran NA. Extreme genome reduction in symbiotic bacteria. Nat Rev Microbiol. 2012;10: 13–26.

95. Moran NA, Bennett GM. The tiniest tiny genomes. Annu Rev Microbiol. 2014;68: 195–215.

96. Strand MR, Burke GR. Polydnaviruses: Evolution and Function. Curr Issues Mol Biol. 2020;34: 163–182.

97. Simmonds TJ, Carrillo D, Burke GR. Characterization of a venom gland-associated rhabdovirus in the parasitoid wasp *Diachasmimorpha longicaudata*. J Insect Physiol. 2016;91-92: 48–55.

98. Chin C-S, Alexander DH, Marks P, Klammer AA, Drake J, Heiner C, et al. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. Nat Methods. 2013;10: 563–569.

99. Myers EW, Sutton GG, Delcher AL, Dew IM, Fasulo DP, Flanigan MJ, et al. A whole-genome assembly of *Drosophila*. Science. 2000;287: 2196–2204.

100. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9: 357–359.

101. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and SAMtools. Bioinformatics. 2009;25: 2078–2079.

102. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014;30: 2068–2069.

103. Carver T, Harris SR, Berriman M, Parkhill J, McQuillan JA. Artemis: an integrated platform for visualization and analysis of high-throughput sequence-based experimental data. Bioinformatics. 2012;28: 464–469.

104. Eddy SR. Accelerated profile HMM Searches. PLoS Comput Biol. 2011;7: e1002195.

105. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013;30: 772–780.

106. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011;29: 644–652.

107. Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics. 2009;25: 1972–1973.

108. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30: 1312–1313.

109. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc. 2012;7: 562–578.

110. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Series B Stat Methodol. 1995;57: 289–300.

111. Lartillot N, Philippe H. A Bayesian mixture model for across-site heterogeneities in the amino-acid replacement process. Mol Biol Evol. 2004;21: 1095–1109.

112. Emms DM, Kelly S. OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. Genome Biol. 2015;16: 157.

Supplemental Table 3.1. Annotated ORFs in the DlEPV genome.

					Best BLAST Hit								
Locus Tag	Start	End	Size (aa)	Gene ID	Accession	Source Species	Size (aa)	Identity (%)	Pfam Domain(s)	VACV Core Gene	Yalta virus Ortholog(s)*	Early Promoter	Putative Function
DLEV001/193	4080	3826	85										Unknown
DLEV002/192	5545	5384	54										Unknown
DLEV003/191	6463	5960	168	BRO protein					Bro-N			Yes	Virulence: BRO Genes
DLEV004/190	7243	6851	131	unknown protein	YP_001649041.1	Helicoverpa armigera granulovirus	684	37					Unknown
DLEV005/189	8847	8389	153										Unknown
DLEV006/188	9621	9208	138									Yes	Virulence: Early Promoter
DLEV007/187	9692	10117	142	deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase)	XP_012261123.1	*Athalia rosae*	151	64	dUTPase		Yalta_053		Replication
DLEV008/186	14962	14165	266									Yes	Virulence: Early Promoter
DLEV009/185	17646	17191	152									Yes	Virulence: Early Promoter
DLEV010	17946	17710	79										Unknown
DLEV011	18885	18370	172									Yes	Virulence: Early Promoter
DLEV012	19683	19330	118	BRO protein					Bro-N			Yes	Virulence: BRO Genes
DLEV013	20800	20048	251						HALZ				Unknown
DLEV014	21247	20822	142	BRO protein	NP_064959.1	Amsacta moorei entomopoxvirus	360	30	Bro-N				Virulence: BRO Genes
DLEV015	21961	21248	238									Yes	Virulence: Early Promoter
DLEV016	22072	22818	249	unknown protein	XP_004224708.1	*Plasmodium cynomolgi* strain B	1017	32	DUF4638			Yes	Virulence: Early Promoter
DLEV017	23517	22813	235	unknown protein	SPJ09464.1	*Plasmodium* sp. DRC-Itaito	781	33	Gal11_ABD1				Unknown
DLEV018	23561	23788	76										Unknown
DLEV019	24984	23797	396	reverse transcriptase	YP_008003836.1	Adoxophyes honmai entomopoxvirus	283	30	RVT_1		Yalta_167	Yes	Transcription/RNA Modification
DLEV020	28703	27114	530	nucleoside triphosphatase/helicase 1	ARF10049.1	Indivirus ILV1	487	28	IBR		Yalta_006	Yes	Transcription/RNA Modification
DLEV021	28779	30068	430	serine/threonine protein kinase	AAO31700.1	Orf virus strain D1701	497	26	Pox_ser-thr_kin	F10L	Yalta_093		Structure/Morphogenesis
DLEV022	31749	31096	218								Yalta_092		Unknown

					Best BLAST Hit								
Locus Tag	**Start**	**End**	**Size (aa)**	**Gene ID**	**Accession**	**Source Species**	**Size (aa)**	**Identity (%)**	**Pfam Domain(s)**	**VACV Core Gene**	**Yalta virus Ortholog(s)***	**Early Promoter**	**Putative Function**
DLEV023	31778	32713	312								Yalta_089		Unknown
DLEV024	32714	34705	664	viral early transcription factor small subunit (VETFS)	NP_064956.1	Amsacta moorei entomopoxvirus	670	37	SNF2_N	D6R	Yalta_024		Transcription/RNA Modification
DLEV025	36868	36287	194						TraC			Yes	Virulence: Early Promoter
DLEV026	36906	38768	621	metalloprotease	YP_009268770.1	Pteropox virus	593	25		G1L	Yalta_131		Structure/Morphogenesis
DLEV027	38769	39014	82										Unknown
DLEV028	42741	40258	828	ribonucleoside-diphosphate reductase large subunit (RNRL)	OJU81738.1	*Chlamydia sp.* 32-24	771	30	Ribonuc_red_lgC		Yalta_121	Yes	Replication
DLEV029	44772	44047	242	RNA polymerase 18kDa subunit (RPO18)	YP_009001517.1	Anomala cuprea entomopoxvirus	180	31	Pox_RNA_pol	D7R	Yalta_120	Yes	Transcription/RNA Modification
DLEV030	45089	44787	101										Unknown
DLEV031	46149	45148	334	ribonucleoside-diphosphate reductase small subunit (RNRS)	AEO98243.1	Emiliania huxleyi virus 203	325	28	Ribonuc_red_sm		Yalta_116	Yes	Replication
DLEV032	47451	46963	163										Unknown
DLEV033	49666	48401	422								Yalta_142		Unknown
DLEV034	49695	50609	305	mRNA capping enzyme small subunit	NP_048195.1	Melanoplus sanguinipes entomopoxvirus	267	33	Pox_mRNA-cap	D12L	Yalta_143		Transcription/RNA Modification
DLEV035	51032	50598	145	late membrane protein	YP_008004034.1	Adoxophyes honmai entomopoxvirus	139	38	Pox_G9-A16	J5L	Yalta_056		Structure/Morphogenesis
DLEV036	51046	51801	252	ssDNA/dsDNA binding protein					Pox_VP8_L4R	L4R	Yalta_059		Structure/Morphogenesis
DLEV037	54427	52940	496	gamma-glutamyl transpeptidase	XP_018319317.1	*Agrilus planipennis*	613	27	G_glu_transpept			Yes	Virulence: Homology
DLEV038	54439	55401	321	alpha/beta fold hydrolase	WP_052830421.1	*Gynuella sunshinyii*	325	29	Hydrolase_4		Yalta_165		Unknown
DLEV039	58130	55491	880	DNA primase	YP_008003627.1	Mythimna separata entomopoxvirus	730	29	D5_N	D5R	Yalta_152		Replication
DLEV040	58168	58845	226	S-S bond formation pathway protein substrate	ARE67656.1	Shearwaterpox virus	213	31	L1R_F9L	F9L	Yalta_151		Structure/Morphogenesis
DLEV041	58847	59077	77										Unknown
DLEV042	60506	59160	449	virion core cysteine protease	YP_008004293.1	Choristoneura biennis entomopoxvirus	459	30	Peptidase_C57	I7L	Yalta_147		Structure/Morphogenesis

					Best BLAST Hit								
Locus Tag	**Start**	**End**	**Size (aa)**	**Gene ID**	**Accession**	**Source Species**	**Size (aa)**	**Identity (%)**	**Pfam Domain(s)**	**VACV Core Gene**	**Yalta virus Ortholog(s)***	**Early Promoter**	**Putative Function**
DLEV043	60524	61945	474										Unknown
DLEV044	62705	63949	415	FEN1-like nuclease	YP_008003980.1	Adoxophyes honmai entomopoxvirus	414	24		G5R	Yalta_145	Yes	Structure/Morphogenesis
DLEV045	66551	65640	304	adenosine triphosphatase/DNA packaging protein	YP_008003707.1	Mythimna separata entomopoxvirus	238	32	Pox_A32	A32L	Yalta_094		Structure/Morphogenesis
DLEV046	68862	67771	364	myristylated membrane protein	NP_048161.1	Melanoplus sanguinipes entomopoxvirus	380	35	Pox_G9-A16	A16L	Yalta_134		Structure/Morphogenesis
DLEV047	69540	68863	226	uracil DNA glycosylase	OUU96308.1	*Flavobacteriales* bacterium TMED84	220	46	UDG	D4R		Yes	Replication
DLEV048	70287	69913	125										Unknown
DLEV049	70664	70314	117	IMV entry/fusion membrane protein	YP_008004356.1	Choristoneura biennis entomopoxvirus	112	29	Pox_A21	A21L	Yalta_138		Structure/Morphogenesis
DLEV050	71837	70950	296									Yes	Virulence: Early Promoter
DLEV051	71889	73685	599	unknown protein	YP_008004224.1	Choristoneura biennis entomopoxvirus	438	28			Yalta_135		Unknown
DLEV052	74098	73709	130									Yes	Virulence: Early Promoter
DLEV053	76814	75423	464								Yalta_144	Yes	Virulence: Early Promoter
DLEV054	77163	76795	123										Unknown
DLEV055	77168	77728	187	RNA polymerase 19kDa subunit (RPO19)	NP_064948.1	Amsacta moorei entomopoxvirus	237	43		A5R	Yalta_133	Yes	Transcription/RNA Modification
DLEV056	78513	78094	140									Yes	Virulence: Early Promoter
DLEV057	78988	78593	132						PIG-P				Unknown
DLEV058	80923	79784	380	RNA helicase	EYB84565.1	*Ancylostoma ceylanicum*	384	59	DEAD, Helicase_C			Yes	Transcription/RNA Modification
DLEV059	82820	81738	361	viral intermediate transcription factor 3 large subunit (VITF3L)	YP_008003912.1	Adoxophyes honmai entomopoxvirus	353	22		A23R	Yalta_095		Transcription/RNA Modification
DLEV060	82832	83284	151	IMV entry/fusion membrane protein	YP_009480602.1	Sea otter poxvirus	133	28		L5R	Yalta_096		Structure/Morphogenesis
DLEV061	85113	84076	346	internal virion protein	YP_009001617.1	Anomala cuprea entomopoxvirus	346	28	Pox_L3_FP4	L3L	Yalta_060		Transcription/RNA Modification

					Best BLAST Hit								
Locus Tag	**Start**	**End**	**Size (aa)**	**Gene ID**	**Accession**	**Source Species**	**Size (aa)**	**Identity (%)**	**Pfam Domain(s)**	**VACV Core Gene**	**Yalta virus Ortholog(s)***	**Early Promoter**	**Putative Function**
DLEV062	85130	86026	299	myristylated membrane protein	YP_008003859.1	Adoxophyes honmai entomopoxvirus	336	27	Pox_G9-A16	G9R	Yalta_067		Structure/Morphogenesis
DLEV063	87113	86793	107	viral membrane-associated, early morphogenesis protein	NP_064943.1	Amsacta moorei entomopoxvirus	81	33		A9L	Yalta_141	Yes	Structure/Morphogenesis
DLEV064	87119	89479	787	viral early transcription factor large subunit (VETFL)	NP_048134.1	Melanoplus sanguinipes entomopoxvirus	760	25	Pox_VERT_large	A7L	Yalta_140	Yes	Transcription/RNA Modification
DLEV065	89488	90594	369								Yalta_139	Yes	Virulence: Early Promoter
DLEV066	90572	91159	196	entry/fusion membrane protein	YP_009001651.1	Anomala cuprea entomopoxvirus	201	42	Pox_LP_H2	H2R	Yalta_125	Yes	Structure/Morphogenesis
DLEV067	95846	91929	1306	RNA polymerase 147kDa subunit (RPO147)	AAN01131.1	Anomala cuprea entomopoxvirus	1318	30	RNA_pol_Rpb1_1, RNA_pol_Rpb1_2, RNA_pol_Rpb1_3, RNA_pol_Rpb1_4, RNA_pol_Rpb1_5	J6R	Yalta_105	Yes	Transcription/RNA Modification
DLEV068	97178	96780	133								Yalta_087		Unknown
DLEV069	97853	97218	212										Unknown
DLEV070	97858	98571	238										Unknown
DLEV071	98708	98442	89										Unknown
DLEV072	100483	98993	497	NAD-dependent DNA ligase	AYV79438.1	Faunusvirus sp.	640	31	DNA_ligase_OB		Yalta_084	Yes	Replication
DLEV073	102995	102645	117									Yes	Virulence: Early Promoter
DLEV074	103031	103531	167								Yalta_080	Yes	Virulence: Early Promoter
DLEV075	104764	103541	408								Yalta_073		Unknown
DLEV076	105366	104824	181	oligoribonuclease	XP_018347894.1	*Trachymyrmex septentrionalis*	180	48	RNase_T				Transcription/RNA Modification
DLEV077	105404	105892	163										Unknown
DLEV078	107981	106539	481	DNA helicase	NP_073495.1	Yaba-like disease virus	479	29	ResIII, Helicase_C	A18R	Yalta_075		Transcription/RNA Modification
DLEV079	108344	107991	118						CRA				Unknown
DLEV080	108700	108359	114						DUF4094, RILP				Unknown

					Best BLAST Hit								
Locus Tag	**Start**	**End**	**Size (aa)**	**Gene ID**	**Accession**	**Source Species**	**Size (aa)**	**Identity (%)**	**Pfam Domain(s)**	**VACV Core Gene**	**Yalta virus Ortholog(s)***	**Early Promoter**	**Putative Function**
DLEV081	108707	110704	666	nucleoside triphosphatase I	NP_048124.1	Melanoplus sanguinipes entomopoxvirus	647	33	NPHI_C, Helicase_C, SNF2_N	D11R	Yalta_079		Transcription/RNA Modification
DLEV082	111550	111146	135						Bro-N			Yes	Virulence: BRO Genes
DLEV083	112971	112156	272	BRO protein	YP_009046672.1	Armadillidium vulgare iridescent virus	231	32	Bro-N, T5orf172			Yes	Virulence: BRO Genes
DLEV084	114229	113669	187	BRO protein	YP_003517810.1	Lymantria xylina nucleopolyhedrovirus	249	37	Bro-N, T5orf172			Yes	Virulence: BRO Genes
DLEV085	114677	114324	118										Unknown
DLEV086	115026	114751	92									Yes	Virulence: Early Promoter
DLEV087	115835	115125	237										Unknown
DLEV088	116526	115861	222										Unknown
DLEV089	117212	116529	228										Unknown
DLEV090	117716	117384	111										Unknown
DLEV091	119106	118663	148									Yes	Virulence: Early Promoter
DLEV092	119955	119158	266	BRO protein					Bro-N			Yes	Virulence: BRO Genes
DLEV093	121604	121050	185									Yes	Virulence: Early Promoter
DLEV094	122914	122243	224	BRO protein					Bro-N			Yes	Virulence: BRO Genes
DLEV095	123585	123016	190						CC2-LZ			Yes	Virulence: Early Promoter
DLEV096	124627	123779	283	BRO protein	AJP09121.1	Heliothis virescens ascovirus 3f	158	37	Bro-N			Yes	Virulence: BRO Genes
DLEV097	126880	126116	255	BRO protein					Bro-N			Yes	Virulence: BRO Genes
DLEV098	128318	127533	262	BRO protein	AOL56971.1	Chrysodeixis includens nucleopolyhedrovirus	499	32	Bro-N			Yes	Virulence: BRO Genes
DLEV099	129352	128576	259						T4SS			Yes	Virulence: Homology
DLEV100	130617	129940	226	BRO protein	AXN77333.1	Heliothis virescens ascovirus 3i	343	27	Bro-N, T5orf172			Yes	Virulence: BRO Genes
DLEV101	131376	130726	217	BRO protein	WP_069467284.1	*Streptomyces rubidus*	316	57	Bro-N			Yes	Virulence: BRO Genes
DLEV102	131920	131444	159										Unknown
DLEV103	132245	131940	102	BRO protein	YP_009121837.1	Spodoptera frugiperda granulovirus	252	40	Bro-N			Yes	Virulence: BRO Genes

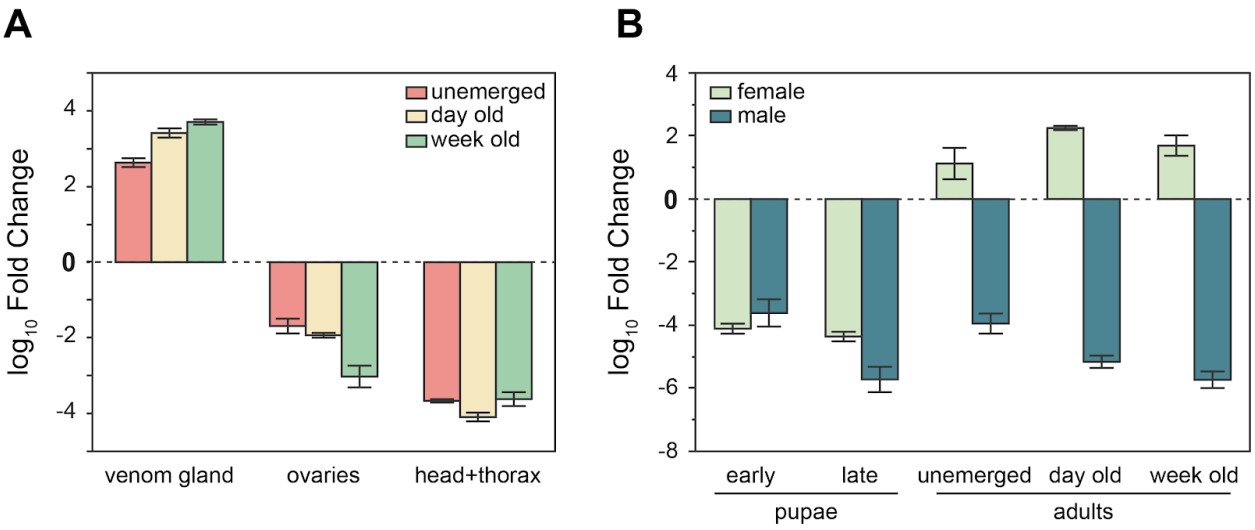
					Best BLAST Hit								
Locus Tag	**Start**	**End**	**Size (aa)**	**Gene ID**	**Accession**	**Source Species**	**Size (aa)**	**Identity (%)**	**Pfam Domain(s)**	**VACV Core Gene**	**Yalta virus Ortholog(s)***	**Early Promoter**	**Putative Function**
DLEV104	133579	133037	181						Uds1				Unknown
DLEV105	134350	133649	234	BRO protein	BBB16628.1	Heliothis virescens ascovirus 3j	496	34	Bro-N, ACCA			Yes	Virulence: BRO Genes
DLEV106	135696	135340	119									Yes	Virulence: Early Promoter
DLEV107	136156	135788	123	BRO protein					Bro-N				Virulence: BRO Genes
DLEV108	137198	136257	314						MIEAP			Yes	Virulence: Early Promoter
DLEV109	137907	137236	224	BRO protein	YP_009121837.1	Spodoptera frugiperda granulovirus	252	31	Bro-N			Yes	Virulence: BRO Genes
DLEV110	140837	139749	363	viral membrane formation protein	NP_064920.1	Amsacta moorei entomopoxvirus	320	28	Phage_int_SAM_6	A11R	Yalta_072		Structure/Morphogenesis
DLEV111	140868	144182	1105	P4a precursor	QGM48880.1	Magpiepox virus	893	30		A10L	Yalta_071		Structure/Morphogenesis
DLEV112	145744	144188	519										Unknown
DLEV113	146491	147072	194	Holliday junction resolvase	YP_008003995.1	Adoxophyes honmai entomopoxvirus	159	29	Pox_A22	A22R	Yalta_069		Replication
DLEV114	149654	147105	850	unknown protein	RUS86699.1	*Elysia chlorotica*	333	27	Phage_XkdX, FlaC_arch, DUF2939		Yalta_068		Unknown
DLEV115	150917	149661	419						RnlB_antitoxin, Phage_T7_tail, CALCOCO1		Yalta_068		Unknown
DLEV116	152432	150933	500								Yalta_068		Unknown
DLEV117	153053	153838	262										Unknown
DLEV118	154369	155607	413	BRO protein	YP_008004169.1	Choristoneura biennis entomopoxvirus	347	30	Bro-N, DUF3627			Yes	Virulence: BRO Genes
DLEV119	156416	156027	130	BRO protein					Bro-N			Yes	Virulence: BRO Genes
DLEV120	156608	156441	56									Yes	Virulence: Early Promoter
DLEV121	157195	156662	178	unknown protein	XP_022536439.1	*Astyanax mexicanus*	1333	32					Unknown
DLEV122	158380	157514	289	BRO protein	YP_003517810.1	Lymantria xylina nucleopolyhedrovirus	249	32	Bro-N				Virulence: BRO Genes
DLEV123	159252	158443	270	BRO protein	AXU41742.1	Spodoptera eridania nucleopolyhedrovirus	281	33	Bro-N			Yes	Virulence: BRO Genes
DLEV124	159401	159817	139										Unknown

					Best BLAST Hit								
Locus Tag	Start	End	Size (aa)	Gene ID	Accession	Source Species	Size (aa)	Identity (%)	Pfam Domain(s)	VACV Core Gene	Yalta virus Ortholog(s)*	Early Promoter	Putative Function
DLEV125	159858	160055	66								Yalta_101	Yes	Virulence: Early Promoter
DLEV126	160511	160044	156	IMV entry/fusion membrane protein	YP_008658542.1	Squirrelpox virus	140	35	Pox_A28	A28L	Yalta_098		Structure/Morphogenesis
DLEV127	160835	160548	96										Unknown
DLEV128	160852	162750	633	poly(A) polymerase large subunit	NP_064820.1	Amsacta moorei entomopoxvirus	573	27	Pox_polyA_pol	E1L	Yalta_100		Transcription/RNA Modification
DLEV129	164286	164474	63								Yalta_097		Unknown
DLEV130	164509	165717	403								Yalta_068		Unknown
DLEV131	166774	167670	299									Yes	Virulence: Early Promoter
DLEV132	168566	167835	244	myristylated membrane protein	YP_009046359.1	Pigeonpox virus	243	37	L1R_F9L	L1R	Yalta_028		Structure/Morphogenesis
DLEV133	168788	168567	74										Unknown
DLEV134	168896	172432	1179	RNA polymerase 132kDa subunit (RPO132)	NP_048226.1	Melanoplus sanguinipes entomopoxvirus	1190	31	RNA_pol_Rpb2_6, RNA_pol_Rpb2_7	A24R	Yalta_051		Transcription/RNA Modification
DLEV135	172457	172753	99										Unknown
DLEV136	172830	173780	317						SWIB			Yes	Virulence: Early Promoter
DLEV137	176579	176247	111										Unknown
DLEV138	177397	176570	276	mRNA decapping enzyme	YP_008003878.1	Adoxophyes honmai entomopoxvirus	282	31		D10R	Yalta_047		Transcription/RNA Modification
DLEV139	179651	177366	762	RAP94 RNA polymerase-associated transcriptional specificity factor	YP_008004477.1	Choristoneura rosaceana entomopoxvirus	822	22	Pox_Rap94	H4L	Yalta_046		Transcription/RNA Modification
DLEV140	179729	180856	376									Yes	Virulence: Early Promoter
DLEV141	181188	182129	314	unknown protein	AUS94114.1	Trichoplusia ni ascovirus 6b	344	28	DUF4419				Unknown
DLEV142	182145	183152	336	DNA topoisomerase type I	NP_064834.1	Amsacta moorei entomopoxvirus	333	49	Topoisom_I, VirDNA-topo-I_N	H6R	Yalta_041		Transcription/RNA Modification
DLEV143	183136	183531	132										Unknown
DLEV144	186023	184968	352	unknown protein	XP_018020522.1	*Hyalella azteca*	339	41	HAUS2				Unknown
DLEV145	186220	188655	812	mRNA capping enzyme large subunit	NP_064917.1	Amsacta moorei entomopoxvirus	872	28	Pox_MCEL, Pox_ATPase-GT	D1R	Yalta_038		Transcription/RNA Modification
DLEV146	188722	190053	444	unknown protein	XP_005059778.1	*Ficedula albicollis*	593	44	DUF4573		Yalta_037	Yes	Virulence: Early Promoter

					Best BLAST Hit								
Locus Tag	**Start**	**End**	**Size (aa)**	**Gene ID**	**Accession**	**Source Species**	**Size (aa)**	**Identity (%)**	**Pfam Domain(s)**	**VACV Core Gene**	**Yalta virus Ortholog(s)***	**Early Promoter**	**Putative Function**
DLEV147	192522	190579	648	P4b precursor	YP_004821457.1	Yokapox virus	646	26	Pox_P4B	A3L	Yalta_036		Structure/Morphogenesis
DLEV148	192529	193305	259	viral late transcription factor (VLTF2)	NP_064829.1	Amsacta moorei entomopoxvirus	259	36	PLATZ	A1L	Yalta_035		Transcription/RNA Modification
DLEV149	195149	194508	214	viral late transcription factor (VLTF3)	YP_008003748.1	Mythimna separata entomopoxvirus	228	30	Pox_VLTF3, A2L_zn_ribbon	A2L	Yalta_031		Transcription/RNA Modification
DLEV150	195170	196810	547	rifampicin resistance protein	YP_009001666.1	Anomala cuprea entomopoxvirus	566	30	Pox_Rif	D13L	Yalta_030		Structure/Morphogenesis
DLEV151	196811	197368	186										Unknown
DLEV152	198686	198309	126										Unknown
DLEV153	199108	198689	140						Hormone_recep				Unknown
DLEV154	199110	199592	161										Unknown
DLEV155	200069	199557	171										Unknown
DLEV156	200613	201146	178									Yes	Virulence: Early Promoter
DLEV157	201995	201657	113									Yes	Virulence: Early Promoter
DLEV158	202722	206045	1108	DNA topoisomerase type II	XP_004994772.1	*Salpingoeca rosetta*	1795	38	DNA_topoisoIV, TOPRIM_C, DNA_gyraseB, HATPase_c, Toprim		Yalta_014	Yes	Transcription/RNA Modification
DLEV159	206800	207879	360								Yalta_111		Unknown
DLEV160	207906	209990	695	RNA helicase/nucleoside triphosphatase II	YP_008003621.1	Mythimna separata entomopoxvirus	714	33	Helicase_C, NPH-II, DEAD	I8R	Yalta_112		Transcription/RNA Modification
DLEV161	211289	211537	83	unknown protein	WP_077952852.1	*Listeria monocytogenes*	333	60					Unknown
DLEV162	212457	211567	297								Yalta_022		Unknown
DLEV163	212566	213138	191						IF4E		Yalta_114		Unknown
DLEV164	213165	214532	456	unknown protein	WP_056620920.1	*Brevundimonas sp.* Root1423	398	25	LCAT		Yalta_113		Unknown
DLEV165	215387	215058	110	FAD-linked sulfhydryl oxidase	NP_048164.1	Melanoplus sanguinipes entomopoxvirus	107	40	Pox_E10	E10R	Yalta_110		Structure/Morphogenesis
DLEV166	215413	215808	132	ATP-dependent DNA ligase	NP_064902.1	Amsacta moorei entomopoxvirus	140	24			Yalta_109		Unknown

					Best BLAST Hit								
Locus Tag	**Start**	**End**	**Size (aa)**	**Gene ID**	**Accession**	**Source Species**	**Size (aa)**	**Identity (%)**	**Pfam Domain(s)**	**VACV Core Gene**	**Yalta virus Ortholog(s)***	**Early Promoter**	**Putative Function**
DLEV167	217252	216371	294	poly(A) polymerase small subunit	YP_008004175.1	Choristoneura biennis entomopoxvirus	294	45	PARP_regulatory	J3R	Yalta_108	Yes	Transcription/RNA Modification
DLEV168	217277	221026	1250	DNA polymerase	YP_005296243.1	Cotia virus SPAn232	1006	30	DNA_pol_B, DNA_pol_B_3	E9L	Yalta_107	Yes	Replication
DLEV169	222817	224868	684	nucleoside triphosphatase/helicase 2	YP_008004504.1	Choristoneura rosaceana entomopoxvirus	867	42	IBR			Yes	Transcription/RNA Modification
DLEV170	225374	224961	138										Unknown
DLEV171	226388	225381	336	unknown protein	WP_080051845.1	*Oceanospirillum multiglobuliferum*	970	44	RhoGEF				Unknown
DLEV172	226454	226675	74						T_hemolysin				Virulence: Homology
DLEV173	226683	227102	140									Yes	Virulence: Early Promoter
DLEV174	227136	228014	293									Yes	Virulence: Early Promoter
DLEV175	228507	229199	231	BRO protein	WP_002988317.1	*Streptococcus pyogenes*	253	31	Bro-N				Virulence: BRO Genes
DLEV176	229254	229910	219	thymidylate kinase	XP_016910867.1	*Apis cerana*	214	44	Thymidylate_kin			Yes	Virulence: Homology
DLEV177	229964	230605	214	BRO protein	YP_008004340.1	Choristoneura biennis entomopoxvirus	220	33	Bro-N				Virulence: BRO Genes
DLEV178	231583	230978	202	deoxynucleoside kinase	XP_024870688.1	*Temnothorax curvispinosus*	241	50	dNK		Yalta_042, Yalta_119	Yes	Virulence: Homology
DLEV179	231652	232365	238	F-box protein	XP_011633068.1	*Pogonomyrmex barbatus*	354	28	FBA, F-box-like				Virulence: Homology
DLEV180	232428	232826	133	BRO protein	AYN44984.1	Spodoptera exigua multiple nucleopolyhedrovirus	473	31	Bro-N			Yes	Virulence: BRO Genes
DLEV181	233229	233675	149	BRO protein	YP_009221130.1	Diadromus pulchellus ascovirus 4a	343	30	Bro-N				Virulence: BRO Genes
DLEV182	233770	234213	148	BRO protein	YP_009001476.1	Anomala cuprea entomopoxvirus	394	32	Bro-N				Virulence: BRO Genes
DLEV183	234309	234809	167									Yes	Virulence: Early Promoter
DLEV184	235205	235672	156										Unknown

*Orthologs between DlEPV and Yalta virus were identified with OrthoFinder v2.3.7 [112] and additional blastp searches between the two genomes.



Supplemental Figure 3.1. Normalized DlEPV abundance in *D. longicaudata* wasps. DlEPV copy number relative to *D. longicaudata* copy number was estimated with qPCR for (A) adult female wasp reproductive tissues, and (B) female and male whole wasps in pupal-adult developmental stages. Venom glands and ovaries from adult females were pooled in triplicate for each biological replicate. DlEPV genome copy number was estimated using the poly(A) polymerase small subunit gene (polyAPol, DLEV167), and *D. longicaudata* copy number with the elongation factor alpha gene (EF1a). qPCR was performed as done previously [34]. The y-axes indicate the $\log_{10}$ fold change of total DlEPV genome copy number over total *D. longicaudata* genome copy number. Permanent integration of the DlEPV genome into the *D. longicaudata* genome would result in a ratio of virus to wasp copy number that is ≥ 1 for all wasp tissues, developmental stages, and sexes, which is equivalent to a $\log_{10}$ abundance fold change of 0. Negative $\log_{10}$ abundance fold change values indicate samples in which the virus to wasp copy number ratio was < 1. Each bar represents the average relative DlEPV copy number across 6 biological replicates, and error bars represent one standard error above and below the mean.

Supplemental Table 3.2. Sequenced EPV genome features.

Genome	ORFs (#)	Total Length (bp)	Average ITR Length (bp)	GC (%)	Coding Density (%)
AMEV	294	232,392	9,458	17.8	95.5
AHEV	247	228,750	5,617	21.0	92.0
CBEV	334	307,691	23,817	19.7	87.0
CREV	296	282,895	13,406	19.5	87.8
MySEV	306	281,182	7,347	19.7	88.6
ACEV	263	245,717	22, 978	20.0	89.8
MSEV	267	236,120	7,201	18.3	91.7
LHEV (partial)	53	46,321	N/A	23.6	90.4
Yalta virus	177	219,929	8,403	25.2	85.8
DlEPV	193	252,940	17,469	30.1	65.1

Supplemental Table 3.3. Poxvirus core gene homologs in EPV and CPV genomes. The 49 poxvirus core genes are shown with corresponding locus tags for homologous ORFs in EPV and CPV genomes. The 16 core genes used to build the phylogeny in Figure 3.2 are highlighted in yellow.

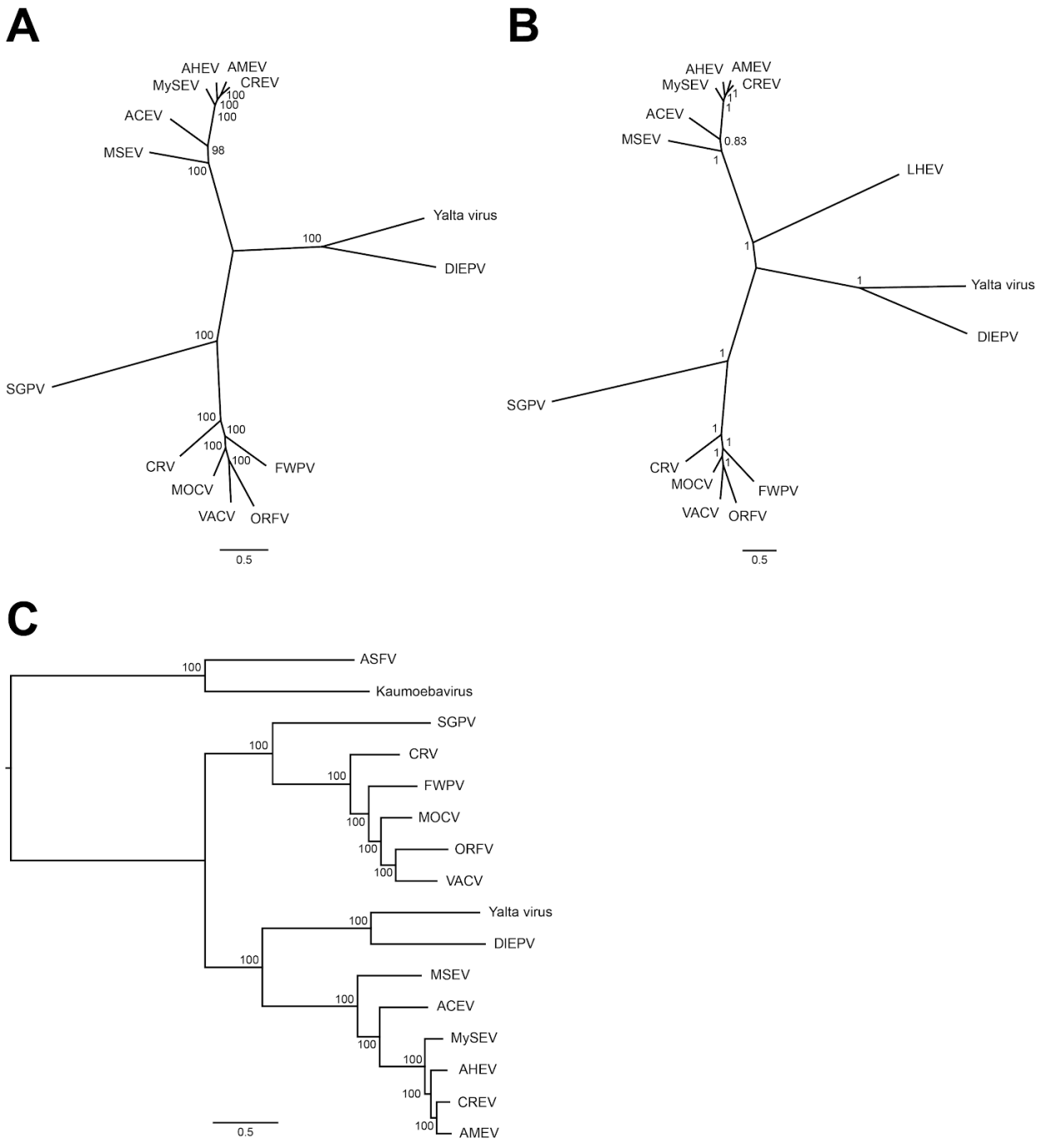
Gene Function	Gene Name	MSEV	ACEV	AMEV	AHEV	CREV	MySEV	Yalta virus	LHEV	DlEPV	VACV	ORFV	CRV	FPV	MOCV	SGPV
S-S bond formation pathway protein substrate	F9L	MseVgp094	ACV218	AMV243	AHEV_221	CHREV_246	MYSEV_268	Yalta_151		DLEV040	VACWR048	ORFVgORF131	CRV036	FPV112	MOCVgp016	AL387_gp092
Essential Ser/Thr kinase morph	F10L	MseVgp173	ACV137	AMV153	AHEV_178	CHREV_166	MYSEV_192	Yalta_093	LHEV_053	DLEV021	VACWR049	ORFVgORF130	CRV037	FPV111	MOCVgp017	AL387_gp067
Poly(A) polymerase catalytic subunit VP55	E1L	MseVgp143	ACV146	AMV038	AHEV_038	CHREV_064	MYSEV_064	Yalta_100	LHEV_037	DLEV128	VACWR057	ORFVgORF018	CRV054	FPV102	MOCVgp031	AL387_gp070
Virion protein	E6R	MseVgp145	ACV163	AMV170	AHEV_163	CHREV_181	MYSEV_213				VACWR062	ORFVgORF022	CRV056	FPV096	MOCVgp037	AL387_gp074
DNA polymerase	E9L	MseVgp036	ACV226	AMV050	AHEV_049	CHREV_048	MYSEV_075	Yalta_107		DLEV168	VACWR065	ORFVgORF025	CRV059	FPV094	MOCVgp039	AL387_gp076
Sulfhydryl oxidase, FAD linked	E10R	MseVgp093	ACV068	AMV114	AHEV_103	CHREV_129	MYSEV_170	Yalta_110	LHEV_012	DLEV165	VACWR066	ORFVgORF026	CRV060	FPV093	MOCVgp040	AL387_gp077
Virion core cysteine protease	I7L	MseVgp189	ACV108	AMV181	AHEV_156	CHREV_191	MYSEV_217	Yalta_147		DLEV042	VACWR076	ORFVgORF035	CRV070	FPV083	MOCVgp049	AL387_gp151
RNA helicase, DExH-NPH-II domain	I8R	MseVgp086	ACV170	AMV081	AHEV_079	CHREV_106	MYSEV_104	Yalta_112	LHEV_013	DLEV160	VACWR077	ORFVgORF036	CRV071	FPV082	MOCVgp050	AL387_gp150
Metalloprotease	G1L	MseVgp056	ACV184	AMV256	AHEV_231	CHREV_258	MYSEV_027	Yalta_131	LHEV_016	DLEV026	VACWR078	ORFVgORF037	CRV072	FPV081	MOCVgp056	AL387_gp149
FEN1-like nuclease	G5R	MseVgp115	ACV109	AMV179	AHEV_157	CHREV_186	MYSEV_215	Yalta_145		DLEV044	VACWR082	ORFVgORF041	CRV076	FPV117	MOCVgp060	AL387_gp146
NlpC/P60 superfamily protein	G6R	MseVgp039	ACV228	AMV041	AHEV_040	CHREV_061	MYSEV_066	Yalta_021			VACWR084	ORFVgORF043	CRV078	FPV119	MOCVgp062	AL387_gp144
Entry-fusion complex component, myristylprotein	G9R	MseVgp121	ACV056	AMV035	AHEV_036	CHREV_067	MYSEV_060	Yalta_067		DLEV062	VACWR087	ORFVgORF046	CRV081	FPV127	MOCVgp068	AL387_gp141
IMV membrane protein	L1R	MseVgp183	ACV097	AMV217	AHEV_135	CHREV_211	MYSEV_240	Yalta_028	LHEV_007	DLEV132	VACWR088	ORFVgORF047	CRV082	FPV128	MOCVgp069	AL387_gp097
Internal virion protein	L3L	MseVgp180	ACV144	AMV069	AHEV_066	CHREV_095	MYSEV_092	Yalta_060		DLEV061	VACWR090	ORFVgORF049	CRV092	FPV130	MOCVgp072	AL387_gp066
ssDNA/dsDNA binding protein VP8	L4R	MseVgp158	ACV096	AMV061	AHEV_061	CHREV_083	MYSEV_084	Yalta_059		DLEV036	VACWR091	ORFVgORF050	CRV093	FPV131	MOCVgp073	AL387_gp065
Entry and fusion IMV protein	L5R	MseVgp129	ACV037	AMV083	AHEV_081	CHREV_108	MYSEV_106	Yalta_096		DLEV060	VACWR092	ORFVgORF051	CRV094	FPV132	MOCVgp074	AL387_gp064

Poly(A) polymerase small subunit VP39	J3R	MseVgp041	ACV180	AMV060	AHEV_057	CHREV_082	MYSEV_083	Yalta_108		DLEV167	VACWR095	ORFVgORF053	CRV096	FPV134	MOCVgp076	AL387_gp062
Putative late 16-kDa membrane protein	J5L	MseVgp142	ACV042	AMV232	AHEV_211	CHREV_235	MYSEV_258	Yalta_056		DLEV035	VACWR097	ORFVgORF055	CRV098	FPV136	MOCVgp078	AL387_gp059
RNA polymerase RPO147	J6R	MseVgp043	ACV230	AMV221	AHEV_199	CHREV_222	MYSEV_244	Yalta_105		DLEV067	VACWR098	ORFVgORF056	CRV099	FPV137	MOCVgp079	AL387_gp165
Entry-fusion complex essential component	H2R	MseVgp060	ACV178	AMV127	AHEV_194	CHREV_149	MYSEV_156	Yalta_125		DLEV066	VACWR100	ORFVgORF058	CRV101	FPV139	MOCVgp083	AL387_gp168
IMV heparin binding surface protein	H3L	MseVgp206	ACV112	AMV248	AHEV_225	CHREV_252	MYSEV_274	Yalta_103, Yalta_115*			VACWR101	ORFVgORF059	CRV103	FPV140	MOCVgp084	
RNA polymerase-associated protein RAP94	H4L	MseVgp118	ACV150	AMV054	AHEV_054	CHREV_073	MYSEV_079	Yalta_046	LHEV_002	DLEV139	VACWR102	ORFVgORF060	CRV104	FPV141	MOCVgp085	AL387_gp169
DNA topoisomerase type I	H6R	MseVgp130	ACV152	AMV052	AHEV_052	CHREV_071	MYSEV_077	Yalta_041	LHEV_004, LHEV_005**	DLEV142	VACWR104	ORFVgORF062	CRV106	FPV143	MOCVgp087	AL387_gp171
mRNA-capping enzyme large subunit	D1R	MseVgp067	ACV140	AMV135	AHEV_190	CHREV_154	MYSEV_178	Yalta_038	LHEV_019	DLEV145	VACWR106	ORFVgORF064	CRV109	FPV146	MOCVgp090	AL387_gp173
Uracil-DNA glycosylase, DNA polymerase processivity factor	D4R	MseVgp208	ACV039	AMV231	AHEV_210	CHREV_234	MYSEV_257			DLEV047	VACWR109	ORFVgORF067	CRV112	FPV062	MOCVgp093	AL387_gp134
NTPase, DNA primase	D5R	MseVgp089	ACV164	AMV087	AHEV_085	CHREV_112	MYSEV_110	Yalta_152		DLEV039	VACWR110	ORFVgORF068	CRV113	FPV058	MOCVgp094	AL387_gp091
Morph, early transcription factor small subunit (VETF-s)	D6R	MseVgp113	ACV110	AMV174	AHEV_159	CHREV_185	MYSEV_214	Yalta_024		DLEV024	VACWR111	ORFVgORF069	CRV114	FPV057	MOCVgp095	AL387_gp090
RNA polymerase RPO18	D7R	MseVgp245	ACV044	AMV230	AHEV_207	CHREV_232	MYSEV_253	Yalta_120		DLEV029	VACWR112	ORFVgORF070	CRV115	FPV056	MOCVgp097	AL387_gp136
mRNA-decapping enzyme	D10R	MseVgp150	ACV147	AMV058	AHEV_055	CHREV_080	MYSEV_081	Yalta_047		DLEV138	VACWR115	ORFVgORF071	CRV117	FPV053	MOCVgp099	AL387_gp137
ATPase, NPH1	D11R	MseVgp053	ACV074	AMV192	AHEV_150	CHREV_196	MYSEV_222	Yalta_079	LHEV_041, LHEV_042**	DLEV081	VACWR116	ORFVgORF072	CRV118	FPV052	MOCVgp100	AL387_gp138
mRNA-capping enzyme small subunit	D12R	MseVgp124	ACV049	AMV093	AHEV_090	CHREV_117	MYSEV_115	Yalta_143		DLEV034	VACWR117	ORFVgORF074	CRV119	FPV051	MOCVgp101	AL387_gp140
Trimeric virion coat protein; rifampin resistance	D13L	MseVgp069	ACV193	AMV122	AHEV_112	CHREV_139	MYSEV_176	Yalta_030	LHEV_022	DLEV150	VACWR118	ORFVgORF075	CRV120	FPV050	MOCVgp102	AL387_gp098
Late transcription factor (VLTF-2)	A1L	MseVgp187	ACV093	AMV047	AHEV_046	CHREV_056	MYSEV_072	Yalta_035	LHEV_027	DLEV148	VACWR119	ORFVgORF076	CRV121	FPV049	MOCVgp103	AL387_gp099
Late transcription factor (VLTF-3)	A2L	MseVgp065	ACV192	AMV205	AHEV_142	CHREV_203	MYSEV_231	Yalta_031	LHEV_028	DLEV149	VACWR120	ORFVgORF077	CRV122	FPV165	MOCVgp104	AL387_gp100

P4b precursor	A3L	MseVgp164	ACV131	AMV147	AHEV_182	CHREV_162	MYSEV_186	Yalta_036	LHEV_036	DLEV147	VACWR122	ORFVgORF079	CRV124	FPV167	MOCVgp106	AL387_gp101
RNA polymerase RPO19	A5R	MseVgp101	ACV100	AMV166	AHEV_168	CHREV_176	MYSEV_202	Yalta_133		DLEV055	VACWR124	ORFVgORF081	CRV126	FPV169	MOCVgp108	AL387_gp103
Early transcription factor large subunit (VETF-L)	A7L	MseVgp063	ACV187	AMV105	AHEV_106	CHREV_127	MYSEV_121	Yalta_140		DLEV064	VACWR126	ORFVgORF083	CRV128	FPV171	MOCVgp110	AL387_gp105
Viral membrane-associated early morphogenesis protein	A9L	MseVgp108	ACV104	AMV161	AHEV_173	CHREV_171	MYSEV_197	Yalta_141		DLEV063	VACWR128	ORFVgORF085	CRV130	FPV173	MOCVgp112	AL387_gp107
P4a precursor	A10R	MseVgp152	ACV143	AMV139	AHEV_187	CHREV_157	MYSEV_181	Yalta_071	LHEV_049	DLEV111	VACWR129	ORFVgORF086	CRV131	FPV174	MOCVgp113	AL387_gp108
Viral membrane formation	A11R	MseVgp151	ACV142	AMV138	AHEV_188	CHREV_156	MYSEV_180	Yalta_072		DLEV110	VACWR130	ORFVgORF087	CRV132	FPV175	MOCVgp114	AL387_gp110
Myristylated protein, essential for entry/fusion	A16L	MseVgp090	ACV066	AMV118	AHEV_099	CHREV_133	MYSEV_131	Yalta_134		DLEV046	VACWR136	ORFVgORF093	CRV139	FPV181	MOCVgp121	AL387_gp118
DNA helicase, transcript release factor	A18R	MseVgp148	ACV148	AMV059	AHEV_056	CHREV_081	MYSEV_082	Yalta_075		DLEV078	VACWR138	ORFVgORF095	CRV141	FPV183	MOCVgp123	AL387_gp120
IMV membrane protein entry/fusion complex component	A21L	MseVgp209	ACV040	AMV249	AHEV_226	CHREV_253	MYSEV_275	Yalta_138		DLEV049	VACWR140	ORFVgORF098	CRV144	FPV186	MOCVgp125	AL387_gp122
Holliday junction resolvase	A22R	MseVgp106	ACV103	AMV162	AHEV_172	CHREV_172	MYSEV_198	Yalta_069		DLEV113	VACWR142	ORFVgORF099	CRV145	FPV187	MOCVgp127	AL387_gp124
Intermediate transcription factor 45-kDa subunit (VITF-3)	A23R	MseVgp052	ACV190	AMV091	AHEV_089	CHREV_116	MYSEV_114	Yalta_095		DLEV059	VACWR143	ORFVgORF100	CRV146	FPV188	MOCVgp128	AL387_gp125
RNA polymerase RPO132	A24R	MseVgp155	ACV085	AMV066	AHEV_068	CHREV_097	MYSEV_094	Yalta_051		DLEV134	VACWR144	ORFVgORF101	CRV147	FPV189	MOCVgp129	AL387_gp126
IMV MP/virus entry	A28L	MseVgp132	ACV071	AMV186	AHEV_152	CHREV_194	MYSEV_220	Yalta_098		DLEV126	VACWR151	ORFVgORF105	CRV149	FPV192	MOCVgp134	AL387_gp127
RNA polymerase RPO35	A29L	MseVgp149	ACV154	AMV051	AHEV_051	CHREV_070	MYSEV_076				VACWR152	ORFVgORF106	CRV150	FPV193	MOCVgp135	AL387_gp128
ATPase/DNA-packaging protein	A32L	MseVgp171	ACV135	AMV150	AHEV_180	CHREV_164	MYSEV_190	Yalta_094	LHEV_054	DLEV045	VACWR155	ORFVgORF108	CRV154	FPV197	MOCVgp140	AL387_gp068

*Homolog has two full-length copies within the genome.

**Homolog is split between two adjacent putative ORFs, likely a frameshift sequencing error.



Supplemental Figure 3.2. Additional poxvirus core gene phylogenies. (A) Maximum likelihood (ML) phylogenetic tree built from a concatenated multiple sequence alignment of the 44 core genes shared by all EPV complete genomes. Methods were the same as used to build the phylogeny in Figure 3.2. Node support (%) was inferred with 1,000 bootstrap iterations. (B) Bayesian inference phylogeny of the concatenated 16 core genes from Figure 3.2 built using

PhyloBayes-MPI v20161021 with the CAT-GTR substitution model [111]. Node support in panel B is labeled with the consensus posterior probability from two independent Markov chain Monte Carlo simulations that ran for 10,000 cycles each with a 1,000 cycle burn-in. (C) ML phylogeny built from a concatenated alignment of the 10 poxvirus core genes shared by all poxviruses and sister group Asfarviridae members African swine fever virus (ASFV, NC_001659) and Kaumoebavirus (NC_034249). Core genes and respective ASFV and Kaumoebavirus accession numbers used to build the phylogeny in panel C include the DNA polymerase E9L (AAA65319.1, ARA71927.1), RNA helicase I8R (AAA65302.1, ARA71975.1), RPO147 J6R (AAA65328.1, ARA71945.1 and ARA71948.1), mRNA-capping enzyme large subunit D1R (AAA65330.1, ARA71993.1), NTPase, DNA primase D5R (AAA65301.1, ARA71965.1), viral early transcription factor (VETF) small subunit D6R (AAA65335.1, ARA72203.1), VETF large subunit A7L (AAA65318.1, ARA71923.1), ATPase NPH1 D11R (AAA65350.1, ARA72259.1), RPO132 A24R (AAA65283.1, ARA72182.1), and ATPase/DNA-packaging protein A32L (AAA65308.1, ARA72015.1). Tree building methods were the same as done for other ML trees.

Supplemental Table 3.4. BRO genes in EPV genomes. BRO genes are defined as those with a Bro-N protein domain. Protein domains were identified using hmmsearch to query genes from each genome against the Pfam database. A maximum *e*-value cutoff of 0.05 was used to isolate significant domain matches.

DlEPV	ACEV	CBEV	CREV	Yalta virus	MSEV	AMEV	MySEV	AHEV
DLEV003	ACV003	CHBEV_089	CHREV_068	Yalta_004	MseVgp023	AMV055	MYSEV_087	N/A
DLEV012	ACV004	CHBEV_091	CHREV_069	Yalta_012	MseVgp024	AMV057		
DLEV014	ACV005	CHBEV_092	CHREV_075	Yalta_025	MseVgp025	AMV175		
DLEV082	ACV006	CHBEV_093	CHREV_076	Yalta_026	MseVgp194	AMV177		
DLEV083	ACV011	CHBEV_099	CHREV_077	Yalta_091	MseVgp195	AMV259		
DLEV084	ACV032	CHBEV_100	CHREV_078	Yalta_123	MseVgp196	AMV262		
DLEV092	ACV198	CHBEV_101	CHREV_079	Yalta_126	MseVgp204			
DLEV094	ACV253	CHBEV_102	CHREV_107	Yalta_127	MseVgp226			
DLEV096	ACV258	CHBEV_139	CHREV_190	Yalta_129	MseVgp229			
DLEV097	ACV259	CHBEV_222	CHREV_237	Yalta_174				
DLEV098	ACV260	CHBEV_270	CHREV_263					
DLEV100	ACV261	CHBEV_297						
DLEV101								
DLEV103								
DLEV105								
DLEV107								
DLEV109								
DLEV118								
DLEV119								
DLEV122								
DLEV123								
DLEV175								
DLEV177								
DLEV180								
DLEV181								
DLEV182								
DLEV191								

Supplemental Table 3.5. Expression of DlEPV genes in wasp venom gland (DL) and parasitized fly (AS) samples. Genes are grouped by their putative function based on similarity to other poxvirus genes. Genes of putative virulence function are subdivided between those with a Bro-N domain (Virulence: BRO Genes), those that were identified by sequence similarity to known virulence genes (Virulence: Homology), and those that have a conserved EPV early gene promoter motif and no other assigned function (Virulence: Early Promoter). Genes with an asterisk indicate those that demonstrated significant differential expression between the two treatments ($q < 0.05$).

Putative Function	Gene ID	AS Mean FPKM ± SD	DL Mean FPKM ± SD	Log2 Fold Change DL over AS
DNA Replication	DLEV007*	1438 ± 334	178 ± 81	-3.01
	DLEV028*	1888 ± 832	388 ± 110	-2.28
	DLEV031*	7825 ± 5003	236 ± 64	-5.05
	DLEV039	2133 ± 858	1941 ± 176	-0.14
	DLEV047*	624 ± 75	65 ± 16	-3.27
	DLEV072*	1925 ± 1243	70 ± 10	-4.79
	DLEV113*	1742 ± 496	2775 ± 334	0.67
	DLEV168*	543 ± 292	64 ± 13	-3.08
	DLEV187*	1496 ± 365	182 ± 70	-3.04
Structure/Morphogenesis	DLEV021*	350 ± 138	1870 ± 182	2.42
	DLEV026*	1021 ± 468	6692 ± 720	2.71
	DLEV035*	768 ± 248	3867 ± 283	2.33
	DLEV036*	14619 ± 7020	70321 ± 6932	2.27
	DLEV040*	1742 ± 262	4966 ± 404	1.51
	DLEV042*	1315 ± 609	14803 ± 1011	3.49
	DLEV044*	4004 ± 3039	68 ± 19	-5.89
	DLEV045*	2645 ± 1210	11620 ± 989	2.14
	DLEV046*	993 ± 420	8520 ± 1038	3.10
	DLEV049*	2495 ± 1251	7288 ± 716	1.55

	DLEV060*	1057 ± 554	5776 ± 617	2.45
	DLEV062*	1035 ± 399	7466 ± 778	2.85
	DLEV063*	392 ± 169	957 ± 142	1.29
	DLEV066*	2105 ± 971	8023 ± 941	1.93
	DLEV110	472 ± 131	462 ± 72	-0.03
	DLEV111*	12026 ± 5775	55952 ± 12475	2.22
	DLEV126*	572 ± 186	5711 ± 473	3.32
	DLEV132*	3562 ± 1528	33045 ± 4142	3.21
	DLEV147*	12645 ± 5449	91623 ± 13910	2.86
	DLEV150*	10858 ± 4329	34698 ± 5021	1.68
	DLEV165*	222 ± 103	837 ± 123	1.91
Transcription/RNA Modification	DLEV019	836 ± 162	1036 ± 126	0.31
	DLEV020*	1756 ± 780	182 ± 22	-3.27
	DLEV024*	1120 ± 557	13183 ± 1672	3.56
	DLEV029*	3826 ± 851	8610 ± 638	1.17
	DLEV034*	767 ± 258	4126 ± 473	2.43
	DLEV055*	1752 ± 189	4903 ± 191	1.48
	DLEV058*	2977 ± 2156	457 ± 65	-2.70
	DLEV059*	1124 ± 589	434 ± 45	-1.37
	DLEV061*	1617 ± 710	21486 ± 1040	3.73
	DLEV064*	540 ± 265	3762 ± 407	2.80
	DLEV067*	1392 ± 185	3709 ± 736	1.41
	DLEV076*	2656 ± 708	223 ± 50	-3.57
	DLEV078*	780 ± 128	1291 ± 103	0.73
	DLEV081*	1312 ± 559	7886 ± 1689	2.59
	DLEV128*	1467 ± 583	5137 ± 559	1.81
	DLEV134	1884 ± 257	2225 ± 489	0.24

	DLEV138*	1337 ± 160	4137 ± 487	1.63
	DLEV139*	482 ± 207	4077 ± 695	3.08
	DLEV142*	405 ± 203	1335 ± 213	1.72
	DLEV145*	1119 ± 118	3734 ± 692	1.74
	DLEV148*	2855 ± 1830	792 ± 64	-1.85
	DLEV149*	879 ± 268	2114 ± 389	1.27
	DLEV158*	1021 ± 283	379 ± 64	-1.43
	DLEV160*	792 ± 356	8267 ± 1069	3.38
	DLEV167*	1652 ± 485	4073 ± 267	1.30
	DLEV169*	311 ± 163	27 ± 4	-3.52
Virulence: BRO Genes	DLEV003*	895 ± 495	222 ± 60	-2.01
	DLEV012*	2401 ± 594	478 ± 100	-2.33
	DLEV014*	2983 ± 1187	1937 ± 169	-0.62
	DLEV082*	1920 ± 1193	646 ± 70	-1.57
	DLEV083*	4930 ± 3859	84 ± 27	-5.87
	DLEV084	42 ± 15	37 ± 19	-0.16
	DLEV092*	3003 ± 2175	39 ± 11	-6.27
	DLEV094*	2288 ± 1359	113 ± 24	-4.34
	DLEV096*	283 ± 139	59 ± 9	-2.27
	DLEV097*	7224 ± 7206	56 ± 18	-7.00
	DLEV098*	2860 ± 2646	62 ± 12	-5.52
	DLEV100*	3295 ± 2806	42 ± 12	-6.30
	DLEV101*	4197 ± 2654	239 ± 38	-4.13
	DLEV103*	5095 ± 3453	768 ± 216	-2.73
	DLEV105*	993 ± 655	88 ± 13	-3.50
	DLEV107*	13867 ± 8198	1411 ± 286	-3.30
	DLEV109*	1154 ± 933	126 ± 23	-3.19

	DLEV118*	348 ± 36	43 ± 8	-3.02
	DLEV119*	6647 ± 4480	61 ± 17	-6.77
	DLEV122	806 ± 254	958 ± 112	0.25
	DLEV123*	1089 ± 207	260 ± 27	-2.07
	DLEV175	630 ± 405	572 ± 98	-0.14
	DLEV177*	1205 ± 224	2065 ± 151	0.78
	DLEV180*	8179 ± 7186	1177 ± 135	-2.80
	DLEV181*	2350 ± 386	7247 ± 1179	1.62
	DLEV182*	831 ± 106	91 ± 19	-3.19
	DLEV191*	939 ± 534	233 ± 60	-2.01
Virulence: Homology	DLEV037*	1398 ± 629	5410 ± 1325	1.95
	DLEV099*	1217 ± 828	53 ± 7	-4.53
	DLEV172*	4618 ± 2081	108 ± 26	-5.41
	DLEV176*	1265 ± 269	105 ± 10	-3.59
	DLEV178*	3509 ± 2522	81 ± 18	-5.44
	DLEV179*	1086 ± 423	166 ± 28	-2.71
Virulence: Early Promoter	DLEV006*	1613 ± 224	246 ± 40	-2.71
	DLEV008*	1013 ± 512	70 ± 8	-3.85
	DLEV009*	1959 ± 975	132 ± 57	-3.90
	DLEV011*	2457 ± 1228	285 ± 115	-3.11
	DLEV015*	1724 ± 1364	307 ± 37	-2.49
	DLEV016*	897 ± 453	164 ± 11	-2.46
	DLEV025*	2048 ± 1513	28 ± 8	-6.19
	DLEV050*	1260 ± 529	186 ± 26	-2.76
	DLEV052*	20090 ± 13456	433 ± 158	-5.54
	DLEV053*	1343 ± 624	9309 ± 706	2.79
	DLEV056*	3229 ± 1840	256 ± 47	-3.66

	DLEV065*	1307 ± 980	39 ± 8	-5.06
	DLEV073*	31730 ± 16272	179234 ± 15436	2.50
	DLEV074*	25226 ± 11820	135181 ± 9485	2.42
	DLEV086*	2726 ± 1248	203 ± 52	-3.75
	DLEV091*	2285 ± 1419	43 ± 12	-5.73
	DLEV093*	656 ± 347	58 ± 14	-3.49
	DLEV095*	6173 ± 4451	231 ± 43	-4.74
	DLEV106*	782 ± 147	138 ± 37	-2.50
	DLEV108	3517 ± 373	4170 ± 320	0.25
	DLEV120*	346 ± 271	5 ± 6	-5.99
	DLEV125	4089 ± 2024	6545 ± 488	0.68
	DLEV131*	1135 ± 482	367 ± 50	-1.63
	DLEV136*	7377 ± 3046	1612 ± 208	-2.19
	DLEV140*	25260 ± 3084	12907 ± 2659	-0.97
	DLEV146	4625 ± 434	5239 ± 734	0.18
	DLEV156*	769 ± 163	81 ± 33	-3.25
	DLEV157*	276 ± 106	57 ± 8	-2.27
	DLEV173*	5312 ± 4330	74 ± 20	-6.17
	DLEV174*	380 ± 87	586 ± 60	0.62
	DLEV183*	4210 ± 1754	290 ± 124	-3.86
	DLEV185*	2546 ± 1299	287 ± 51	-3.15
	DLEV186*	959 ± 457	73 ± 15	-3.71
	DLEV188	46 ± 14	58 ± 30	0.33
Unknown	DLEV001*	196 ± 54	813 ± 66	2.05
	DLEV002*	1935 ± 521	4464 ± 835	1.21
	DLEV004*	2171 ± 998	379 ± 74	-2.52
	DLEV005*	919 ± 408	46 ± 12	-4.32

DLEV010*	1132 ± 226	287 ± 53	-1.98
DLEV013*	3723 ± 1820	43342 ± 7052	3.54
DLEV017*	3164 ± 1265	16411 ± 1403	2.37
DLEV018*	16627 ± 8595	101597 ± 7179	2.61
DLEV022*	1933 ± 621	5624 ± 1002	1.54
DLEV023*	2620 ± 479	6266 ± 423	1.26
DLEV027*	6878 ± 3113	98526 ± 10455	3.84
DLEV030*	1324 ± 698	5835 ± 512	2.14
DLEV032*	5909 ± 2536	229 ± 83	-4.69
DLEV033*	1866 ± 870	8067 ± 1381	2.11
DLEV038*	537 ± 84	3534 ± 532	2.72
DLEV041*	1880 ± 1197	315 ± 29	-2.58
DLEV043*	1482 ± 730	8235 ± 1030	2.47
DLEV048*	1104 ± 728	170 ± 28	-2.70
DLEV051*	1430 ± 403	3837 ± 614	1.42
DLEV054	1232 ± 903	15146 ± 2597	3.62
DLEV057*	2523 ± 1132	22138 ± 2493	3.13
DLEV068*	2048 ± 341	4670 ± 344	1.19
DLEV069*	2417 ± 1409	11355 ± 910	2.23
DLEV070*	676 ± 287	5774 ± 822	3.09
DLEV071	1351 ± 323	2852 ± 279	1.08
DLEV075*	866 ± 99	3119 ± 355	1.85
DLEV077*	31340 ± 14340	144080 ± 21496	2.20
DLEV079*	13981 ± 7310	50643 ± 5408	1.86
DLEV080*	7923 ± 4485	56621 ± 4577	2.84
DLEV085*	288 ± 114	2558 ± 203	3.15
DLEV087*	1180 ± 346	404 ± 45	-1.55

	DLEV088*	1506 ± 666	814 ± 117	-0.89
	DLEV089*	232 ± 53	157 ± 21	-0.56
	DLEV090*	636 ± 134	104 ± 22	-2.62
	DLEV102*	1065 ± 1006	28 ± 4	-5.24
	DLEV104*	6478 ± 5296	418 ± 88	-3.95
	DLEV112*	447 ± 295	67 ± 12	-2.74
	DLEV114*	382 ± 119	8573 ± 1036	4.49
	DLEV115*	1785 ± 892	3413 ± 736	0.93
	DLEV116	10893 ± 4458	14406 ± 2269	0.40
	DLEV117*	971 ± 233	469 ± 51	-1.05
	DLEV121*	48 ± 13	19 ± 6	-1.29
	DLEV124*	11722 ± 5902	82869 ± 6351	2.82
	DLEV127*	19124 ± 8866	120280 ± 11603	2.65
	DLEV129*	665 ± 522	2661 ± 374	2.00
	DLEV130*	1423 ± 965	52692 ± 4856	5.21
	DLEV133*	725 ± 388	4328 ± 729	2.58
	DLEV135*	36153 ± 21678	179208 ± 15640	2.31
	DLEV137	1109 ± 417	7297 ± 1620	2.72
	DLEV141*	3120 ± 2466	66 ± 6	-5.55
	DLEV143*	1815 ± 811	9943 ± 658	2.45
	DLEV144*	1132 ± 545	4218 ± 695	1.90
	DLEV151	11242 ± 3215	13742 ± 1109	0.29
	DLEV152*	1116 ± 312	395 ± 48	-1.50
	DLEV153*	1080 ± 440	8516 ± 652	2.98
	DLEV154*	2728 ± 2027	583 ± 67	-2.23
	DLEV155	265 ± 75	1799 ± 115	2.76
	DLEV159*	8665 ± 3995	58607 ± 8451	2.76

	DLEV161*	17283 ± 4211	2967 ± 325	-2.54
	DLEV162*	1577 ± 627	4337 ± 969	1.46
	DLEV163*	1424 ± 235	2032 ± 229	0.51
	DLEV164*	20096 ± 9137	95019 ± 10808	2.24
	DLEV166*	37168 ± 16529	234307 ± 18472	2.66
	DLEV170*	2358 ± 940	42421 ± 3997	4.17
	DLEV171*	1285 ± 530	3700 ± 904	1.53
	DLEV184*	5159 ± 3859	59 ± 25	-6.44
	DLEV189*	1204 ± 509	64 ± 14	-4.22
	DLEV190*	2236 ± 1105	346 ± 61	-2.69
	DLEV192*	1972 ± 637	4480 ± 700	1.18
	DLEV193*	204 ± 47	796 ± 84	1.96

Supplemental Table 3.6. Re-annotation of the LHEV genome segment. Feature table of LHEV ORFs including the 11 ORFs previously annotated by Viljakainen *et al.* 2018 [43] (accession NC_040577).

>Feature ref|NC_040577.1|

271	1764	gene		
			locus_tag	EXJ30_gp01
			db_xref	GeneID:41702288
271	1764	CDS		
			product	putative tryptophan repeat family protein
			protein_id	ref|YP_009552022.1|
2476	1649	gene		
			locus_tag	EXJ30_gp02
			db_xref	GeneID:41702291
2476	1649	CDS		
			product	putative RNA polymerase-associated transcription-specificity factor RAP94
			protein_id	ref|YP_009552023.1|
			note	similar to vaccinia virus H4
2564	3529	gene		
			locus_tag	LHEV_003
2564	3529	CDS		
			transl_table	1
			product	hypothetical protein

3584	4099	gene		
			gene	TOP1_1
			locus_tag	LHEV_004
3584	4099	CDS		
			transl_table	1
			gene	TOP1_1
			locus_tag	LHEV_004
			product	putative DNA topoisomerase type I
			note	similar to vaccinia virus H6
4312	4554	gene		
			gene	TOP1_2
			locus_tag	LHEV_005
4312	4554	CDS		
			transl_table	1
			gene	TOP1_2
			locus_tag	LHEV_005
			product	putative DNA topoisomerase type I
			note	similar to vaccinia virus H6
4547	4987	gene		
			locus_tag	LHEV_006
4547	4987	CDS		
			transl_table	1

			product	hypothetical protein
5726	4992	gene		
			locus_tag	EXJ30_gp03
			db_xref	GeneID:41702292
5726	4992	CDS		
			product	putative virion membrane protein M25
			protein_id	ref|YP_009552024.1|
			note	similar to vaccinia virus L1
5787	6038	gene		
			locus_tag	LHEV_008
5787	6038	CDS		
			transl_table	1
			product	hypothetical protein
7431	6040	gene		
			locus_tag	LHEV_009
7431	6040	CDS		
			transl_table	1
			locus_tag	LHEV_009
			product	putative DNA polymerase beta/AP endonuclease
7928	7398	gene		
			locus_tag	LHEV_010
7928	7398	CDS		

			transl_table	1
			locus_tag	LHEV_010
			product	putative DNA topoisomerase subunit
8363	7974	gene		
			locus_tag	LHEV_011
8363	7974	CDS		
			transl_table	1
			product	hypothetical protein
8692	8414	gene		
			locus_tag	LHEV_012
8692	8414	CDS		
			transl_table	1
			locus_tag	LHEV_012
			product	putative FAD-linked sulfhydryl oxidase
			note	similar to vaccinia virus E10
8734	10992	gene		
			gene	NPH2
			locus_tag	LHEV_013
8734	10992	CDS		
			transl_table	1
			gene	NPH2
			locus_tag	LHEV_013

			product	putative RNA helicase, NPH-II
			note	similar to vaccinia virus I8
11756	10947	gene		
			locus_tag	LHEV_014
11756	10947	CDS		
			transl_table	1
			product	hypothetical protein
12155	11811	gene		
			locus_tag	LHEV_015
12155	11811	CDS		
			transl_table	1
			product	hypothetical protein
12778	12263	gene		
			locus_tag	LHEV_016
12778	12263	CDS		
			transl_table	1
			locus_tag	LHEV_016
			product	putative metalloprotease
			note	similar to vaccinia virus G1
13522	12968	gene		
			locus_tag	LHEV_017
13522	12968	CDS		

			transl_table	1
			product	hypothetical protein
14114	13593	gene		
			locus_tag	LHEV_018
14114	13593	CDS		
			transl_table	1
			product	hypothetical protein
15457	14138	gene		
			locus_tag	EXJ30_gp04
			db_xref	GeneID:41702284
15457	14138	CDS		
			product	putative mRNA-capping enzyme catalytic subunit
			protein_id	ref|YP_009552025.1|
			note	similar to vaccinia virus D1
15484	16341	gene		
			locus_tag	LHEV_020
15484	16341	CDS		
			transl_table	1
			product	hypothetical protein
16701	16273	gene		
			locus_tag	LHEV_021
16701	16273	CDS		

			transl_table	1
			product	hypothetical protein
18431	16734	gene		
			locus_tag	EXJ30_gp05
			db_xref	GeneID:41702285
18431	16734	CDS		
			product	putative rifampicin resistance protein
			protein_id	ref|YP_009552026.1|
			note	similar to vaccinia virus D13
18494	19945	gene		
			locus_tag	LHEV_023
18494	19945	CDS		
			transl_table	1
			product	hypothetical protein
20357	19923	gene		
			locus_tag	LHEV_024
20357	19923	CDS		
			transl_table	1
			product	hypothetical protein
22882	20642	gene		
			locus_tag	LHEV_025
22882	20642	CDS		

			transl_table	1
			product	hypothetical protein
22888	23502	gene		
			gene	VLTF2
			locus_tag	LHEV_026
22888	23502	CDS		
			transl_table	1
			gene	VLTF2
			locus_tag	LHEV_026
			product	putative viral late transcription factor 2
			note	similar to vaccinia virus A1
24343	23663	gene		
			gene	VLTF3
			locus_tag	LHEV_027
24343	23663	CDS		
			transl_table	1
			gene	VLTF3
			locus_tag	LHEV_027
			product	putative viral late transcription factor 3
			note	similar to vaccinia virus A2
24384	25673	gene		
			locus_tag	LHEV_028

24384	25673	CDS		
			transl_table	1
			product	hypothetical protein
26351	25674	gene		
			locus_tag	LHEV_029
26351	25674	CDS		
			transl_table	1
			product	hypothetical protein
28110	26398	gene		
			locus_tag	EXJ30_gp06
			db_xref	GeneID:41702290
28110	26398	CDS		
			product	putative DNA ligase
			protein_id	ref|YP_009552027.1|
28763	28446	gene		
			locus_tag	LHEV_031
28763	28446	CDS		
			transl_table	1
			product	hypothetical protein
28968	28765	gene		
			locus_tag	LHEV_032
28968	28765	CDS		

			transl_table	1
			product	hypothetical protein
29374	28973	gene		
			locus_tag	LHEV_033
29374	28973	CDS		
			transl_table	1
			product	hypothetical protein
29477	29325	gene		
			locus_tag	LHEV_034
29477	29325	CDS		
			transl_table	1
			product	hypothetical protein
31570	29645	gene		
			locus_tag	LHEV_035
31570	29645	CDS		
			transl_table	1
			product	putative major core protein 4b
			note	similar to vaccinia virus A3
33305	31593	gene		
			locus_tag	EXJ30_gp07
			db_xref	GeneID:41702286
33305	31593	CDS		

			product	putative poly(A) polymerase catalytic subunit
			protein_id	ref|YP_009552028.1|
			note	similar to vaccinia virus E1
33443	33628	gene		
			locus_tag	LHEV_037
33443	33628	CDS		
			transl_table	1
			product	hypothetical protein
33600	33797	gene		
			locus_tag	LHEV_038
33600	33797	CDS		
			transl_table	1
			product	hypothetical protein
34720	34328	gene		
			locus_tag	LHEV_039
34720	34328	CDS		
			transl_table	1
			product	hypothetical protein
36085	34724	gene		
			locus_tag	EXJ30_gp08
			db_xref	GeneID:41702287
36085	34724	CDS		

			product	putative nucleoside triphosphatase I
			protein_id	ref|YP_009552029.1|
			note	similar to vaccinia virus D11
36624	36253	gene		
			locus_tag	LHEV_041
36624	36253	CDS		
			transl_table	1
			locus_tag	LHEV_041
			product	putative nucleoside triphosphatase I
			note	similar to vaccinia virus D11
36650	36970	gene		
			locus_tag	LHEV_042
36650	36970	CDS		
			transl_table	1
			product	hypothetical protein
37001	37258	gene		
			locus_tag	LHEV_043
37001	37258	CDS		
			transl_table	1
			product	hypothetical protein
37283	37660	gene		
			locus_tag	LHEV_044

37283	37660	CDS		
			transl_table	1
			product	hypothetical protein
37687	38028	gene		
			locus_tag	LHEV_045
37687	38028	CDS		
			transl_table	1
			product	hypothetical protein
38054	38401	gene		
			locus_tag	LHEV_046
38054	38401	CDS		
			transl_table	1
			product	hypothetical protein
39449	38406	gene		
			locus_tag	LHEV_047
39449	38406	CDS		
			transl_table	1
			product	hypothetical protein
39493	42060	gene		
			locus_tag	EXJ30_gp09
			db_xref	GeneID:41702294
39493	42060	CDS		

			product	putative major core protein 4a precursor
			protein_id	ref|YP_009552030.1|
			note	similar to vaccinia virus A10
42440	42057	gene		
			locus_tag	LHEV_049
42440	42057	CDS		
			transl_table	1
			product	hypothetical protein
42784	42560	gene		
			locus_tag	LHEV_050
42784	42560	CDS		
			transl_table	1
			product	hypothetical protein
43571	43410	gene		
			locus_tag	LHEV_051
43571	43410	CDS		
			transl_table	1
			product	hypothetical protein
44939	43572	gene		
			locus_tag	EXJ30_gp10
			db_xref	GeneID:41702293
44939	43572	CDS		

			product	putative serine/threonine-protein kinase 2
			protein_id	ref|YP_009552031.1|
			note	similar to vaccinia virus F10
44988	45743	gene		
			locus_tag	EXJ30_gp11
			db_xref	GeneID:41702289
44988	45743	CDS		
			product	putative protein A32
			prot_desc	similar to variola virus
			protein_id	ref|YP_009552032.1|

CHAPTER 4

A Viral Mutualist Employs Post-Hatch Transmission for Vertical and Horizontal Spread Among Parasitoid Wasps[3]

[3] Coffman KA, Burke GR. To be submitted to *Proceedings of the Royal Society B: Biological Sciences*.

4.1 ABSTRACT

Heritable microbial symbionts within insects display a wide variety of transmission strategies to travel from one host generation to the next. Two major types of vertical transmission include passage of symbionts within insect eggs, or transovarial transmission, as well as symbiont acquisition following egg hatching, or post-hatch transmission. Parasitoid wasps, one of the most diverse groups of insects, maintain several heritable associations with viruses that are highly beneficial for wasp survival during their development as parasites of other insects. Most of these beneficial viral elements are strictly transmitted through the wasp germline as integrated proviruses within wasp genomes. However, a beneficial poxvirus inherited by *Diachasmimorpha longicaudata* wasps, known as Diachasmimorpha longicaudata entomopoxvirus (DlEPV), is not integrated into the wasp genome and therefore, may employ different transmission routes to infect future generations of wasps. Here, we uncovered a previously undocumented form of post-hatch transmission for a facultative mutualistic virus, which entails external acquirement and localization of the virus within the adult wasp venom gland. We showed that this route is highly effective for vertical and horizontal transmission of the virus within *D. longicaudata* wasps. Furthermore, the highly beneficial phenotype provided by DlEPV during parasitism was also transmitted with extremely high efficiency, indicating an effective mode of symbiont spread to the advantage of infected wasps. These results provide novel insight into how beneficial viruses can be transmitted and spread among insects.

4.2 INTRODUCTION

Insects are well-known for their widespread and complex associations with microbes [1]. The crucial roles that microbes play in insect biology often result in hereditary associations, in which

microbes are passed down from insect parents to offspring [2]. This type of microbial inheritance, known as vertical transmission, can strengthen a symbiotic relationship by aligning the fitness of the microbe to that of its insect host, encouraging coevolution of both partners [3]. Obligate symbiotic associations, such as the bacterium *Buchnera aphidicola* within pea aphids, often display strict, transovarial vertical transmission, in which bacteria are incorporated into developing embryos within the female insect [4,5]. Other insect symbionts, like those found within hemipterans in the suborder Heteroptera, acquire their symbionts after egg-hatching by ingesting microbes that the mother has provided near the site of oviposition [6]. This form of post-hatch symbiont acquisition allows for external vertical transmission, also known as pseudo-vertical transmission [7].

One of the most diverse groups of insects is the order Hymenoptera, which contains over 150,000 described species that are composed mostly of parasites of other arthropods, known as parasitoid wasps [8]. Relatively few heritable symbionts have been studied in detail within parasitoid wasps, apart from a fungal symbiont within the species *Comperia merceti* [9,10] and bacteria, such as *Wolbachia*, *Cardinium*, *Rickettsia*, and *Arsenophonus*, that manipulate the reproduction systems of various wasp species [11]. These bacteria are not beneficial for parasitoid hosts, but rather alter the reproductive strategies of infected wasps to further their own transmission, causing cytoplasmic incompatibility, parthenogenesis, feminization, or male-killing phenotypes within wasp hosts [12–18]. *Wolbachia*, *Cardinium*, and *Rickettsia* display transovarial vertical transmission within parasitoids, whereby the bacteria invade the eggs of wasps prior to oviposition [16,18,19]. *Arsenophonus* has a more unusual transmission strategy within *Nasonia vitripennis* wasps: the bacteria are not observed within wasp eggs but are injected with wasp venom into the fly host during oviposition and are acquired by the next generation of wasps as larvae that

feed upon *Arsenophonus*-infected host tissue [20,21]. The bacteria then localize to the venom gland in adult female wasps [21]. This route represents a unique mode of pseudo-vertical transmission that has also been described in a virus that manipulates the reproductive strategy within the parasitoid wasp *Leptopilina boulardi*, known as Leptopilina boulardi filamentous virus (LbFV) [22]. Wasps that are infected with LbFV lay multiple eggs within the same host, a behavior known as superparasitism, while uninfected wasps lay only one egg per host [23]. LbFV also displays pseudo-vertical transmission, because uninfected wasps can become stably infected by developing within the same host as infected wasps [24,25], presumably through a similar route as *Arsenophonus* bacteria.

All of the above examples of heritable symbionts impose a fitness cost to the parasitoids that transmit them. However, an increasing number of beneficial viruses have also been characterized in parasitoid wasp lineages that are important factors for successful parasitism and survival of wasp offspring within host insects [11,26,27]. These viral elements are produced in massive quantities within wasp reproductive tissues and accompany wasp eggs into the host during oviposition, where they alter host physiology through processes like host immune suppression to the advantage of wasps developing within [28]. Many of these viruses are transmitted vertically through the germline of wasps as endogenous viral elements (EVEs) within wasp genomes [29]. Parasitoid EVEs identified to date have all undergone substantial genomic reorganization within wasp genomes, including viral gene dispersal and core gene loss, that restricts virus replication to occur only within wasp tissue [30–35]. Because EVEs no longer maintain a replicative genome apart from their associated wasps, they do not represent true symbionts in the way other microbial symbionts are classified; nevertheless, they are highly beneficial entities that are required for the survival of thousands of parasitoid species [36].

A poxvirus produced by the wasp *Diachasmimorpha longicaudata*, known as Diachasmimorpha longicaudata entomopoxvirus (DlEPV), represents a rare example of a true viral mutualistic symbiont, as it provides a strong fitness benefit to *D. longicaudata* wasps and maintains a complete, exogenous genome that replicates in wasps and fruit fly hosts of the wasp [37]. DlEPV has been detected within *D. longicaudata* eggs that were oviposited within *Anastrepha suspensa* fly hosts, suggesting that transmission is vertical and occurs transovarially within female wasps [37]. However, alternative modes of DlEPV transmission have not yet been investigated. In this study, we established a line of *D. longicaudata* wasps that were not infected with DlEPV to explore additional ways in which DlEPV and its beneficial phenotype can be transmitted among wasps. While the uninfected *D. longicaudata* colony, denoted DlEPV(-), survived at significantly lower rates than the infected, DlEPV(+) colony, reintroduction of the virus into fly hosts during parasitism by DlEPV(-) wasps resulted in a full recovery of DlEPV within the venom glands of female progeny. Furthermore, reacquisition of DlEPV caused a complete recovery of parasitism success within a single generation. These findings therefore support a post-hatch transmission strategy for the DlEPV system, which represents the first beneficial virus shown to utilize such a mode of transmission.

4.3 RESULTS

Cured line of wasps demonstrates the highly beneficial role of DlEPV as a facultative symbiont. To further understand the transmission dynamics of DlEPV within the *D. longicaudata* system, we used RNAi-based elimination of DlEPV to establish a stable line of uninfected wasps. We deprived *D. longicaudata* wasps of their resident DlEPV population using previously established methods to target the expression of three DlEPV core genes for knockdown during

virus replication in the female wasp venom gland. RNAi knockdown was an extremely efficient approach for inhibiting DlEPV replication, and total DlEPV abundance was reduced by 99.99% in wasps treated with DlEPV-specific double stranded RNA (dsRNA) [37]. The female progeny of DlEPV-specific dsRNA-treated wasps contained negligible amounts of virus, and these wasps were used to found a stable colony of uninfected, or DlEPV(-), wasps. This virus-free colony was established in November 2019 and has been maintained for over 10 generations to date.

We screened wasps from both the original infected colony, referred to here as DlEPV(+), as well as the DlEPV(-) colony for the presence of three DlEPV genes with PCR, including the poly(A) polymerase small subunit gene (PAP-S, DLEV167), the DNA polymerase gene (DNAP, DLEV168), and the structural P4b capsid gene (DLEV147) [38]. PCR screening of DlEPV(+) wasps showed a clear presence of DlEPV DNA within female wasps, and to a lesser extent, also in male wasps (Figure 4.1A). These results agree with previous quantitative PCR (qPCR) measurements of DlEPV abundance within our infected colony, with $>10^9$ viral genome copies found within adult female wasps and $<10^4$ copies within adult male wasps [37]. In contrast, DlEPV was not detected in any females or males from the DlEPV(-) wasp colony, confirming their uninfected status (Figure 4.1A).

We next performed assays using female wasps from both DlEPV(+) and DlEPV(-) lines to measure differences in parasitism success associated with viral infection status. Similar to prior assays that measured second generation dsRNA-treated wasps [37], we found that wasps in our DlEPV(-) colony emerged from *A. suspensa* fly hosts at a significantly reduced average rate of 4% compared to DlEPV(+) wasps, which emerged at an average rate of 43% (Figure 4.1B). In addition, flies showed a significant increase in emergence when parasitized by DlEPV(-) wasps compared to DlEPV(+) wasps, demonstrating the pathogenic role of DlEPV within fly hosts (Figure 4.1C).

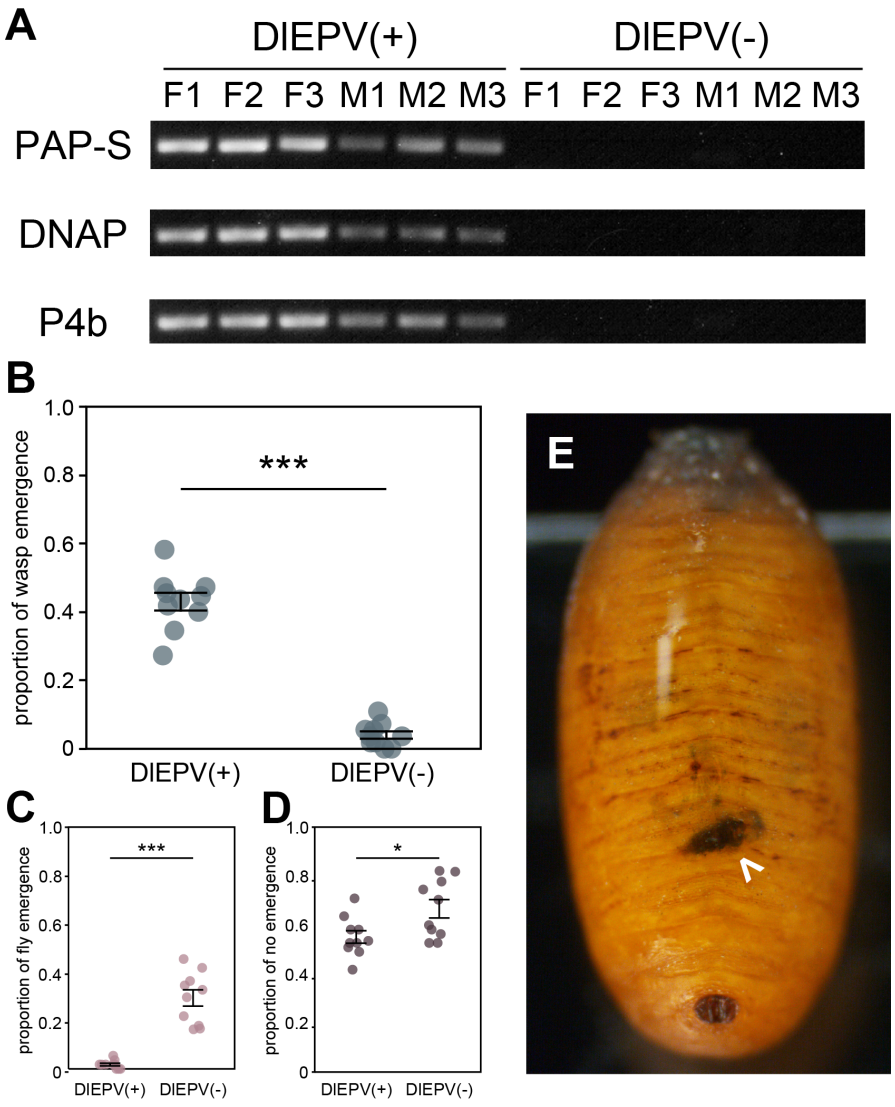


Figure 4.1. Establishment of cured *D. longicaudata* wasps display reduced survival during parasitism. (A) PCR was used to screen female (F) and male (M) wasps from both infected DlEPV(+) and uninfected DlEPV(-) colonies for DlEPV infection. DlEPV genes amplified include the poly(A) polymerase small subunit (PAP-S), the DNA polymerase (DNAP), and the P4b major capsid gene. (B-D) Parasitism assays measured the average emergence rates of (B) wasp progeny, (C) parasitized flies, and (D) neither wasp nor fly following oviposition by DlEPV(+) or DlEPV(-) wasps. Average proportions of wasp, fly, or no emergence were calculated using 10 replicate assay trials per treatment. Each trial is indicated by a dot and represents the fate of >50 singly parasitized flies after oviposition by a group of six female wasps. Statistical significance of *t*-tests in panels B-D is indicated as follows: *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$. (E) Fly pupa at 72 h after oviposition by a DlEPV(-) wasp. Arrowhead indicates the melanized first instar wasp larva visible underneath the fly puparium.

The average rate of no emergence (i.e. neither wasp nor fly emerged) was significantly higher in DlEPV(-) assays, which reflects the increased failure of wasps to emerge without the virus (Figure 4.1D). Visual inspection of fly pupae after oviposition by DlEPV(-) wasps indicated an abundance of flies that contained a melanized first instar wasp larva underneath the fly puparium (Figure 4.1E), suggesting that wasps are killed by the host immune system at this stage when not accompanied by DlEPV. These results thus provide further support for the highly beneficial function of DlEPV for infected *D. longicaudata* wasps.

DlEPV is reacquired by cured wasps within DlEPV-infected fly hosts. Alternative modes of DlEPV transmission were next explored through investigation of whether DlEPV can be reacquired by developing DlEPV(-) wasp progeny during parasitism. We allowed DlEPV(-) wasps to oviposit within fly hosts, and afterwards injected parasitized flies with one oviposition equivalent (approximately 10^7 viral genome copies) of purified DlEPV from either an unaltered "active" virus stock or a UV-inactivated "inactive" stock (Figure 4.2A). qPCR was then used to quantify DlEPV copy number in the venom glands of female progeny to ascertain whether the virus introduced into active DlEPV-injected flies had recolonized the venom gland of DlEPV(-) wasp progeny as adults. The venom glands of wasps that developed in the presence of active DlEPV contained an average of >6 billion viral genome copies, while those that developed with inactive DlEPV remained relatively clear of virus, although sparse amplification (<100 copies) was detected on average (Figure 4.2B). These results thus demonstrate that DlEPV can be reacquired by developing *D. longicaudata* wasps through their external environment and localize within the venom gland before eclosion. Furthermore, the amount of reacquired DlEPV found within the venom glands of active virus-treated DlEPV(-) wasps is consistent with that previously

described in DlEPV(+) wasps [37], which signifies that a complete reversal of infection status can occur in a single generation.

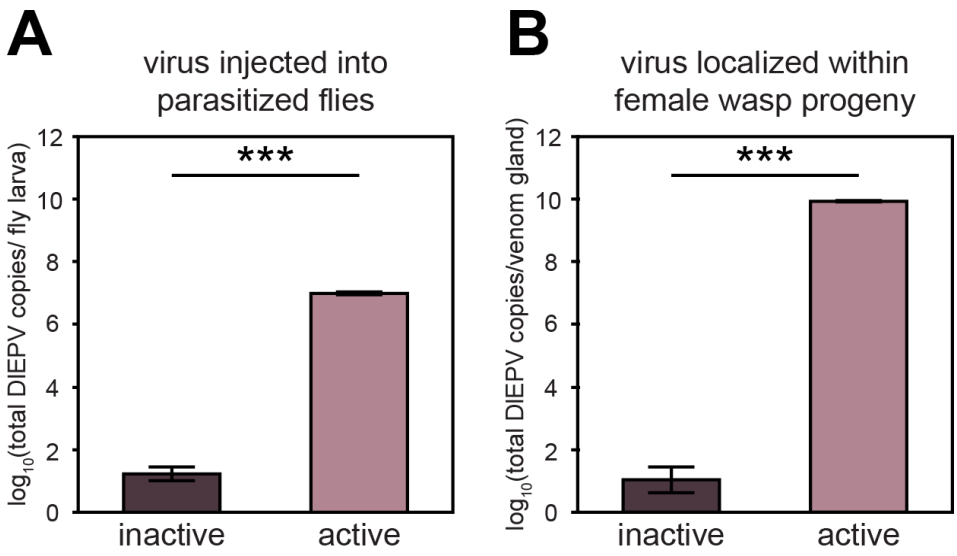


Figure 4.2. Manual introduction of DlEPV during parasitism by DlEPV(-) wasps elicits a full recovery of venom gland viral load in adult progeny. qPCR was used to measure the amount of unaltered (active) or UV-inactivated (inactive) DlEPV (A) initially injected into flies that had been parasitized by DlEPV(-) wasps immediately prior to injection, and (B) in the venom glands of resulting female wasp progeny after eclosion. Venom glands were pooled in triplicate for each biological replicate. Each bar represents the average $\log_{10}$-transformed viral copy number per specimen from 6 biological replicate samples, and error bars represent one standard error above and below the mean. Statistical significance of *t*-tests in panels A and B is as indicated in Figure 4.1.

We next tested whether DlEPV(-) wasps could naturally reacquire the virus during superparasitism with DlEPV(+) wasps. For this experiment, we took advantage of the haplodiploid nature of parasitoid wasp reproduction, in which female wasps that are not mated (i.e. virgin females) will produce only haploid male eggs, while mated wasps can produce either haploid male

or diploid female eggs [39]. We allowed mated DlEPV(-) females to oviposit within the same host as virgin DlEPV(+) wasps in three replicate experiments, such that any female progeny from these superparasitism events would be from the DlEPV(-) background. We then screened female progeny for DlEPV with PCR and measured viral abundance with qPCR to determine whether DlEPV had been reacquired by DlEPV(-) wasp progeny. Our results showed that 100% of screened DlEPV(-) female progeny were positive for DlEPV and contained a full viral load of >1 billion viral copies when they developed under superparasitism conditions with DlEPV(+) progeny (Table 4.1). Thus, either manual or natural inoculation of fly hosts with DlEPV caused uninfected wasp progeny to re-acquire the virus.

Table 4.1. Superparasitism as an efficient means for spread of DlEPV to uninfected wasps.

Replicate trial	Positive no. female progeny/ total no. female progeny	DlEPV Infection Frequency (%)	Mean female DlEPV copy number ± SE
1	6/6	100	5.76e+9 ± 3.10e+8
2	2/2	100	5.90e+9 ± 1.30e+9
3	5/5	100	4.66e+9 ± 6.34e+8

DlEPV reacquisition rescues parasitism success of developing wasps. To determine if wasps that reacquire DlEPV also recover the beneficial function of the virus during parasitism, we conducted assays similar to those done before to measure whether active DlEPV-treated wasps that have regained their venom gland viral population, now referred to as DlEPV(R+), have improved parasitism success compared to inactive DlEPV-treated wasps that largely remain uninfected, referred to as DlEPV(R-). Our results demonstrate that the vast majority of DlEPV(R-) wasp progeny failed to survive within fly hosts and showed a low adult emergence rate of 1.3%,

which is similar to the emergence rate of the main DlEPV(-) colony (Figure 4.3A). However, DlEPV(R+) wasp progeny that were once again accompanied by DlEPV during development survived at significantly higher proportions and emerged as adults at an average rate of 52.3% (Figure 4.3A). Fly emergence and no emergence rates were also similar in DlEPV(R+) and DlEPV(R-) treatments to the original DlEPV(+) and DlEPV(-) colonies, respectively (Figure 4.3B to C). These findings show that the beneficial effects of DlEPV during parasitism are fully reinstated in the same generation as the virus is reacquired.

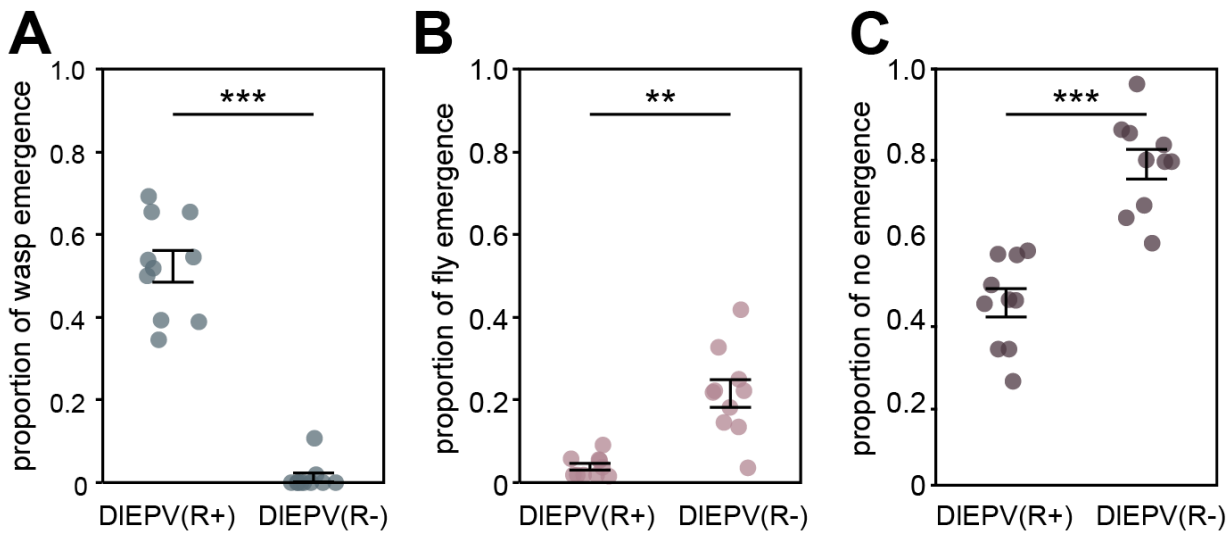


Figure 4.3. Reacquisition of DlEPV causes an immediate reversal of parasitism success. (A-C) Results from parasitism assays conducted with originally uninfected wasps that had reacquired DlEPV, or DlEPV(R+), compared to wasps that remained uninfected, or DlEPV(R-). Proportional (A) wasp emergence, (B) fly emergence, and (C) no emergence rates, as well as statistical significance are as indicated in Figure 4.1.

4.4 DISCUSSION

DlEPV represents an anomaly compared to other beneficial viral elements inherited by parasitoid wasps, in that it is not integrated into the *D. longicaudata* genome and maintains replicative autonomy while supplying a considerable advantage to developing wasps [37]. These features indicate that DlEPV is a true mutualistic symbiont, and therefore, we might expect some aspects of the DlEPV system to resemble other insect-microbe symbioses more closely than they do parasitoid-EVE associations. In contrast to the strict germline transmission that all EVEs share, beneficial microbial symbionts in insects are transmitted to future generations through myriad mechanisms and often exhibit more than one mode of transmission within the same host [40,41]. Therefore, we investigated alternative modes of DlEPV transmission in this study and identified a novel mode of transmission for a beneficial virus, characterized by virus particles that are externally acquired by wasp progeny during development and migrate to the venom gland of adult female wasps where they undergo rapid virus replication. While similar routes of transmission have been demonstrated for heritable pathogenic microbes in parasitoids [21,23,42], DlEPV is the first known mutualistic microbe to display this form of post-hatch transmission.

DlEPV is highly beneficial but not essential for *D. longicaudata* parasitism success. Our ability to establish and maintain a stable colony of uninfected *D. longicaudata* wasps demonstrates that DlEPV is not obligately required by wasps for successful parasitism. Nevertheless, wasps that are infected with DlEPV survive to adulthood at almost 40% greater frequency than those without the virus, as shown in our DlEPV(+) and DlEPV(-) colony parasitism assays. An important factor of these assays was our use of solitary parasitism events (i.e. one wasp egg laid per host) to measure wasp emergence rate with and without DlEPV. It therefore remains unclear whether DlEPV is as responsible for parasitism success during superparasitism events. Superparasitism can be costly

for solitary parasitoids under some situations, since only one wasp will ultimately survive within each host insect [43]. However, wild *D. longicaudata* populations have been observed to show both solitary and superparasitism behavior, and flies that are modestly superparasitized (2-3 eggs per host) are more likely to produce a single surviving wasp than solitary parasitism [44,45]. Therefore, superparasitism appears to be adaptive for this parasitoid species and could cause a general exhaustion of the host immune system that facilitates the survival of one wasp, as has been shown in other parasitoid systems [43]. Our DlEPV(-) colony is maintained with robust parasitism success by allowing for superparasitism during daily oviposition opportunities, so it is conceivable that other virulence factors introduced by wasp mothers or developing wasp larvae can also benefit parasitism success when introduced to the host at higher doses. DlEPV may therefore act more as a fail-safe plan for *D. longicaudata* when superparasitism is not attainable. Further study regarding the contribution of DlEPV on *D. longicaudata* survival during superparasitism events would thus provide more insight on the dimensions of benefit provided by this symbiont. Also of interest would be to explore potential differences in oviposition behavior by DlEPV(+) and DlEPV(-) wasps, since it may be more advantageous for wasps without the virus to promote superparasitism at the expense of increased reproductive costs, while virus-infected wasps would not need to expend supernumerary eggs per host due to the beneficial contributions of the virus.

The venom gland population and beneficial phenotype of DlEPV can be re-established through post-hatch acquisition. We showed in this study that DlEPV(-) wasps can regain a stable DlEPV infection by developing within a virus-infected host. Both manual injection of DlEPV during DlEPV(-) parasitism, as well as superparasitism between DlEPV(-) and DlEPV(+) wasps resulted in a full recovery of DlEPV copy number in the venom gland of DlEPV(-) wasps. In addition, wasps that had regained DlEPV showed an immediate reversal of parasitism success rate,

with an average DlEPV(R+) wasp emergence rate of >50% compared to DlEPV(R-) wasps that had not reacquired the virus.

The reproduction-manipulating virus LbFV is similarly infectious as it can be transmitted to uninfected *L. boulardi* wasps through superparasitism events [23,25]. However, LbFV infection and the resulting transfer of behavioral manipulation is gradual among uninfected wasps. Generally, <50% of uninfected *L. boulardi* wasps from various source populations acquired LbFV after reintroduction of the virus within the host, and the superparasitism behavior caused by LbFV was only partially transferred to the next generation [25]. Later generations of wasps showed perfect LbFV transmission to offspring and displayed a much stronger superparasitism phenotype [25]. DlEPV, in comparison, exhibited an immediate and absolute infection frequency to previously uninfected wasps, as DlEPV was detected in all screened DlEPV(R+) wasps and massive quantities of virus were measured within dissected venom glands or whole body tissue. Additionally, wasp emergence rate of DlEPV(R+) wasps closely mirrored that of DlEPV(+) wasps. This difference in infectivity between DlEPV and LbFV could be correlated to the difference in fitness consequences caused by the two viruses within their respective wasp species. As a mutualist, it is likely adaptive for *D. longicaudata* to efficiently acquire DlEPV, since the virus provides such a strong advantage to developing wasps. In contrast, LbFV exerts a fitness cost to *L. boulardi* wasps, and therefore, may become fixed within new populations more slowly. The ability of DlEPV to replicate exponentially within the fruit fly hosts of *D. longicaudata* may also contribute to the rapid spread of this virus to uninfected wasps, whereas the ability of LbFV to replicate within the *Drosophila* hosts of *L. boulardi* wasps has yet to be determined to our knowledge.

DlEPV is capable of both vertical and horizontal transmission. As demonstrated by the virus transmission route uncovered here, DlEPV is capable of both vertical and horizontal transmission strategies among *D. longicaudata* wasps. Pseudo-vertical transmission is supported through this route when a wasp mother oviposits an egg within a fly host and her progeny consumes the virus-infected fly tissue, and horizontal transmission is supported through superparasitism events involving uninfected wasps developing within the same host as infected wasps. This post-hatch mode of transmission adds to previous findings that suggest virus particles are transmitted transovarially within wasp eggs [37]. Several insect-microbe symbioses have demonstrated mixed modes of transmission, including *Wolbachia* bacteria that cause parthenogenesis within *Trichogramma* parasitoid wasps. *Wolbachia* is primarily transmitted transovarially within *Trichogramma* eggs, but the bacteria can also be horizontally transmitted to uninfected wasps of the same species and related species during superparasitism and multiparasitism events, respectively [46,47]. Similar to *Trichogramma* wasps, *D. longicaudata* is a generalist parasitoid species that oviposits within several genera of tephritid fruit flies and engages in multiparasitism behavior with other fruit fly parasitoid species [48–50]. It is therefore possible that DlEPV could also undergo parasitoid host switches, in which unrelated parasitoid species that develop within the same host as infected *D. longicaudata* could become infected.

Our findings also suggest that mixed vertical and horizontal transmission of DlEPV could cause rapid spread of the virus within natural populations, given the remarkable infection efficiency of the virus and the large fitness advantage conferred to wasps that have the virus over wasps that are uninfected. Other facultative symbionts of insects have been shown to rise in relative abundance within natural or laboratory insect populations due to the beneficial function provided by the symbiont and the competitive advantage that infected insects have compared to uninfected

insects [51–53]. For example, the defensive symbiont *Hamiltonella defensa* protects aphid hosts from attack by parasitoid wasps [54]. In the presence of parasitoid pressure, *H. defensa* frequency climbed to almost 100% within small-scale population cages containing infected and uninfected aphids [51]. Similar cage experiments conducted with DlEPV(+) and DlEPV(-) *D. longicaudata* wasps could therefore offer more resolution on the population dynamics of this symbiont.

The precise route of DlEPV post-hatch transmission remains to be elucidated. While we have demonstrated in this study that DlEPV can be externally acquired by *D. longicaudata* wasps, the exact route that DlEPV traverses to eventually colonize the venom gland is still unknown. Possibly the most likely path involves the consumption of virus-infected fly tissue by the wasp larva during parasitism and internal migration of virions to the venom gland during pupal development. A similar symbiont migration pattern following ingestion by a parasitoid has been demonstrated for *Arsenophonus* bacteria within developing *N. vitripennis* wasps. *Arsenophonus* is detected at highest abundance within the oral region of wasp larvae, then is found dispersed throughout the wasp hemocoel after pupation, and is localized within female reproductive tissues at the adult stage [21]. Part of this path appears to be shared by DlEPV as previous findings show that large quantities of virus are consumed by *D. longicaudata* larvae [37]. Therefore, DlEPV may undergo a similar journey through the developing wasp as *Arsenophonus*: DlEPV virions could be sequestered somewhere within the wasp larva as fly tissue is consumed, released within the hemolymph during the pupal stage, infect the female venom gland as it forms at the end of pupal development, and replicate to high abundance during eclosion.

An alternative route of DlEPV transmission to the venom gland could occur by means of surface virions that exist on the cuticle of wasps as they develop within the virus-infected host remains. Virions stuck to the wasp cuticle could become incorporated into the venom gland as it

forms, because this tissue is of ectodermal origin and therefore lined by cuticle [55]. This scenario is perhaps less likely than internal migration, as it requires DlEPV particles to exist extracellularly for a substantial amount of time before localization within the venom gland. Other insect poxviruses persist in the outside environment by producing a protein matrix, known as a spheroid, that occludes several virions within and protects the virions from harsh conditions, such as UV inactivation [56]. DlEPV, however, does not contain the gene necessary to produce spheroids, and microscopy has shown that DlEPV virions are not embedded within occlusion bodies [57]. Future experiments could rule out this scenario by surface sterilization of DlEPV(+) wasp pupae before venom gland formation or adding virions to the cuticle of DlEPV(-) wasp pupae. The presence of normal virus replication in the venom gland of surface-sterilized DlEPV(+) wasps combined with a lack of virus replication in the venom gland of surface-contaminated DlEPV(-) wasps would further support that DlEPV transmission is internal rather than external.

Migration of bacterial symbionts during insect development can be controlled either by the host or the symbiont [58]. Obligate symbionts, like *B. aphidicola* in aphids, are often transported to insect reproductive tissues by host factors, such as specialized cells that carry the bacteria from the symbiont-housing organ to the ovaries [4,5]. Mechanisms like this are thought to evolve due to both the essential function of the symbiont for the insect and the limited functional capabilities of many obligate symbionts with reduced genomes [2]. In contrast, symbionts that are facultative and/or pathogenic, such as *Wolbachia*, circulate in the hemolymph and often invade host reproductive tissues themselves [59,60]. DlEPV likely falls within the latter scenario and drives its own transmission, due to its facultative role for *D. longicaudata* wasps, its complete genome, and its preserved ancestral function as a pathogen of fly hosts [37]. Nonetheless, many questions

still remain regarding the mechanisms by which DlEPV is sequestered from the external environment, migrates to the venom gland, and is stimulated for virus replication.

4.5 MATERIALS AND METHODS

Insect colonies. *A. suspensa* flies and DlEPV(+) *D. longicaudata* wasps were reared as reported previously [61]. The DlEPV(-) wasp colony was initially established by injecting female wasp pupae from the DlEPV(+) colony with 1 µg of a dsRNA cocktail targeting the DlEPV RNA polymerase 147 kDa large subunit gene (RPO147), the DNA polymerase gene (DNAP), and the P4b structural capsid gene, as in [37]. Resulting dsRNA-treated adult wasps were allowed to mate with DlEPV(+) males and oviposit for 8 h daily into third instar fly larvae to increase colony size over time. Successive generations of female and male DlEPV(-) wasps were kept separate from DlEPV(+) wasps but were reared using the same methods.

DNA isolation and PCR screening for DlEPV. DNA was isolated from whole-body female and male adult wasps of both DlEPV(+) and DlEPV(-) colonies using a phenol:chloroform extraction method, as in [37]. DNA was eluted in 30 µL water, and each sample was diluted 1:10 prior to PCR screening. Standard PCR was performed using gene-specific primer sequences for PAP-S, DNAP, and P4b genes (Table 4.2) and with the following thermocycling conditions: 1 cycle of 95^{o}C for 2 min, 35 cycles of 95^{o}C for 20 sec, 55^{o}C for 20 sec, and 65^{o}C for 45 sec, and a final extension at 95^{o}C for 7 min. PCR products were then loaded on 1% agarose gels and subjected to electrophoresis for 45 min at 120V to visualize product bands.

Table 4.2. Primer sequences used in this study.

Primer Set	Forward Sequence (5' – 3')	Reverse Sequence (5' – 3')
PAP-S PCR	GCTCCAGTAAAACCGTTTCC	GGCTTTGGATCGTAAAACCA
P4b PCR	CGTGGGGAAACTGATATGCT	GGATTCCCCTCCAGTTTGTT
DNAP PCR	AAAATTGGAATCGGGTGGAT	TTGCGAAAGTTGGTTGTGAG

Parasitism success assays. Assays measuring the parasitism success of DlEPV(+)/DlEPV(-) wasps and DlEPV(R+)/DlEPV(R-) wasps were performed as previously described [37]. Briefly, *A. suspensa* second instar larvae were collected from within tubs of artificial fly diet and presented to groups of six female *D. longicaudata* wasps of one treatment for 4 h of oviposition. Afterwards, flies were placed back into fresh fly diet to continue larval development. 2 d later, resulting fly pupae were examined and only those containing a single oviposition scar were kept for observation. Four weeks post parasitism, the number of emerged wasps, emerged flies, and unemerged pupal cases were each counted, and emergence rates were calculated by dividing the number of specimens in each category by the total number of singly-scarred flies in the trial. Statistically significant differences between average emergence rates between DlEPV(+) and DlEPV(-) or DlEPV(R+) and DlEPV(R-) treatments were analyzed with *t*-tests assuming equal variance using JMP Pro 14 software.

Virus purification and parasitized fly injections. DlEPV virions were isolated from DlEPV(+) wasp venom gland tissue through filter purification, and UV-inactivation was performed on half of the produced active virus stock to generate the inactive virus stock, as in [37]. Third instar fly larvae were then offered to DlEPV(-) wasps for 2 h, and immediately afterwards, virions of active or inactive treatment were injected at a dose of 10^7 viral copies/µL into parasitized

flies. Injected flies were transferred to standard rearing conditions, and venom glands were dissected from resulting female progeny within 24 h of eclosion for viral abundance measurements.

Viral transmission via superparasitism experiment. Virgin female DlEPV(+) wasps were collected by separating female wasps from male wasps at the pupal stage. Female wasp pupae were identified by their distinctively long ovipositor, which was visible through the fly puparia that encased them. Virgin females were kept in a separate cage for 7 d following eclosion, then were offered third instar fly larvae to oviposit within for 4 h. Parasitized flies bearing at least one oviposition scar were then offered to groups of 6 mated DlEPV(-) females for 4 h to promote superparasitism between DlEPV(+) and DlEPV(-) wasp progeny. Flies were then transferred to standard rearing conditions until female progeny emerged as adults, approximately 17 d later. Upon emergence, female progeny were surface sterilized by vortex mixing each wasp in 1mL 5% bleach for 1 min, followed by three rounds of vortex mixing in 1mL water. Wasps were then collected as whole-body samples or were dissected for venom gland tissue specifically. Whole body wasps were subjected to PCR screening for DlEPV using the methods described before, and samples that displayed amplification of the viral PAP-S gene were scored as positive for DlEPV infection.

Quantitative PCR estimation of viral abundance and statistical analysis. Viral abundance was estimated from all collected samples using qPCR measurements of viral copy number for the PAP-S gene, as has been done previously [37]. Mean copy numbers were $\log_{10}$-transformed prior to statistical analysis. Statistical differences in average copy number between treatments were calculated with *t*-tests assuming equal variance using JMP Pro 14.

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4.7 REFERENCES

1. Douglas AE. Multiorganismal insects: diversity and function of resident microorganisms. Annu Rev Entomol. 2015;60: 17–34.

2. Moran NA, McCutcheon JP, Nakabachi A. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet. 2008;42: 165–190.

3. Douglas AE. The symbiotic habit. Princeton University Press; 2010.

4. Wilkinson TL, Fukatsu T, Ishikawa H. Transmission of symbiotic bacteria *Buchnera* to parthenogenetic embryos in the aphid *Acyrthosiphon pisum* (Hemiptera: Aphidoidea). Arthropod Struct Dev. 2003;32: 241–245.

5. Koga R, Meng X-Y, Tsuchida T, Fukatsu T. Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte–embryo interface. Proc Natl Acad Sci. 2012;109: E1230–E1237.

6. Salem H, Florez L, Gerardo N, Kaltenpoth M. An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. Proc R Soc Lond B Biol Sci. 2015;282: 20142957.

7. Wilkinson DM, Sherratt TN. Horizontally acquired mutualisms, an unsolved problem in ecology? Oikos. 2001;92: 377–384.

8. Huber JT. Biodiversity of Hymenoptera. In: Foottit RG, Adler PH, editors. Insect Biodiversity. Chichester, UK: John Wiley & Sons, Ltd; 2017. pp. 419–461.

9. Lebeck LM. Extracellular symbiosis of a yeast-like microorganism within *Comperia merceti* (Hymenoptera: Encyrtidae). Symbiosis. 1989;7: 51–66.

10. Gibson CM, Hunter MS. Inherited fungal and bacterial endosymbionts of a parasitic wasp and its cockroach host. Microb Ecol. 2009;57: 542–549.

11. Dicke M, Cusumano A, Poelman EH. Microbial symbionts of parasitoids. Annu Rev Entomol. 2020;65: 171–190.

12. Gherna RL, Werren JH, Weisburg W, Cote R, Woese CR, Mandelco L, et al. *Arsenophonus nasoniae* gen. nov., sp. nov., the causative agent of the son-killer trait in the parasitic wasp *Nasonia vitripennis*. Int J Syst Evol Microbiol. 1991;41: 563–565.

13. Rousset F, Bouchon D, Pintureau B, Juchault P, Solignac M. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. Proc R Soc Lond B Biol Sci. 1992;250: 91–98.

14. Stouthamer R, Breeuwert JA, Luck RF, Werren JH. Molecular identification of microorganisms associated with parthenogenesis. Nature. 1993;361: 66–68.

15. Vavre F, Fleury F, Varaldi J, Fouillet P, Bouleatreau M. Evidence for female mortality in *Wolbachia*-mediated cytoplasmic incompatibility in haplodiploid insects: epidemiologic and evolutionary consequences. Evolution. 2000;54: 191–200.

16. Zchori-Fein E, Gottlieb Y, Kelly SE, Brown JK, Wilson JM, Karr TL, et al. A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. Proc Natl Acad Sci. 2001;98: 12555–12560.

17. Hunter MS, Perlman SJ, Kelly SE. A bacterial symbiont in the *Bacteroidetes* induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. Proc R Soc Lond B Biol Sci. 2003;270: 2185–2190.

18. Hagimori T, Abe Y, Date S, Miura K. The first finding of a *Rickettsia* bacterium associated with parthenogenesis induction among insects. Curr Microbiol. 2006;52: 97–101.

19. Stouthamer R, Werren JH. Microbes associated with parthenogenesis in wasps of the genus *Trichogramma*. J Invertebr Pathol. 1993;61: 6–9.

20. Huger AM, Skinner SW, Werren JH. Bacterial infections associated with the son-killer trait in the parasitoid wasp *Nasonia* (= *Mormoniella*) *vitripennis* (Hymenoptera: Pteromalidae). J Invertebr Pathol. 1985;46: 272–280.

21. Nadal-Jimenez P, Griffin JS, Davies L, Frost CL, Marcello M, Hurst GDD. Genetic manipulation allows *in vivo* tracking of the life cycle of the son-killer symbiont, *Arsenophonus nasoniae*, and reveals patterns of host invasion, tropism and pathology. Environ Microbiol. 2019;21: 3172–3182.

22. Varaldi J, Martinez J, Patot S, Lepetit D, Fleury F, Gandon S. Chapter 17 - An Inherited Virus Manipulating the Behavior of its Parasitoid Host: Epidemiology and Evolutionary Consequences. In: Beckage NE, Drezen J-M, editors. Parasitoid Viruses. San Diego: Academic Press; 2012. pp. 203–214.

23. Varaldi J, Fouillet P, Ravallec M, López-Ferber M, Boulétreau M, Fleury F. Infectious behavior in a parasitoid. Science. 2003;302: 1930.

24. Varaldi J, Ravallec M, Labrosse C, Lopez-Ferber M, Boulétreau M, Fleury F. Artifical transfer and morphological description of virus particles associated with superparasitism behaviour in a parasitoid wasp. J Insect Physiol. 2006;52: 1202–1212.

25. Patot S, Lepetit D, Charif D, Varaldi J, Fleury F. Molecular detection, penetrance, and transmission of an inherited virus responsible for behavioral manipulation of an insect parasitoid. Appl Environ Microbiol. 2009;75: 703–710.

26. Lawrence PO. Non-poly-DNA viruses, their parasitic wasps, and hosts. J Insect Physiol. 2005;51: 99–101.

27. Roossinck MJ. The good viruses: viral mutualistic symbioses. Nat Rev Microbiol. 2011;9: 99–108.

28. Strand MR, Burke GR. Polydnaviruses: nature's genetic engineers. Annu Rev Virol. 2014;1: 333–354.

29. Drezen J-M, Leobold M, Bézier A, Huguet E, Volkoff A-N, Herniou EA. Endogenous viruses of parasitic wasps: variations on a common theme. Curr Opin Virol. 2017;25: 41–48.

30. Bézier A, Annaheim M, Herbinière J, Wetterwald C, Gyapay G, Bernard-Samain S, et al. Polydnaviruses of braconid wasps derive from an ancestral nudivirus. Science. 2009;323: 926–930.

31. Volkoff A-N, Jouan V, Urbach S, Samain S, Bergoin M, Wincker P, et al. Analysis of virion structural components reveals vestiges of the ancestral ichnovirus genome. PLoS Pathog. 2010;6: e1000923.

32. Burke GR, Walden KKO, Whitfield JB, Robertson HM, Strand MR. Widespread genome reorganization of an obligate virus mutualist. PLoS Genet. 2014;10: e1004660.

33. Pichon A, Bézier A, Urbach S, Aury JM, Jouan V, Ravallec M, et al. Recurrent DNA virus domestication leading to different parasite virulence strategies. Sci Adv. 2015;1: e1501150.

34. Béliveau C, Cohen A, Stewart D, Periquet G, Djoumad A, Kuhn L, et al. Genomic and proteomic analyses indicate that banchine and campoplegine polydnaviruses have similar, if not identical, viral ancestors. J Virol. 2015;89: 8909–8921.

35. Burke GR, Simmonds TJ, Sharanowski BJ, Geib SM. Rapid viral symbiogenesis via changes in parasitoid wasp genome architecture. Mol Biol Evol. 2018;35: 2463–2474.

36. Strand MR, Burke GR. Polydnaviruses: Evolution and Function. Curr Issues Mol Biol. 2020;34: 163–182.

37. Coffman KA, Harrell TC, Burke GR. A mutualistic poxvirus exhibits convergent evolution with other heritable viruses in parasitoid wasps. J Virol. 2020;94: e02059–19.

38. Coffman KA, Burke GR. Genomic analysis reveals an exogenous viral symbiont with dual functionality in parasitoid wasps and their hosts. PLoS Pathog. 2020; In press.

39. Quicke DLJ. Parasitic wasps. London, UK: Chapman & Hall; 1997.

40. Perlmutter JI, Bordenstein SR. Microorganisms in the reproductive tissues of arthropods. Nat Rev Microbiol. 2020;18: 97–111.

41. Ebert D. The epidemiology and evolution of symbionts with mixed-mode transmission. Annu Rev Ecol Evol Syst. 2013;44: 623–643.

42. Gibson CM, Hunter MS. Negative fitness consequences and transmission dynamics of a heritable fungal symbiont of a parasitic wasp. Appl Environ Microbiol. 2009;75: 3115–3119.

43. van Alphen JJ, Visser ME. Superparasitism as an adaptive strategy for insect parasitoids. Annu Rev Entomol. 1990;35: 59–79.

44. Gonzalez PI, Montoya P, Perez-Lachaud G, Cancino J, Liedo P. Superparasitism in mass reared *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae), a parasitoid of fruit flies (Diptera: Tephritidae). Biol Control. 2007;40: 320–326.

45. Montoya P, Ruiz L, Perez-Lachaud G, Cancino J, Liedo P. Field superparasitism by *Diachasmimorpha longicaudata* attacking *Anastrepha* spp. larvae on mango fruits. Biol Control. 2013;64: 160–165.

46. Huigens ME, Luck RF, Klaassen RH, Maas MF, Timmermans MJ, Stouthamer R. Infectious parthenogenesis. Nature. 2000;405: 178–179.

47. Huigens ME, de Almeida RP, Boons PAH, Luck RF, Stouthamer R. Natural interspecific and intraspecific horizontal transfer of parthenogenesis–inducing *Wolbachia* in *Trichogramma* wasps. Proc R Soc Lond B Biol Sci. 2004;271: 509–515.

48. Van den Bosch R, Haramoto FH. Competition among parasites of the oriental fruit fly. Proc Hawaii Entomol Soc. 1952;15: 201–206.

49. Bautista RC, Harris EJ. Effects of multiparasitism on the parasitization behavior and progeny development of oriental fruit fly parasitoids (Hymenoptera: Braconidae). J Econ Entomol. 1997;90: 757–764.

50. Wang X-G, Messing RH, Bautista RC. Competitive superiority of early acting species: a case study of opiine fruit fly parasitoids. Biocontrol Sci Technol. 2003;13: 391–402.

51. Oliver KM, Campos J, Moran NA, Hunter MS. Population dynamics of defensive symbionts in aphids. Proc R Soc Lond B Biol Sci. 2008;275: 293–299.

52. Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ. Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. Science. 2010;329: 212–215.

53. Himler AG, Adachi-Hagimori T, Bergen JE, Kozuch A, Kelly SE, Tabashnik BE, et al. Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. Science. 2011;332: 254–256.

54. Oliver KM, Russell JA, Moran NA, Hunter MS. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc Natl Acad Sci. 2003;100: 1803–1807.

55. Edson KM, Vinson SB. A comparative morphology of the venom apparatus of female braconids (Hymenoptera: Braconidae). Can Entomol. 1979;111: 1013–1024.

56. Perera S, Li Z, Pavlik L, Arif B. Entomopoxviruses. In: Asgari S, Johnson KN, editors. Insect Virology. Norfolk, UK: Caister Academic Press; 2010. pp. 83–102.

57. Lawrence PO. Morphogenesis and cytopathic effects of the Diachasmimorpha longicaudata entomopoxvirus in host haemocytes. J Insect Physiol. 2005;51: 221–233.

58. Bright M, Bulgheresi S. A complex journey: transmission of microbial symbionts. Nat Rev Microbiol. 2010;8: 218–230.

59. Dobson SL, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F, et al. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochem Mol Biol. 1999;29: 153–160.

60. Fast EM, Toomey ME, Panaram K, Desjardins D, Kolaczyk ED, Frydman HM. *Wolbachia* enhance *Drosophila* stem cell proliferation and target the germline stem cell niche. Science. 2011;334: 990–992.

61. Simmonds TJ, Carrillo D, Burke GR. Characterization of a venom gland-associated rhabdovirus in the parasitoid wasp *Diachasmimorpha longicaudata*. J Insect Physiol. 2016;91-92: 48–55.

CHAPTER 5

Host Range of a Parasitoid Wasp is Correlated with Host Susceptibility to its Mutualistic Viral Symbiont[4]

[4] Coffman KA, Gillette NE, Geib SM, Burke GR. To be submitted to *Molecular Ecology*.

5.1 ABSTRACT

Parasitoid wasps are one of the most species-rich groups of animals on Earth. The widespread diversification of parasitoid wasps is linked to their ability to successfully develop as parasites of nearly all types of insects. While most parasitoid species specialize for development within one or a few host species, the wasp *Diachasmimorpha longicaudata* is a generalist that can survive within multiple genera of tephritid fruit fly hosts, including many of the most destructive fruit fly pests known to agriculture. Therefore, *D. longicaudata* has been widely released to suppress pest populations as part of biological control efforts in the tropics and subtropics. In this study, we compared the relative success of *D. longicaudata* parasitism within three invasive fruit fly species that are serious pests on the Hawaiian Islands: the Mediterranean fruit fly *Ceratitis capitata*, the oriental fruit fly *Bactrocera dorsalis*, and the melon fly *Zeugodacus cucurbitae*. Our results demonstrated that *D. longicaudata* wasps survived at high proportions within *C. capitata* and *B. dorsalis*, while *Z. cucurbitae* was not a compatible host for *D. longicaudata*. We then explored the role of a symbiotic poxvirus carried by *D. longicaudata* wasps in determining host permissiveness for this parasitoid species. We found that permissive hosts *C. capitata* and *B. dorsalis* were highly susceptible to virus infection, resulting in rapid virus replication and high fly mortality. However, the nonpermissive host *Z. cucurbitae* largely overcame virus infection, demonstrating significantly lower mortality and no virus replication. Further investigation revealed that each fly species had a distinct tolerance for viral infection, at which flies appeared to mount an effective immune response to the virus. These results suggest that virus activity may contribute to the ability of *D. longicaudata* to parasitize various host fly species, and that virus suppression could be important for effective host defense against *D. longicaudata* attack.

5.2 INTRODUCTION

Parasitoid wasps, which are obligate parasites of other arthropods, represent one of the most diverse groups of animals known to exist [1–3]. These insects are free-living as adults, but immature wasps develop by feeding on or within other insect hosts [4]. The overwhelming success of parasitoid wasps can largely be attributed to their ability to effectively exploit virtually all types of insects as hosts and some non-insect arthropods [1]. Effective parasitism often involves specialization of a parasitoid species to optimize its developmental strategy toward one or a few host species [5]. Parasitoids that develop inside the body of their host, known as endoparasitoids, display more extreme forms of specialization, as they must directly combat the host immune system in order to survive [6,7]. Endoparasitoids have thus evolved numerous tactics to manipulate host physiology for increased parasitism success, including virulence factors that are produced by adult female wasps and injected into host insects during oviposition [8]. These maternal factors include venoms composed of unique cocktails of virulence proteins that can alter host immune and developmental systems for the benefit of developing wasps [9]. In some cases, additional factors like virus-derived particles are also produced within female wasps and cause similar detrimental effects when delivered to host insects [10].

A growing number of independent viral elements have been documented within parasitoid wasp lineages, and many represent stable heritable associations that provide substantial benefit, or more often, are absolutely required by the wasps that produce them [11–15]. Most examples that have been genetically characterized are known to exist as endogenous viral elements (EVEs) within wasp genomes that are activated toward the end of wasp development and produce high densities of virus or virus-like particles (VLPs) within the reproductive tissues of the associated parasitoid [13,14,16–19]. However, due to viral genome rearrangements that have occurred in all

known parasitoid-EVE systems, the virus particles or VLPs can not replicate outside of the wasp [20,21]. Therefore, the virulence caused by these viral elements is mainly due to their infection and delivery of virulence genes and/or proteins into host insect cells [22].

Recent genomic sequencing of a heritable poxvirus produced in the venom gland of *Diachasmimorpha longicaudata* wasps, known as Diachasmimorpha longicaudata entomopoxvirus (DlEPV), revealed that this virus is not an EVE but maintains an exogenous genome that successfully replicates within both *D. longicaudata* wasps and the tephritid fruit fly host, *Anastrepha suspensa* [23,24]. DlEPV replication was highly virulent within *A. suspensa* hosts, while *D. longicaudata* wasps were unaffected by virus replication [23]. Furthermore, we showed that elimination of DlEPV from *D. longicaudata* wasps caused a severe drop in parasitism success, although a small proportion of wasps survived without the virus [23]. Therefore, DlEPV appears to be a pathogen to *A. suspensa* flies, but displays a highly beneficial, if not facultative, relationship with *D. longicaudata* wasps.

D. longicaudata is well-known for its widespread use as a biological control (biocontrol) agent to suppress various tephritid fruit fly populations in the tropics and subtropics [25]. In 1948, *D. longicaudata* was introduced to Hawaii along with many other parasitoid species to control invasive populations of the oriental fruit fly, *Bactrocera dorsalis*, the melon fly, *Zeugodacus cucurbitae*, and the Mediterranean fruit fly, *Ceratitis capitata* [26]. *D. longicaudata* was one of few parasitoid species to become established on the islands, and together with the species *Fopius arisanus*, led to significant reductions in *B. dorsalis* and *C. capitata* populations [27], representing one of the most successful fruit fly biocontrol programs worldwide [28]. Unlike many parasitoids that can only develop within one or a few closely related host species, referred to as specialists, *D. longicaudata* is a generalist parasitoid that can successfully develop within fly species from several

genera of tephritids, including *A. suspensa*, *B. dorsalis*, and *C. capitata* [25,28]. Here, we investigated the potential role of DlEPV in determining the host range of *D. longicaudata* wasps by exploring the effects of the virus in three tropical fruit fly species that display varying permissiveness to *D. longicaudata* parasitism. Our results suggest that DlEPV could be a major contributing factor to the ability of *D. longicaudata* to parasitize a wide range of hosts, indicating that a microbial symbiont could be a novel means of niche expansion for this parasitoid wasp.

5.3 RESULTS

Tropical fruit flies show variation in permissiveness to *D. longicaudata* parasitism. *C. capitata*, *B. dorsalis*, and *Z. cucurbitae* have all posed major threats to agricultural fruit production since their introductions to Hawaii within the last century [29]. While *B. dorsalis* and *Z. cucurbitae* belong to closely related genera within the Tephritidae, *C. capitata* is distantly related in comparison [30] (Figure 5.1A). However, all three species demonstrate host overlap in Hawaii and are known to infest a wide variety of fleshy fruits and vegetables [29,31]. Since its release on the islands, *D. longicaudata* has been reported to parasitize both *C. capitata* and *B. dorsalis*, but *Z. cucurbitae* is not known to be a suitable host for this parasitoid species [26,32].

We first investigated the ability of *D. longicaudata* to develop within these three fruit fly pest species by conducting parasitism success assays that measured the rates of adult wasp emergence, adult fly emergence, and no emergence after fly larvae were subjected to oviposition by female *D. longicaudata*. Parasitism within both *C. capitata* and *B. dorsalis* flies led to considerable wasp emergence rates, with the highest average wasp emergence rate of 63.2% for *C. capitata* and 49.6% for *B. dorsalis* (Figure 5.1B). These two fly species are therefore confirmed as permissive hosts for *D. longicaudata*. In contrast, parasitism within *Z. cucurbitae* flies failed to produce a single

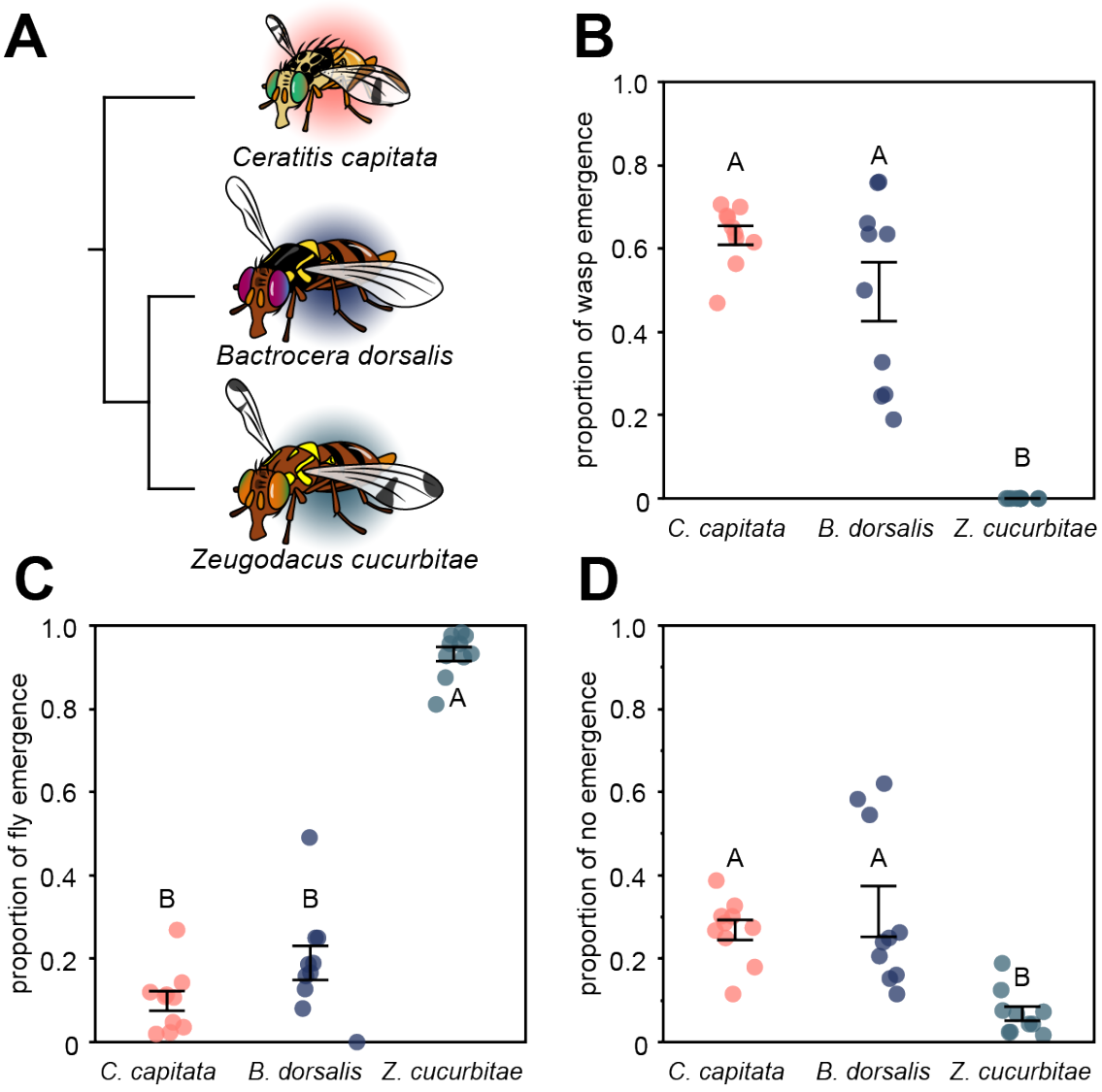

Figure 5.1. Parasitism success of *D. longicaudata* among tropical fruit fly species. (A) The Mediterranean fruit fly (*Ceratitis capitata*), the oriental fruit fly (*Bactrocera dorsalis*), and the melon fly (*Zeugodacus cucurbitae*) represent the three most pestiferous fruit fly species that have been introduced to Hawaii during the last century [29]. *B. dorsalis* and *Z. cucurbitae* represent closely related species, while *C. capitata* is more distantly related within the family Tephritidae [30]. (B-D) Parasitism assays measured the average emergence rates of (B) wasp progeny, (C) parasitized flies, and (D) neither wasp nor fly following oviposition by *D. longicaudata* within the three fruit fly species. Average proportions of wasp, fly, or no emergence were calculated using 10 replicate assay trials per treatment. Each trial is indicated by a dot and represents the fate of > 40 singly parasitized flies after oviposition by a group of six female wasps. Statistical significance of *t*-tests in panels B-D is indicated as follows: *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$.

adult wasp across all trials and thus, differed significantly from *C. capitata* and *B. dorsalis* ($F_{2,27}$ = 60.29, $p < 0.0001$) (Figure 5.1B). These results demonstrate that although *D. longicaudata* will successfully oviposit into *Z. cucurbitae*, it is a nonpermissive host. Average fly emergence rates for *C. capitata* and *B. dorsalis* were severely hindered when parasitized by *D. longicaudata*, at 9.9% and 19.0%, respectively (Figure 5.1C), indicating the forfeit of fly survival over wasp development caused by successful parasitism. *Z. cucurbitae*, however, displayed a significantly higher average emergence rate of 93.2% after parasitism ($F_{2,27}$ = 247.61, $p < 0.0001$) (Figure 5.1C). Rates of no emergence between *C. capitata* and *B. dorsalis* were also not significantly different at 26.9 and 31.4%, respectively, although *Z. cucurbitae* showed a significantly lower non-emergence rate of 6.8% ($F_{2,27}$ = 11.26, p = 0.0003) (Figure 5.1D). This difference in no emergence rate, which is likely the result of increased mortality of both developing wasp and parasitized fly within *C. capitata* and *B. dorsalis* hosts, suggests that the act of parasitism, even when not successful, is less costly for *Z. cucurbitae* survival.

Permissiveness to parasitism is correlated with replication of DlEPV within host flies. Due to the striking difference of *D. longicaudata* parasitism success within *Z. cucurbitae* compared to *C. capitata* and *B. dorsalis*, we next explored whether the activity of DlEPV within these same fly species during parasitism was connected to the observed difference in wasp permissiveness. We allowed *D. longicaudata* wasps to oviposit within each fly species, and then used quantitative PCR (qPCR) to measure DlEPV copy number over time during parasitism as a direct estimate of viral abundance within fly tissue. For *C. capitata*, we observed a significant difference in average viral copy number during parasitism, characterized by an initial introduction of nearly 10^7 viral copies at 0 h post parasitism (hpp), followed by a slight drop within 24 hpp, and a significant increase to > 10^8 copies by 120 hpp ($F_{5,42}$ = 3.18, P = 0.0158) (Figure 5.2A). *B. dorsalis* flies

showed a more robust increase of viral copy number throughout parasitism, as viral abundance significantly rose by > 4 orders of magnitude from 0-120 hpp ($F_{5,42}$ = 4.81, P = 0.0015) (Figure 5.2B). In contrast, viral abundance showed a significant decrease during parasitism within *Z. cucurbitae* flies, as approximately 10^6 viral copies were introduced at 0 hpp, and mean copy number fell to < 10^4 copies by 120 hpp ($F_{5,42}$ = 3.94, P = 0.0051) (Figure 5.2C). These data therefore support a correlation between host compatibility for parasitism and virus replication capability within the fly hosts.

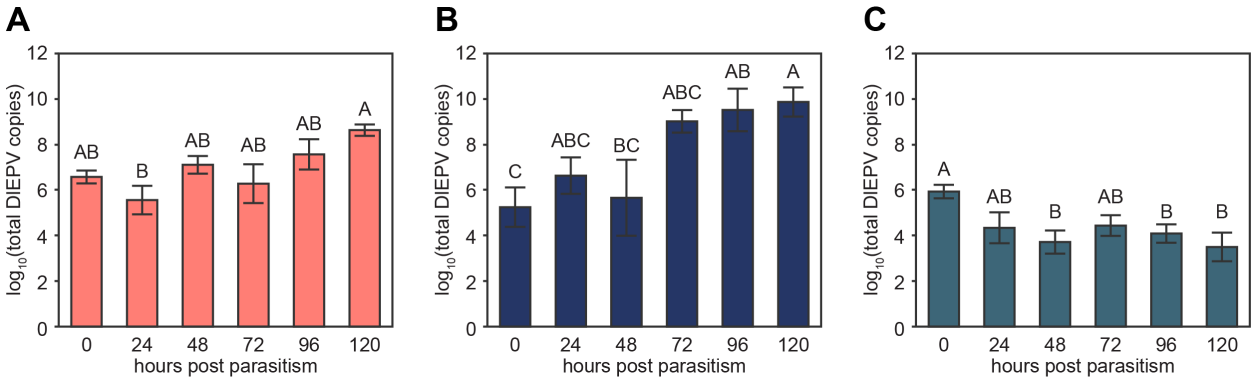

Figure 5.2. Fruit fly permissiveness to parasitism is associated with successful DlEPV replication. DlEPV abundance was estimated using qPCR measurements of the DlEPV poly(A) polymerase small subunit gene during *D. longicaudata* parasitism within (A) *C. capitata*, (B) *B. dorsalis*, and (C) *Z. cucurbitae* flies. Each bar in panels (A-C) represents the mean $\log_{10}$-transformed viral copy number across 8 biological replicate fly samples per time point. Error bars represent one standard error above and below the mean value. Statistical differences between mean copy numbers in each graph are indicated by the letter(s) above each bar.

DlEPV injection demonstrates a gradation of fly susceptibility to viral infection. The pronounced differences in DlEPV replication uncovered by our viral abundance measurements throughout parasitism pose intriguing questions regarding the involvement of the virus in host

compatibility for *D. longicaudata*. However, the concurrent effects of parasitism, in general, throughout the analysis likely caused variability in our results with respect to the amount of virus introduced into each fly species and the observed virus replication patterns. Additionally, any potential virulence caused by the virus would be obscured by parasitism due to developing wasps' active consumption of fly tissue over time. Therefore, we next investigated whether the effects of DlEPV in the absence of parasitism could offer more resolution on the correlation between viral infection and host permissiveness within the three fruit fly species.

We injected nonparasitized fly larvae of all three species with several doses of purified DlEPV and measured whether virus-infected fly larvae survived to adulthood. We also injected separate larvae with UV-inactivated virus at the same three doses as a control treatment. Percentage emergence was then normalized by dividing the unaltered, or "active" DlEPV emergence rate by the UV-treated, or "inactive" DlEPV emergence rate for each species and dose (Figure 5.3, Supplemental Table 5.1). *C. capitata*, which was the most permissive fly species to parasitism by *D. longicaudata*, displayed the highest susceptibility to DlEPV infection. No *C. capitata* flies emerged as adults when inoculated with either 1 oviposition equivalent of DlEPV (approximately 10^7 viral genome copies) or 0.2 oviposition equivalents (Figure 5.3). At the lowest dosage, 0.1 oviposition equivalents, a normalized emergence rate of 10.8% was observed, indicating that a low proportion of *C. capitata* flies can recover from DlEPV infection at this dose. Similar to *C. capitata*, *B. dorsalis* flies displayed a complete failure to emerge after injection with 1 oviposition equivalent of DlEPV, although 1.4% *B. dorsalis* normalized survival was observed after injection with 0.2 oviposition equivalents of virus (Figure 5.3). Furthermore, 42.5% *B. dorsalis* flies emerged when treated with the 0.1 oviposition equivalents, demonstrating that nearly half of infected flies overcame virus infection when inoculated with a low viral dose (Figure 5.3). *Z.*

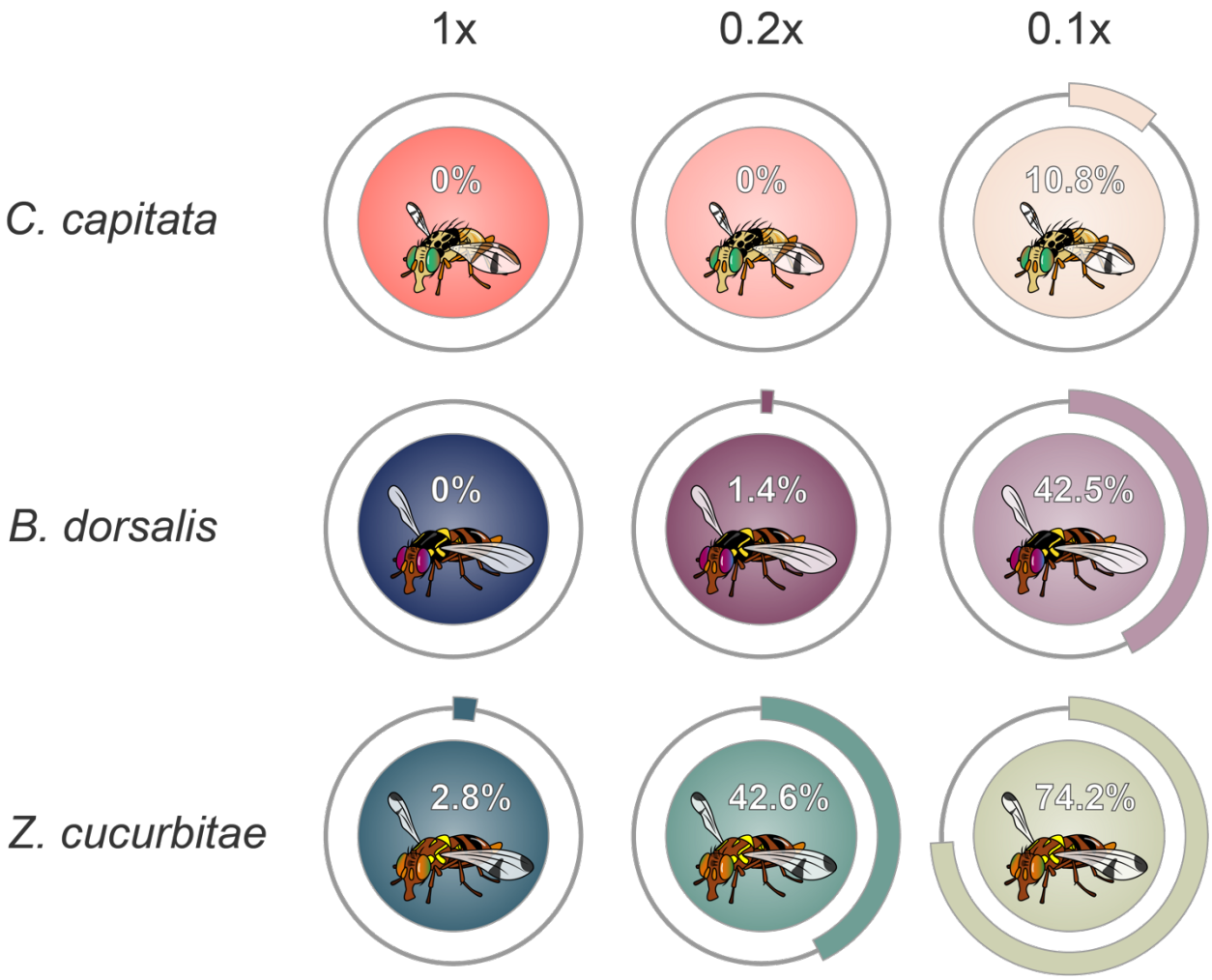

Figure 5.3. DlEPV injection causes differential virulence within fly species. The emergence rate of adult *C. capitata*, *B. dorsalis*, and *Z. cucurbitae* flies was measured after larvae were injected with 1 oviposition equivalent (1x), 0.2 oviposition equivalents (0.2x), or 0.1 oviposition equivalents (0.1x) of purified DlEPV. The outer ring length of each pie chart and the numerical label within indicates the percentage of emerged flies after treatment with active virus normalized by the percentage of emerged flies after treatment with inactive virus. Raw emergence data are provided in Supplemental Table 5.1.

cucurbitae flies exhibited the lowest overall susceptibility to DlEPV, since a substantial proportion of flies survived virus infection at all 3 doses: 2.8% flies emerged at 1 oviposition equivalent,

42.6% at 0.2 oviposition equivalents, and 74.2% at 0.1 oviposition equivalents (Figure 5.3). Taken together, these results demonstrate a gradation of fruit fly susceptibility to DlEPV, in which the virus is most virulent within *C. capitata*, less virulent within *B. dorsalis*, and least virulent to *Z. cucurbitae*.

DlEPV abundance patterns after injection show an added dimension of viral activity within flies. Next, we investigated viral abundance patterns in non-parasitized flies by injecting fly larvae with the same three doses of DlEPV used above and measuring viral copy number over time with qPCR. DlEPV abundance was also measured after injection with UV-inactivated DlEPV as a control, which resulted in limited viral DNA amplification over time for any dose or species (Supplemental Figure 5.1). For the active DlEPV treatment, a significant interaction was observed between time and dose effects within all fly species (*C. capitata* interaction effect: $F_{10,54} = 15.19$, $P < 0.0001$; *B. dorsalis* interaction effect: $F_{10,54} = 30.87$, $P < 0.0001$; *Z. cucurbitae* interaction effect: $F_{10,54} = 5.77$, $P < 0.0001$). Within *C. capitata* flies, injection with either 1 or 0.2 oviposition equivalents of active DlEPV resulted in rapid virus replication, as viral copy number significantly rose to $> 10^{10}$ copies by 120 h post injection (hpi) (1 oviposition equivalent: $F_{5,18} = 131.46$, $P < 0.0001$; 0.2 oviposition equivalents: $F_{5,18} = 267.52$, $P < 0.0001$) (Figure 5.4A). However, injection of *C. capitata* with 0.1 oviposition equivalents resulted in a diminished virus replication pattern, in which a significant drop in viral copy number was observed at 24 hpi, and subsequent amplification of the virus only reached approximately 10^6 copies by 120 hpi (0.1 oviposition equivalent: $F_{5,18} = 14.06$, $P < 0.0001$) (Figure 5.4A). These results suggest that *C. capitata* can form a detectable immune response when infected with a low dose of DlEPV that suppresses overall virus replication performance. When *B. dorsalis* larvae were injected with 1 oviposition

equivalent of DlEPV, we observed a pattern of rapid virus replication similar to *C. capitata* ($F_{5,18}$ = 207.64, $P < 0.0001$) (Figure 5.4B). Interestingly, *B. dorsalis* demonstrated a significant drop in

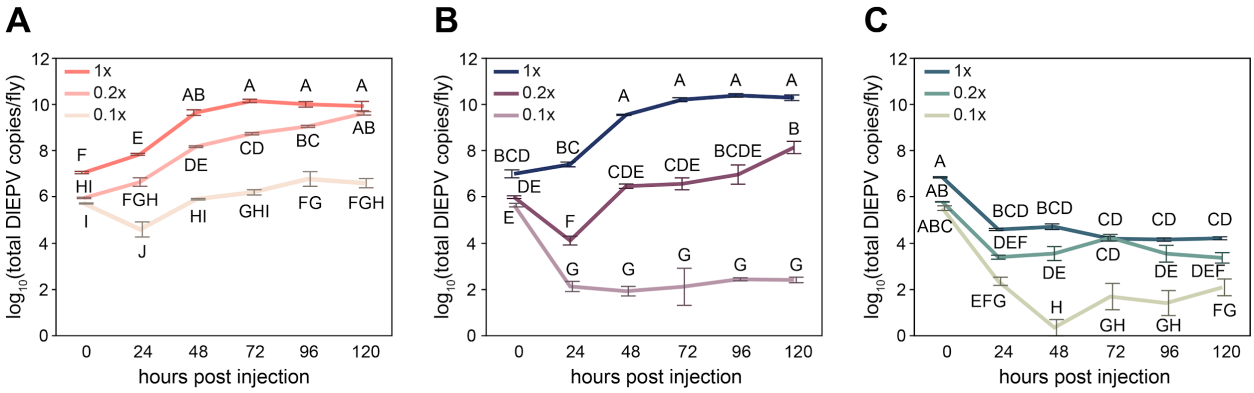

Figure 5.4. DlEPV replication patterns after injection demonstrate varying tolerance to virus replication. qPCR was used to measure DlEPV abundance over time after (A) *C. capitata*, (B) *B. dorsalis*, and (C) *Z. cucurbitae* flies were injected with either 1, 0.2, or 0.1 oviposition equivalents of purified DlEPV. The average $\log_{10}$-transformed DlEPV copy numbers in each graph were determined from 4 replicate fly samples for each time point and viral dose. Error bars are as indicated in Figure 5.2, and letter(s) above mean data points indicate significantly distinct values when all means were compared with Tukey's HSD independently of either main effect.

viral copy number at 24 hpi when injected with 0.2 oviposition equivalents, followed by a subdued replication curve similar to viral abundance activity in *C. capitata* when injected with 0.1 oviposition equivalents ($F_{5,18}$ = 29.37, $P < 0.0001$) (Figure 5.4B). This suggests that *B. dorsalis* can mount a noticeable immune response at a higher dose than *C. capitata*. Furthermore, DlEPV was almost entirely abolished by 24 hpi in *B. dorsalis* when injected with 0.1 oviposition equivalents, demonstrating that the immune response of *B. dorsalis* was successful at largely clearing viral infection at this dose ($F_{5,18}$ = 15.94, $P < 0.0001$) (Figure 5.4B). In contrast to the DlEPV replication observed within *C. capitata* and *B. dorsalis* at multiple initial doses, we

observed no virus replication within *Z. cucurbitae* after injection with any of the three doses (Figure 5.4C). Moreover, a significant decrease in viral copy number was observed throughout infection with all 3 doses of virus (1 oviposition equivalent: $F_{5,18} = 171.40$, $P < 0.0001$; 0.2 oviposition equivalents: $F_{5,18} = 17.86$, $P < 0.0001$; 0.1 oviposition equivalents: $F_{5,18} = 20.22$, $P < 0.0001$) (Figure 5.4C), as viral abundance dropped by over 99% in all cases (Figure 5.4C). These results therefore support that the immune response within *Z. cucurbitae* is the most efficient at overcoming DlEPV infection.

5.4 DISCUSSION

***D. longicaudata* displays varying compatibility with tropical fruit flies.** Our parasitism assay results demonstrated that *C. capitata* and *B. dorsalis* are compatible hosts for *D. longicaudata*, due to the > 50% average wasp emergence rate observed for both permissive fly species. *Z. cucurbitae*, however, served as an incompatible host that failed to produce any adult wasps across all replicate trials. These findings are in agreement with previous sampling data from wild populations on Hawaii that found *C. capitata* and *B. dorsalis* to be frequently parasitized by *D. longicaudata* within infested fruits, while *Z. cucurbitae* was not observed to be successfully parasitized by this parasitoid species [26,32]. However, the host range pattern of *D. longicaudata* established in this study is somewhat peculiar with respect to both host fly taxonomy and ecology, which are two factors thought to be important for determining parasitoid host range [5].

First, if taxonomy was to dictate the host range of *D. longicaudata*, we would expect that permissive hosts would be more closely related to one another than to non-permissive hosts. Of the three fly species investigated here, *C. capitata* is by far the most distantly related in the group, while *B. dorsalis* and *Z. cucurbitae* (formerly *Bactrocera cucurbitae* Coquillett) belong to sister

genera, both within the Dacini tribe [30]. Furthermore, prior studies have established that fruit flies within the genus *Anastrepha* are also compatible hosts for *D. longicaudata*, and previously reported parasitism assays using *A. suspensa* flies yielded > 60% average *D. longicaudata* survival [23]. *Anastrepha* constitutes an even more distantly related tephritid lineage that belongs to a separate subfamily compared to the three fly species used in this investigation [33]. Therefore, the incompatibility of *Z. cucurbitae* as a host for *D. longicaudata* is fairly anomalous with respect to taxonomy, given the compatibility of the closely related *B. dorsalis* combined with the widespread compatibility of this parasitoid for hosts across the tephritid family.

Second, if shared ecology of fly hosts contributes to *D. longicaudata* host range, we would expect there to be differences between the ecology of *Z. cucurbitae* that are otherwise shared by *C. capitata* and *B. dorsalis*. One of the primary characteristics shared by all three fly species is their highly polyphagous nature, which has caused them to become serious pests in agriculture around the world [31]. *C. capitata* has the largest known host range of the invasive flies in Hawaii, and *B. dorsalis* develops within many of the same fruits and vegetables [34,35]. *Z. cucurbitae* is similarly destructive towards many fruits and vegetables but appears to have a greater preference for hosts within the Cucurbitaceae family, such as watermelon, tomato, cucumber, and squash [36]. However, host overlap between the three species has been documented in Hawaii [32]. Also, the distributions of these three species all occur widely throughout South Asia, suggesting host overlap occurs on a larger scale [31,37]. Furthermore, *A. suspensa* is native to Central America and has not spread across the Pacific, indicating that this species, which is also a suitable host for *D. longicaudata*, maintains a unique distribution and likely some distinct host preferences compared to *B. dorsalis* and *Z. cucurbitae* [31]. *D. longicaudata* therefore attacks flies with a wide range of host preferences and geographical distributions, many of which overlap with *Z.*

cucurbitae, suggesting that host ecology also does not strongly support the *D. longicaudata* host range pattern observed here. Consequently, we explored other factors, such as microbial symbionts, that could help shape the host range of this parasitoid species.

DlEPV activity is strongly associated with *D. longicaudata*-host compatibility. We investigated the symbiotic virus DlEPV as an alternative potential factor that may affect *D. longicaudata* host range given the highly beneficial role that this virus plays for *D. longicaudata* during parasitism [23]. Our viral abundance measurements during *D. longicaudata* parasitism within the three fly species showed a remarkable correlation between host permissiveness and replicative ability of DlEPV, which was corroborated by the virulence and replication patterns of the virus after manual injection. The connection between permissiveness to *D. longicaudata* parasitism and virulence associated with DlEPV replication uncovered here suggests that viral activity may contribute to maintaining these species as viable hosts for *D. longicaudata*. Similar correlations between parasitoid permissiveness and viral activity have been observed in some EVE associations, such as in the wasp *Campoletis sonorensis* and its EVE, known as Campoletis sonorensis ichnovirus (CsIV). A prior study of CsIV demonstrated that the ability of the virus to maintain prolonged virulence gene expression within permissive caterpillar hosts of *C. sonorensis* was not observed during parasitism within non-permissive hosts of the wasp [38]. Similarly, the EVE carried by the wasp *Microplitis demolitor*, named Microplitis demolitor bracovirus (MdBV), displayed decreased expression of most virulence genes within the non-permissive host *Trichoplusia ni* compared to the permissive host *Chrysodeixis includens* [39]. Taken together, these studies suggest that EVEs are similarly involved in shaping the host range of the parasitoid that produces them. Viral contributions to parasitoid host range could therefore be a feature of convergent evolution between EVEs and DlEPV. However, due to the obligate and endogenous

nature of EVEs, they are viewed more as extensions of parasitoid genomes rather than true endosymbionts that evolve apart from their associated wasps [40]. EVEs therefore could be a means for further specialization of the wasps that inherit them, leading to further speciation within EVE-producing wasp lineages [41]. In contrast, DlEPV is an exogenous virus and plays a facultative role for *D. longicaudata* survival [23,24]. These distinctions not only suggest that DlEPV is the first true viral symbiont with a demonstrated link to parasitoid host range, but also that DlEPV may be responsible for expanding *D. longicaudata* host range rather than restricting it.

Dynamics of DlEPV infection suggest differences in the strength of host antiviral responses. Manual injection of DlEPV into non-parasitized flies at multiple doses provided a nuanced look at the effects of DlEPV on the different fly species, and revealed that each species used in this study has a distinct level of tolerance for viral infection. Our results showed a strong correlation between effective DlEPV replication and fly susceptibility after viral injection, with *C. capitata* displaying the most consistent evidence of virus replication and lowest survival rate among the tested viral doses and *Z. cucurbitae* showing no virus replication and partial survival at all given doses. Furthermore, each species was able to survive viral infection in some capacity for at least one of the administered doses, which was always associated with a significant drop in viral abundance at 24 hpi. From these findings, we hypothesize that DlEPV infection induces active antiviral responses from all 3 fly species that have varying success depending on the initial viral dose and the strength of the immune response. Future transcriptome sequencing of flies during DlEPV infection could provide a better understanding of how the virus affects each host species and reciprocally, how flies respond to viral infection at varying doses. Differential expression analysis from transcriptomic data such as this could also be used to identify specific DlEPV genes

that are the target of a successful immune response by searching for viral genes that are downregulated during an effective immune response compared to a different dose or host species when the immune system is overwhelmed.

The mechanism(s) used by these fly species to reduce or eliminate DlEPV infection is currently unclear, although the host tropism of poxviruses has been extensively studied and may offer some insight. Unlike many viruses that are blocked by non-permissive hosts at the level of entry into the host cell, poxviruses are widely known for their ability to infect cells from off-target hosts, and host compatibility is instead determined during subsequent processes of the replication cycle [42]. For example, vertebrate-infecting poxviruses, known as chordopoxviruses, can successfully enter and begin viral gene expression within insect cells before virus replication is eventually stalled [43]. Therefore, DlEPV infection is likely targeted by fly hosts after entry into cells but before virus replication can occur. Future investigation of viral gene expression during infection within each species could narrow down the stage of DlEPV replication targeted by flies, as poxvirus gene expression occurs sequentially, and viral genes are categorized into early, intermediate, and late stages based on their chronological activity throughout the virus replication cycle [44]. We could therefore use reverse transcriptase qPCR (RT-qPCR) to measure expression of select early, intermediate, and late DlEPV genes during the first 24 h of infection to identify where virus replication breaks down when an effective fly immune response is elicited.

Our results also clearly demonstrate that *Z. cucurbitae* flies are by far the most capable of thwarting parasitism by *D. longicaudata*, as well as inhibiting DlEPV replication, compared to *C. capitata* and *B. dorsalis*. Our DlEPV injection results show that *Z. cucurbitae* can mount an effective immune response to viral infection of at least 10-fold greater initial concentration compared to the other flies evaluated here. The link between *Z. cucurbitae* immunity towards

DlEPV and non-permissiveness towards *D. longicaudata* suggests that this fly species could specifically target DlEPV as a method to inhibit *D. longicaudata* parasitism. Since parasitoid-host compatibility factors, such as host taxonomy or ecology, do not clearly explain the ability of *Z. cucurbitae* to ward off *D. longicaudata*, potential factors external to the innate immune response of *Z. cucurbitae* that allow such a strong response toward DlEPV would be interesting to explore. For example, microbial symbionts with defensive roles during parasitoid attack have repeatedly been identified in other insects, such as the bacteria *Hamiltonella defensa* in aphids and *Spiroplasma poulsonii* in *Drosophila*. These bacterial symbionts reside within the hemolymph of host insects and enable hosts to resist parasitism [45,46]. In many cases, these symbionts are thought to restrict the host range of the parasitoid toward insects without symbiont-mediated protection [47]. Furthermore, complex microbe-microbe interactions could also occur during *D. longicaudata* parasitism within *Z. cucurbitae* and contribute to host incompatibility. The symbiotic bacterium *Wolbachia*, which is widespread across insects, protects *Wolbachia*-infected hosts, such as *Drosophila melanogaster*, against viral infection by reducing the titer of several RNA viruses [48,49]. Therefore, possible interactions between DlEPV and resident microbes within *Z. cucurbitae* could be involved in the unusual host range pattern observed here.

Concluding Remarks. Our collective results in this study demonstrate that the activity of a facultative viral symbiont is connected to the host range of its associated parasitoid. The inferred function of DlEPV in upholding the wide host range of *D. longicaudata* has novel implications for the history of this parasitoid species as an effective fruit fly biocontrol agent. *D. longicaudata* remains one of the most important parasitoid species released for the control of tropical fruit flies on a global scale [25,28]. The parasitism behavior of *D. longicaudata* as a generalist has likely contributed to its consistent ability to become established in numerous introduced locations, such

as Hawaii, the continental United States, South America, and various islands throughout the Pacific [25,28]. The findings of this investigation thus insinuate that DlEPV could be responsible for the reliable establishment of new *D. longicaudata* populations, and by extension, the wide scale success of *D. longicaudata* for pest management programs. Additionally, the parasitoid *F. arisanus*, which is a highly successful biocontrol agent of tephritid fruit flies in its own right, is also a generalist species and maintains a heritable association with a recently discovered EVE, known as Fopius arisanus endogenous nudivirus (FaENV) [14]. Therefore, beneficial viruses serve as a previously hidden aspect of existing fruit fly parasitoid systems that are likely fundamental to the success of current pest management strategies, and further study of these systems could lead to innovative biocontrol tactics against destructive fruit fly pests.

5.5 MATERIALS AND METHODS

Insects. *D. longicaudata* parasitoid wasps, as well as *C. capitata*, *B. dorsalis*, and *Z. cucurbitae* fruit flies used in this study were obtained from USDA-ARS laboratory colonies kept at the Daniel K. Inouye U.S. Pacific Basin Agricultural Research Center in Hilo, Hawaii, which were reared as previously described [50,51].

Parasitism assays. Assays that measured the parasitism success of *D. longicaudata* developing within each of the three fly species were conducted in a similar manner to that reported previously [23]. Fly larvae of a given species were retrieved from within larval diet containers at the second instar stage and placed between two pieces of organza fabric restricted within a 2.5 in flexible embroidery hoop. Groups of six adult female wasps that were at least 7 d old and naive (i.e. had not yet laid eggs) were offered fly larvae for 4 h to allow for oviposition. Afterward, fly larvae were placed back into fresh diet and kept in standard rearing conditions until they pupated,

approximately 48-72 h later. At this time, fly pupae were inspected for those that bore a single oviposition scar on the pupal casing, known as the puparium, indicating that a single wasp egg had been deposited within. Singly scarred flies were then kept in standard rearing conditions for 4 weeks, after which the proportions of emerged adult wasps, adult flies, and puparia from which nothing emerged were recorded. The rate of occurrence for each category was calculated by dividing the number of insects in the category by the total number of singly scarred flies in the trial. Significant differences in average outcome rate between the three fly species were statistically analyzed using one-way ANOVA, and multiple comparison testing was done using Tukey's HSD with JMP Pro 14 software.

Virus injections. DlEPV was filter-purified from the pooled venom gland tissue of 100 female *D. longicaudata* wasps using a previously performed protocol [23]. Resulting purified virus particles were eluted in 400 µL phosphate-buffered saline (PBS), and the inactive virus stock was generated by exposing half of the prepared active virus stock to shortwave (254nm) UV energy for 10 min using a UVP HL-2000 HybriLinker. Both active and inactive virus stocks were stored at -80^{o}C. A DlEPV dose of 1 oviposition equivalent/µL was obtained by making a 1:20 dilution of the virus stock, and successive dilutions were made from this dose to generate the 0.2 and 0.1 oviposition equivalent doses. Third instar fly larvae were each injected with 1 µL of virus from each treatment and dose, then kept in standard rearing conditions for 4 weeks. The proportions of adult flies that emerged after injection with DlEPV were then calculated by dividing the number of adult flies that had emerged by the total number of larvae injected for each treatment and dose.

DNA isolation and qPCR estimation of viral abundance. DlEPV abundance was estimated over time by collecting fly samples during parasitism by *D. longicaudata* or after manual injection of purified virus. Third instar fly larvae were used for initial oviposition or injection in both time

course analyses. DNA was extracted from whole-body fly samples using the NucleoMag 96 Tissue Kit (Macherey-Nagel) performed on a KingFisher Flex instrument (Thermo). DNA samples were each eluted in 100 µL elution buffer consisting of 5mM Tris/HCl (pH 8.5). Viral copy number was then determined for each sample with qPCR using specific primers for the DlEPV poly(A) polymerase small subunit gene, as done previously [23]. Copy numbers were $\log_{10}$-transformed, then subjected to statistical analysis with JMP Pro 14. One-way ANOVA was used to test for significantly different mean copy numbers across timepoints during parasitism, and Tukey's HSD was used for multiple comparison tests. For injection data, two-way ANOVA was used to test for differences in means between levels of either timepoint or dosage, as well as the interaction between the two effects.

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5.7 REFERENCES

1. LaSalle J, Gauld ID. Hymenoptera: their biodiversity, and their impact on the diversity of other organisms. In: LaSalle J, Gauld ID, editors. Hymenoptera and Biodiversity. Wallingford, UK: CAB International; 1993. pp. 1–26.

2. Huber JT. Biodiversity of Hymenoptera. In: Foottit RG, Adler PH, editors. Insect Biodiversity. Chichester, UK: John Wiley & Sons, Ltd; 2017. pp. 419–461.

3. Forbes AA, Bagley RK, Beer MA, Hippee AC, Widmayer HA. Quantifying the unquantifiable: why Hymenoptera, not Coleoptera, is the most speciose animal order. BMC Ecol. 2018;18: 21.

4. Quicke DLJ. Parasitic wasps. London, UK: Chapman & Hall; 1997.

5. Godfray HCJ. Parasitoids: Behavioral and Evolutionary Ecology. Princeton, NJ: Princeton University Press; 1994.

6. Shaw MR. Parasitoid host ranges. In: Hawkins BA, Sheehan W, editors. Parasitoid Community Ecology. Oxford, UK: Oxford University Press; 1994. pp. 111–144.

7. Whitfield JB. Mutualistic viruses and the evolution of host ranges in endoparasitoid Hymenoptera. In: Hawkins BA, Sheehan W, editors. Parasitoid Community Ecology. Oxford, UK: Oxford University Press; 1994. pp. 163–176.

8. Strand MR, Pech LL. Immunological basis for compatibility in parasitoid host relationships. Annu Rev Entomol. 1995;40: 31–56.

9. Mrinalini, Werren JH. Parasitoid Wasps and Their Venoms. In: Gopalakrishnakone P, Malhotra A, editors. Evolution of Venomous Animals and Their Toxins. Dordrecht, NL: Springer; 2017. pp. 187–212.

10. Asgari S, Rivers DB. Venom proteins from endoparasitoid wasps and their role in host-parasite interactions. Annu Rev Entomol. 2011;56: 313–335.

11. Strand MR, Burke GR. Polydnaviruses: nature's genetic engineers. Annu Rev Virol. 2014;1: 333–354.

12. Lawrence PO. Non-poly-DNA viruses, their parasitic wasps, and hosts. J Insect Physiol. 2005;51: 99–101.

13. Pichon A, Bézier A, Urbach S, Aury JM, Jouan V, Ravallec M, et al. Recurrent DNA virus domestication leading to different parasite virulence strategies. Sci Adv. 2015;1: e1501150.

14. Burke GR, Simmonds TJ, Sharanowski BJ, Geib SM. Rapid viral symbiogenesis via changes in parasitoid wasp genome architecture. Mol Biol Evol. 2018;35: 2463–2474.

15. Burke GR, Hines HM, Sharanowski BJ. Endogenization from diverse viral ancestors is common and widespread in parasitoid wasps. bioRxiv [Preprint]. 2020; 2020.06.17.148684.

16. Bézier A, Annaheim M, Herbinière J, Wetterwald C, Gyapay G, Bernard-Samain S, et al. Polydnaviruses of braconid wasps derive from an ancestral nudivirus. Science. 2009;323: 926–930.

17. Volkoff A-N, Jouan V, Urbach S, Samain S, Bergoin M, Wincker P, et al. Analysis of virion structural components reveals vestiges of the ancestral ichnovirus genome. PLoS Pathog. 2010;6: e1000923.

18. Burke GR, Walden KKO, Whitfield JB, Robertson HM, Strand MR. Widespread genome reorganization of an obligate virus mutualist. PLoS Genet. 2014;10: e1004660.

19. Béliveau C, Cohen A, Stewart D, Periquet G, Djoumad A, Kuhn L, et al. Genomic and proteomic analyses indicate that banchine and campoplegine polydnaviruses have similar, if not identical, viral ancestors. J Virol. 2015;89: 8909–8921.

20. Drezen J-M, Leobold M, Bézier A, Huguet E, Volkoff A-N, Herniou EA. Endogenous viruses of parasitic wasps: variations on a common theme. Curr Opin Virol. 2017;25: 41–48.

21. Burke GR. Common themes in three independently derived endogenous nudivirus elements in parasitoid wasps. Curr Opin Insect Sci. 2019;32: 28–35.

22. Gauthier J, Drezen J-M, Herniou EA. The recurrent domestication of viruses: major evolutionary transitions in parasitic wasps. Parasitology. 2018;145: 713–723.

23. Coffman KA, Harrell TC, Burke GR. A mutualistic poxvirus exhibits convergent evolution with other heritable viruses in parasitoid wasps. J Virol. 2020;94: e02059–19.

24. Coffman KA, Burke GR. Genomic analysis reveals an exogenous viral symbiont with dual functionality in parasitoid wasps and their hosts. PLoS Pathog. 2020; In press.

25. Ovruski S, Aluja M, Sivinski J, Wharton R. Hymenopteran parasitoids on fruit-infesting Tephritidae (Diptera) in Latin America and the Southern United States: diversity, distribution, taxonomic status and their use in fruit fly biological control. Integr Pest Manage Rev. 2000;5: 81–107.

26. Bess HA, Van Den Bosch R, Haramoto FH. Fruit fly parasites and their activities in Hawaii. Proc Hawaiian Entomol Soc. 1961;17: 367–378.

27. Haramoto FH, Bess HA. Recent studies on the abundance of the oriental and Mediterranean fruit flies and the status of their parasites. Proc Hawaiian Entomol Soc. 1970;20: 551–566.

28. Vargas RI, Leblanc L, Harris EJ, Manoukis NC. Regional suppression of *Bactrocera* fruit flies (Diptera: Tephritidae) in the Pacific through biological control and prospects for future introductions into other areas of the world. Insects. 2012;3: 727–742.

29. Vargas RI, Mau RFL, Jang EB, Faust RM, Wong L. The Hawaii Fruit Fly Areawide Pest Management Programme. In: Opender K, Cuperus GW, Elliott N, editors. Areawide Pest Management: Theory and Implementation. Wallingford, UK: CAB International; 2008. pp. 300–325.

30. Virgilio M, Jordaens K, Verwimp C, White IM, De Meyer M. Higher phylogeny of frugivorous flies (Diptera, Tephritidae, Dacini): localised partition conflicts and a novel generic classification. Mol Phylogenet Evol. 2015;85: 171–179.

31. White IM, Elson-Harris MM. Fruit Flies of Economic Significance: Their Identification and Bionomics. Wallingford, UK: CAB International; 1992.

32. Nishida T, Haramoto F. Immunity of *Dacus cucurbitae* to attack by certain parasites of *Dacus dorsalis*. J Econ Entomol. 1953;46: 61–64.

33. Han HY, McPheron BA. Molecular phylogenetic study of Tephritidae (Insecta: Diptera) using partial sequences of the mitochondrial 16S ribosomal DNA. Mol Phylogenet Evol. 1997;7: 17–32.

34. Liquido NJ, Cunningham RT, Shinoda LA. Host plants of the Mediterranean fruit fly, Diptera: Tephritidae, an annotated world review. Misc Publ Entomol Soc Am. 1991;77: 1–52.

35. Allwood AJ, Chinajariyawong A, Kritsaneepaiboon S, Drew RA, Hamacek EL, Hancock DL, et al. Host plant records for fruit flies (Diptera: Tephritidae) in Southeast Asia. Raffles Bull Zool. 1999;47: 1–92.

36. Harris EJ, Takara JM, Nishida T. Distribution of the melon fly, *Dacus cucurbitae* (Diptera: Tephritidae), and host plants on Kauai, Hawaiian Islands. Environ Entomol. 1986;15: 488–493.

37. Vargas RI, Piñero JC, Leblanc L. An overview of pest species of *Bactrocera* fruit flies (Diptera: Tephritidae) and the integration of biopesticides with other biological approaches for their management with a focus on the Pacific region. Insects. 2015;6: 297–318.

38. Cui L, Soldevila AI, Webb BA. Relationships between polydnavirus gene expression and host range of the parasitoid wasp *Campoletis sonorensis*. J Insect Physiol. 2000;46: 1397–1407.

39. Bitra K, Burke GR, Strand MR. Permissiveness of lepidopteran hosts is linked to differential expression of bracovirus genes. Virology. 2016;492: 259–272.

40. Strand MR, Burke GR. Polydnaviruses: Evolution and Function. Curr Issues Mol Biol. 2020;34: 163–182.

41. Branca A, Gitau C, Dupas S. Chapter 10 - Maintenance of Specialized Parasitoid Populations by Polydnaviruses. In: Beckage NE, Drezen J-M, editors. Parasitoid Viruses. San Diego: Academic Press; 2012. pp. 127–135.

42. McFadden G. Poxvirus tropism. Nat Rev Microbiol. 2005;3: 201–213.

43. Li Y, Yuan S, Moyer RW. The non-permissive infection of insect (gypsy moth) LD-652 cells by vaccinia virus. Virology. 1998;248: 74–82.

44. Broyles SS. Vaccinia virus transcription. J Gen Virol. 2003;84: 2293–2303.

45. Oliver KM, Russell JA, Moran NA, Hunter MS. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc Natl Acad Sci. 2003;100: 1803–1807.

46. Xie J, Vilchez I, Mateos M. *Spiroplasma* bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma*. PLoS One. 2010;5: e12149.

47. Monticelli LS, Outreman Y, Frago E, Desneux N. Impact of host endosymbionts on parasitoid host range—from mechanisms to communities. Curr Opin Insect Sci. 2019;32: 77–82.

48. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. *Wolbachia* and virus protection in insects. Science. 2008;322: 702.

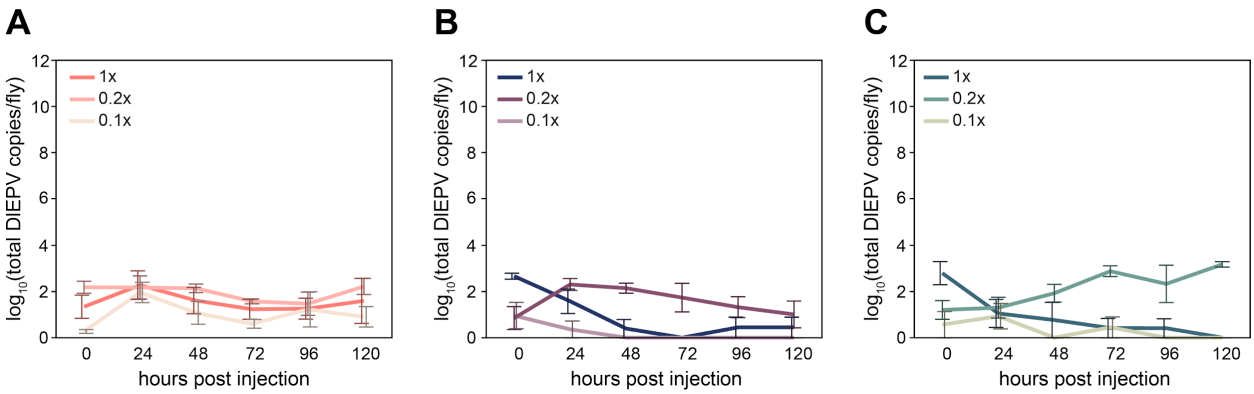
49. Teixeira L, Ferreira A, Ashburner M. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biol. 2008;6: e2.

50. Vargas RI. Mass Production of Tephritid Fruit Flies. In: Robinson AS, Hooper G, editors. Fruit Flies: Their Biology, Natural Enemies and Control. Amsterdam, NL: Elsevier; 1989. pp. 141–151.

51. Wong TTY, Ramadan MM. Mass Rearing Biology of Larval Parasitoids (Hymenoptera: Braconidae: Opiinae) of Tephritid Flies (Diptera: Tephritidae) in Hawaii. In: Anderson TE, Leppla NC, editors. Advances in Insect Rearing for Research and Pest Management. Boulder, CO: Westview Press; 1992. pp. 405–426.

Supplemental Table 5.1. Fruit fly emergence data after injection with DlEPV.

Fly species	DlEPV dose (oviposition equivalents)	Active DlEPV no. adult flies/total no. flies injected	Active DlEPV emergence rate	Inactive DlEPV no. adult flies/total no. flies injected	Inactive DlEPV emergence rate	Normalized active emergence rate
Ceratitis capitata	1	0/83	0.0%	54/88	61.4%	0.0%
	0.2	0/84	0.0%	6080	75.0%	0.0%
	0.1	7/82	8.5%	77/97	79.4%	10.8%
Bactrocera dorsalis	1	0/78	0.0%	76/94	80.9%	0.0%
	0.2	1/80	1.3%	76/88	86.4%	1.4%
	0.1	31/87	35.6%	78/93	83.9%	42.5%
Zeugodacus cucurbitae	1	2/80	2.5%	72/80	90.0%	2.8%
	0.2	30/85	35.3%	72/87	82.8%	42.6%
	0.1	57/89	64.0%	76/88	86.4%	74.2%



Supplemental Figure 5.1. UV-inactivated DlEPV shows limited activity after injection into fruit flies. qPCR was used to measure viral abundance over time when inactive DlEPV was injected into fly larvae in order to confirm that the inactive treatment was a negative control for virus activity. Mean values and error bars are as indicated in Figure 5.4.

CHAPTER 6

Conclusions

Beneficial viruses inherited by parasitoid wasps are not commonly viewed in the same context as heritable bacteria and other microbial symbionts of insects, perhaps due to their non-cellular nature or their generally antagonistic activities within parasitoid host insects. The rise of insect genome sequencing has further distanced parasitoid viruses from endosymbionts, because all known beneficial viruses that have been genetically characterized are endogenous viral elements (EVEs) that exist exclusively within wasp genomes. Accordingly, parasitoid EVEs can no longer be considered as symbionts of parasitoid wasps, because they are not autonomous entities. In this dissertation, I have used an integrative approach to establish a novel insect-virus system that effectively bridges the gap between parasitoid EVEs and traditional microbial symbionts.

In Chapters 2 and 3, I demonstrated that the poxvirus inherited by *Diachasmimorpha longicaudata* wasps, known as Diachasmimorpha longicaudata entomopoxvirus (DlEPV), displays convergent evolution with parasitoid EVEs but is a rare example of a beneficial virus that maintains replicative autonomy within *D. longicaudata* wasps and fruit fly hosts. I also showed that the independent replication of DlEPV within wasps and parasitized hosts is in agreement with its exogenous and complete genome, although due to its lack of integration, DlEPV must utilize a novel method for persistence within its associated parasitoid compared to EVEs. These combined

results distinguish DlEPV as a true viral symbiont, which has not been empirically demonstrated for a virus before and likely has major repercussions in terms of the evolution and maintenance of this virus within its associated parasitoid lineage. Chapters 4 and 5 further explore the dynamics of DlEPV in light of the new perspective with which we can view this symbiotic virus. My results revealed a unique mechanism of beneficial virus transmission that allows for rapid spread of DlEPV to new wasps. In addition, I showed that DlEPV displays an intriguing correlation to *D. longicaudata* host range that could contribute to the widespread establishment and success of *D. longicaudata* as a biological control agent.

In summary, this dissertation serves as a first step for extended investigation into the roles that mutualistic viral symbionts play within insects. Insights from this work, along with other examples of symbiotic viruses, should be included in our collective understanding of microbial symbiosis, as they can be subjected to similar evolutionary forces and therefore, may inform how related systems operate. Future directions of study for this system will likely involve the deduction of specific mechanisms that facilitate DlEPV interactions with parasitoids and fruit flies, including the molecular basis for post-hatch transmission and controlled virus replication in wasps, as well as the specific DlEPV genes and their targets within flies that contribute to the beneficial phenotype provided to developing wasps.