Journal of Virology®

GENETIC DIVERSITY AND EVOLUTION

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

Kelsey A. Coffman,[a] Taylor C. Harrell,[a] Gaelen R. Burke[a]

[a]Department of Entomology, University of Georgia, Athens, Georgia, USA

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.

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

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

Citation Coffman KA, Harrell TC, Burke GR. 2020. A mutualistic poxvirus exhibits convergent evolution with other heritable viruses in parasitoid wasps. J Virol 94:e02059-19. https://doi.org/10.1128/JVI.02059-19.

Editor Joanna L. Shisler, University of Illinois at Urbana Champaign

Address correspondence to Kelsey A. Coffman, kcoffman@uga.edu.

Received 6 December 2019
Accepted 28 January 2020
Accepted manuscript posted online 5 February 2020
Published 31 March 2020

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.

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 (Fig. 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 ($F_{4,25} = 43.27$, $P < 0.0001$) (Fig. 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 ($F_{4,45} = 27.71$, $P < 0.0001$) (Fig. 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: $F_{2,15} = 108.63$, $P < 0.0001$) (Fig. 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: $F_{2,15} = 8.46$, $P = 0.0035$) (Fig. 1B). Furthermore, there was a relative lack of DlEPV in prepupal gut contents compared to

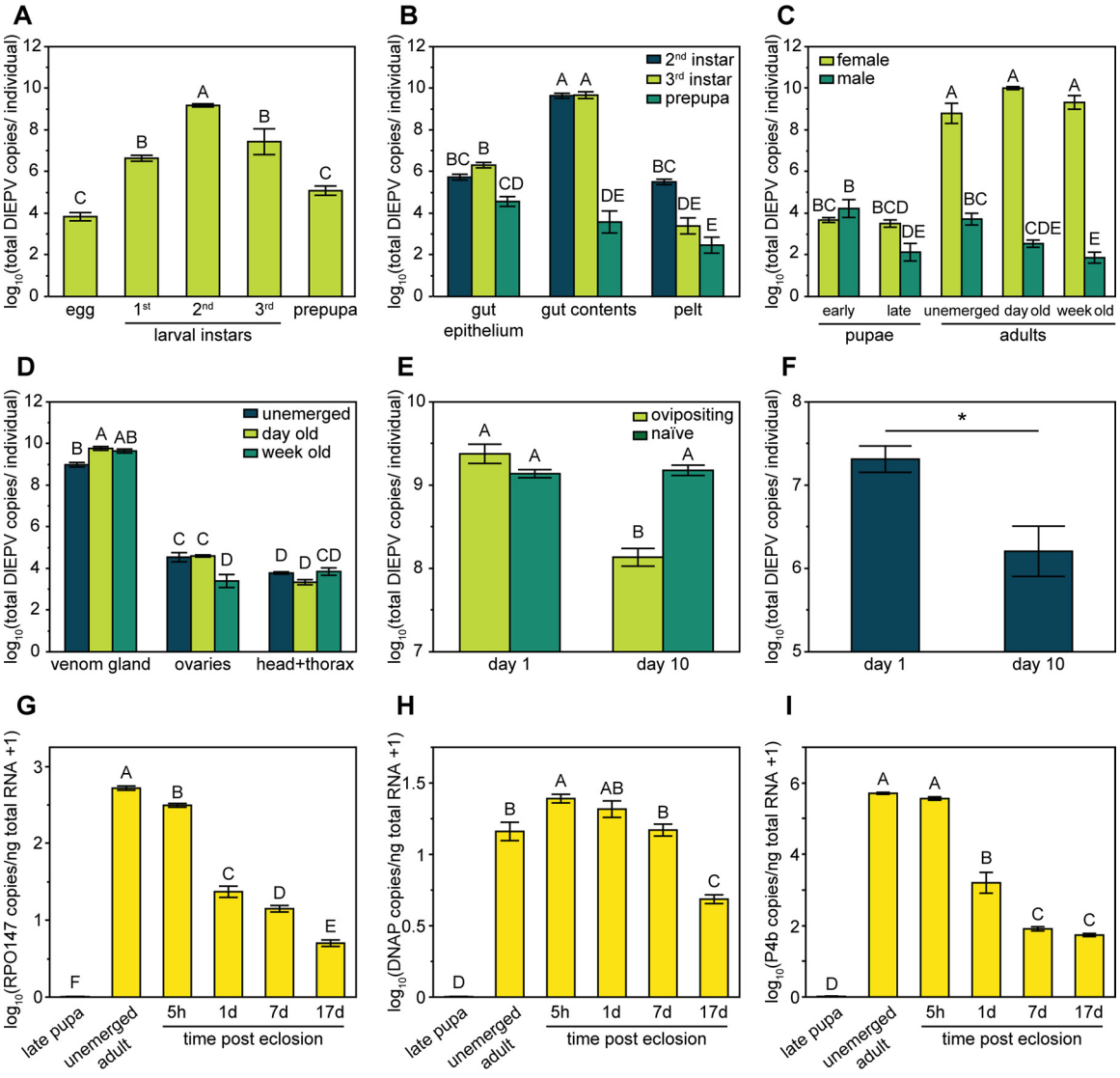

FIG 1 DIEPV replication and transcriptional activity within *D. longicaudata* wasps. DIEPV abundance was estimated via qPCR in egg and larval wasp stages (A), larval wasp gut tissues (B), male and female pupal through adult wasp stages (C), and adult female reproductive tissues (D). Eggs, first-instar, 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) DIEPV copy number per wasp venom gland (replicates pooled in triplicates) at day 1 and day 10. (F) DIEPV 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 DIEPV genome copy number per individual averaged from 6 biological replicates. Expression of DIEPV genes measured with RT-qPCR in female wasp venom glands from late pupa to 17-day-old adult. Profiled DIEPV 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: *, $P < 0.01$.

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 (Fig. 1B).

DIEPV 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 ($F_{4,50} = 70.45$, $P < 0.0001$) (Fig. 1C). We detected a small amount of virus within male and female wasps throughout early and late pupal stages (Fig. 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 emergence (eclosion) and into adulthood (female life stage effect: $F_{4,25} = 131.47$, $P < 0.0001$) (Fig. 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 (Fig. 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 ($F_{4,45} = 12.53$, $P < 0.0001$) (Fig. 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 (Fig. 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 ($F_{1,20} = 53.33$, $P < 0.0001$) (Fig. 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: $F_{1,10} = 0.26$, $P = 0.62$) (Fig. 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 (Fig. 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 (Fig. 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 $F_{5,30} = 329.93$, $P < 0.0001$) (Fig. 1G to I). These expression data confirm that high-level viral replication is

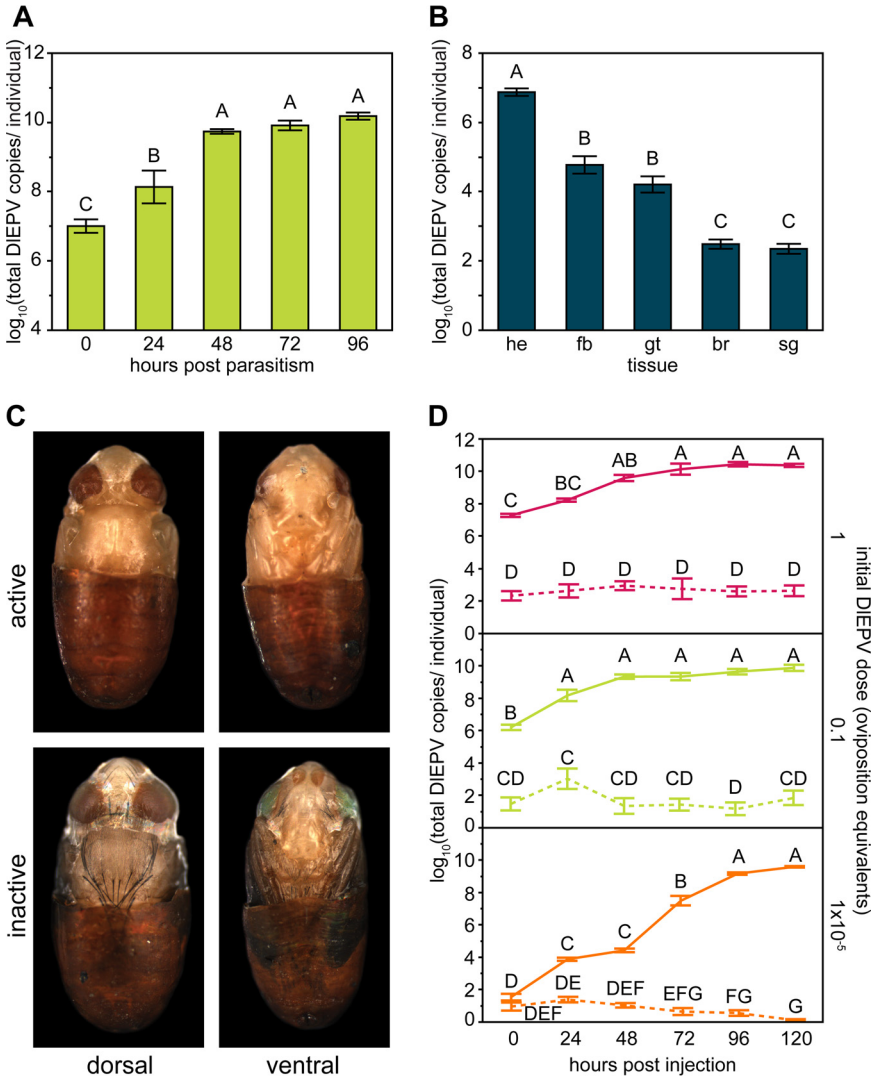

FIG 2 DIEPV replication and phenotypic effects within *A. suspensa* flies. DIEPV abundance was estimated with qPCR in parasitized fly tissue after oviposition by 1-week-old naive wasps. Absolute DIEPV copy number within whole flies throughout parasitism (A) and larval fly tissues at 4 hpp (B), including 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 DIEPV compared to the normal progression of development observed in flies injected with the same dose of UV-inactivated DIEPV. Background debris from dissections in images was removed using Adobe Photoshop CC 2019. (D) qPCR estimation of DIEPV abundance in flies injected with one of three doses of either active or UV-inactivated DIEPV: (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 DIEPV, while dashed lines indicate viral copy number in flies injected with UV-inactivated DIEPV. Mean copy numbers, error bars, and statistical significance are indicated as defined in the legend for Fig. 1.

not continuous and demonstrate that female wasps have a finite amount of DIEPV to utilize throughout their lifetime.

Virus replication also occurs in parasitized hosts and primarily infects host hemocytes. DIEPV 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 DIEPV genome copies were injected into each fly larva (Fig. 2A). DIEPV 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 ($F_{4,25} = 31.58$, $P < 0.0001$) (Fig. 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 ($F_{4,25} = 102.25$, $P < 0.0001$) (Fig. 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) (Fig. 2B), the antagonistic impact of this virus within fly hosts has not yet been directly demonstrated. We therefore investigated the effects 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 (Fig. 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 (Fig. 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: $F_{5,60} = 222.02$, $P < 0.0001$) (Fig. 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: $F_{5,30} = 62.50$, $P < 0.0001$) (Fig. 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 (Fig. 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 (Fig. 3A to C). Furthermore, DlEPV genome copy number was reduced by >99.99% in DlEPV dsRNA-treated wasps, indicating that

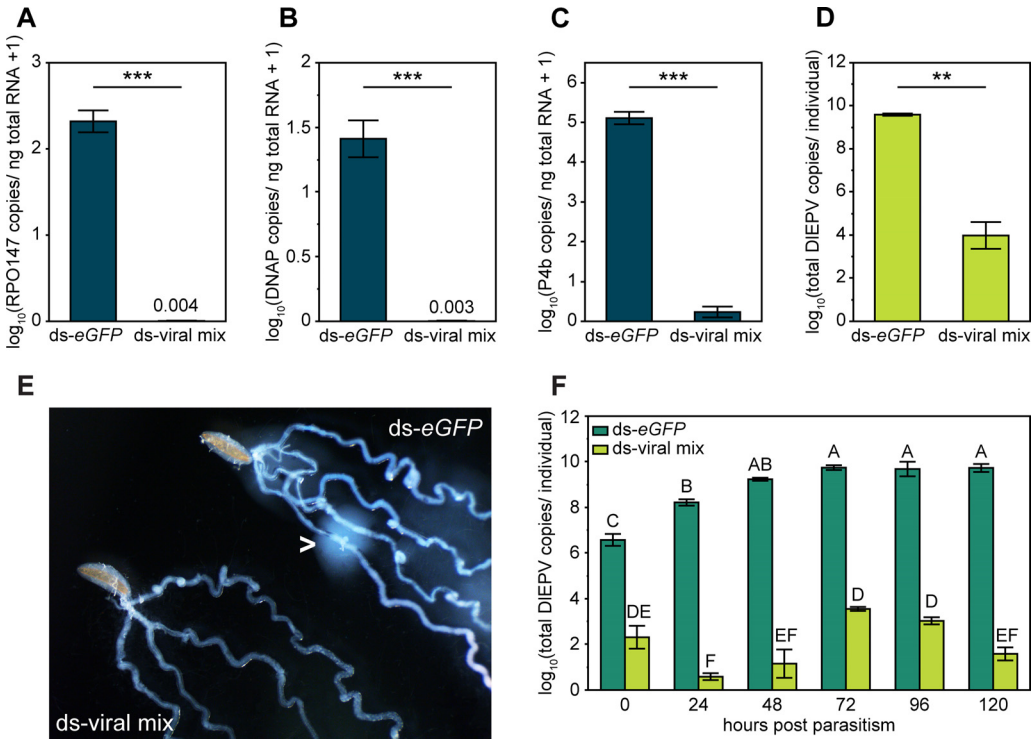

FIG 3 RNAi knockdown of three DlEPV genes reduces viral abundance within both *D. longicaudata* wasps and in *A. suspensa* fly hosts during parasitism. RT-qPCR estimation of viral gene expression after early female wasp pupae were injected with a double-stranded RNA (dsRNA) cocktail specific for RPO147 (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 Fig. 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 Fig. 1. Error bars in each graph represent one standard error above and below the mean.

the knockdown of RPO147, DNAP, and P4b gene expression deprives wasps of the vast majority of virus that is normally present (Fig. 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 ($F_{5,60} = 13.01$, $P < 0.0001$) (Fig. 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: $F_{5,30} = 9.99$, $P < 0.0001$) (Fig. 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.

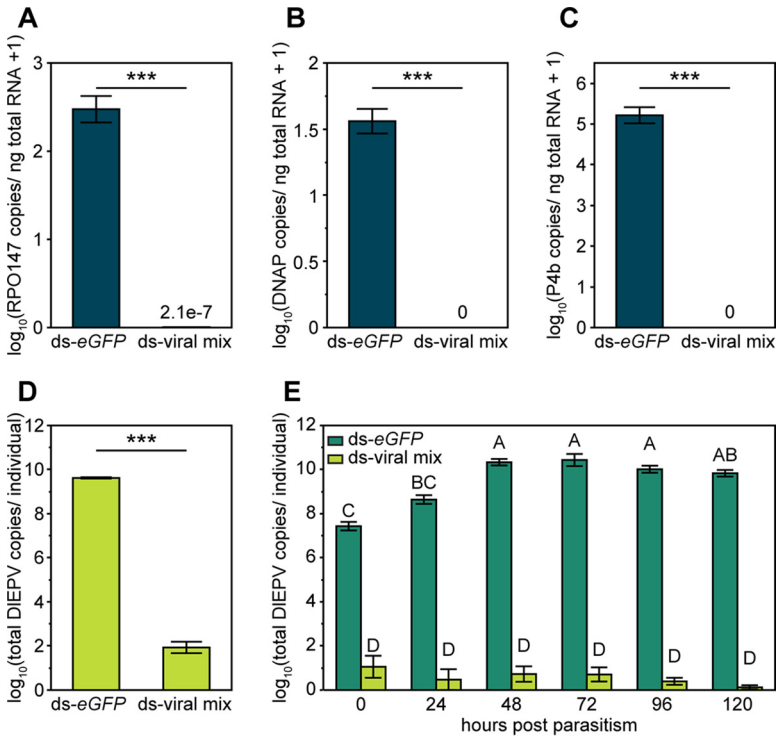

FIG 4 RNAi knockdown phenotype persists in second-generation wasps. (A to C) RT-qPCR estimation of viral gene expression in female progeny of dsRNA-treated wasps. Venom glands were sampled from newly emerged daughters of control (ds-*egfp*) and virus-deprived (ds-viral mix) wasps as described in the legend for Fig. 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 Fig. 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 Fig. 3.

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 (Fig. 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 magnitude further reduced compared to that in the parental generation, with each venom gland containing an average of 100 viral genome copies (Fig. 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 ($F_{5,60} = 11.64$, $P < 0.0001$) (Fig. 4E). Almost no DlEPV was introduced and no viral replication occurred in flies during parasitism (second-generation ds-viral mix time effect: $F_{5,30} = 0.85$, $P = 0.53$) (Fig. 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

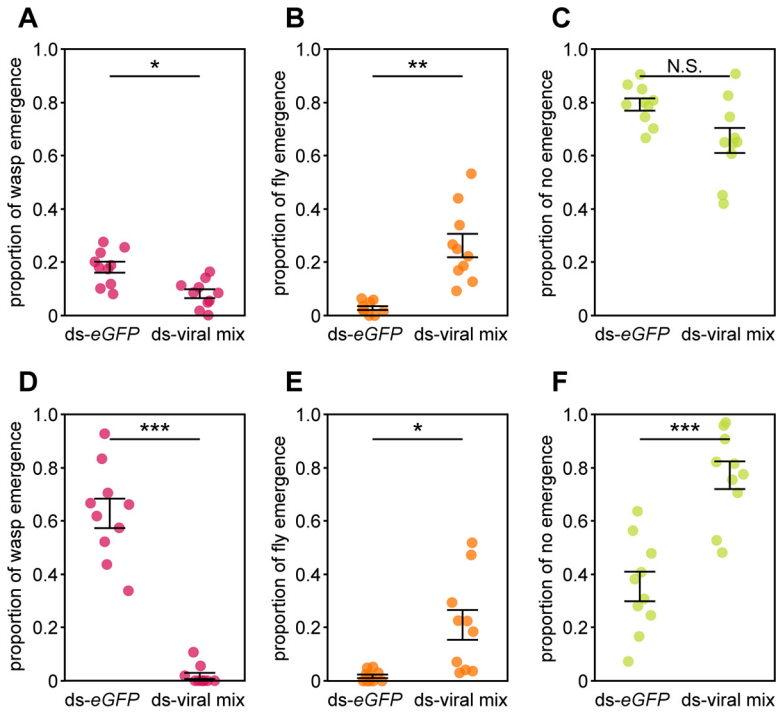

FIG 5 Progeny of second-generation dsRNA-treated wasps without accompanying virus show heavily reduced parasitism success. Proportional emergence rates of wasps (A and D), flies (B and E), or no emergence (C and F) after oviposition by second-generation dsRNA-treated wasps. Flies were offered to wasps 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 Fig. 3.

a fitness benefit to wasps (Fig. 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 (Fig. 5B). Surprisingly, high proportions (>60%) of "no emergence" were observed in both treatments of these assays (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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.

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 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.

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

TABLE 1 Primer sequences used in this study

	Sequence (5′→3′)	
Primer set	**Forward**	**Reverse**
PolyAPol qPCR	GCTCCAGTAAAACCGTTTCC	GGCTTTGGATCGTAAAACCA
RPO147 RT-qPCR	AACGATGCGTTGGTGATTTT	CAAGATGCCCAAAGATGGAC
DNAP RT-qPCR	AAAATTGGAATCGGGTGGAT	TTGCGAAAGTTGGTTGTGAG
P4b RT-qPCR	CGTGGGGAAACTGATATGCT	GGATTCCCCTCCAGTTTGTT
RPO147 RNAi	TAATACGACTCACTATAGGGTGGTGTTCACAAAGGCAAAA	TAATACGACTCACTATAGGGTGAGTGATCCAGCGTTACCA
DNAP RNAi	TAATACGACTCACTATAGGGGCCACTGGTGCCAAAACTAT	TAATACGACTCACTATAGGGCCAAGCATTTCTCCGATTTC
P4b RNAi	TAATACGACTCACTATAGGGCCACACTTTTGGCTCGTACA	TAATACGACTCACTATAGGGATATTGGCTTCTGCGGTTTG

in 30 μl water. qPCR primers were designed with Primer3 (56) (Table 1). We estimated DlEPV genome copy number using the DlEPV putative 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 (C_T) 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). 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) and

venom gland DNA as the template. The three resulting types of DIEPV-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 $\times$ 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.

ACKNOWLEDGMENTS

We thank Sarah Waldron, Tu Dang, and Hannah Boomgarden for insect colony maintenance, Sherilyn Harper for assistance with venom gland repeated oviposition experiments, and Tyler Simmonds and Michael Strand for meaningful discussions regarding this work.

This research was supported by the U.S. National Science Foundation DEB-1622986, IOS-1748862, and DEB-1916788 (G.R.B.), the USDA National Institute of Food and Agriculture Hatch project (1013423 to G.R.B.), and the University of Georgia's Research Foundation, Office for the Vice President for Research, and Agricultural Experiment Station (G.R.B.).

REFERENCES

1. Strand MR, Pech LL. 1995. Immunological basis for compatibility in parasitoid host relationships. Annu Rev Entomol 40:31–56. https://doi.org/10.1146/annurev.en.40.010195.000335.
2. Asgari S, Rivers DB. 2011. Venom proteins from endoparasitoid wasps and their role in host-parasite interactions. Annu Rev Entomol 56:313–335. https://doi.org/10.1146/annurev-ento-120709-144849.
3. Drezen J-M, Leobold M, Bézier A, Huguet E, Volkoff A-N, Herniou EA. 2017. Endogenous viruses of parasitic wasps: variations on a common theme. Curr Opin Virol 25:41–48. https://doi.org/10.1016/j.coviro.2017.07.002.
4. Burke GR, Strand MR. 2012. Polydnaviruses of parasitic wasps: domestication of viruses to act as gene delivery vectors. Insects 3:91–119. https://doi.org/10.3390/insects3010091.
5. Bézier A, Annaheim M, Herbinière J, Wetterwald C, Gyapay G, Bernard-Samain S, Wincker P, Roditi I, Heller M, Belghazi M, Pfister-Wilhem R, Periquet G, Dupuy C, Huguet E, Volkoff A-N, Lanzrein B, Drezen J-M. 2009. Polydnaviruses of braconid wasps derive from an ancestral nudivirus. Science 323:926–930. https://doi.org/10.1126/science.1166788.
6. Volkoff A-N, Jouan V, Urbach S, Samain S, Bergoin M, Wincker P, Demettre E, Cousserans F, Provost B, Coulibaly F, Legeai F, Béliveau C, Cusson M, Gyapay G, Drezen J-M. 2010. Analysis of virion structural components reveals vestiges of the ancestral ichnovirus genome. PLoS Pathog 6:e1000923. https://doi.org/10.1371/journal.ppat.1000923.
7. Herniou EA, Huguet E, Thézé J, Bézier A, Periquet G, Drezen J-M. 2013. When parasitic wasps hijacked viruses: genomic and functional evolution of polydnaviruses. Philos Trans R Soc Lond B Biol Sci 368:20130051. https://doi.org/10.1098/rstb.2013.0051.
8. Beckage NE. 2012. Polydnaviruses as endocrine regulators, p 163–168. *In* Beckage NE, Drezen J-M (ed), Parasitoid viruses. Academic Press, San Diego, CA.
9. Strand MR. 2012. Polydnavirus gene products that interact with the host immune system, p 149–161. *In* Beckage NE, Drezen J-M (ed), Parasitoid viruses. Academic Press, San Diego, CA.
10. Pichon A, Bézier A, Urbach S, Aury JM, Jouan V, Ravallec M, Guy J, Cousserans F, Thézé J, Gauthier J, Demettre E, Schmieder S, Wurmser F, Sibut V, Poirie M, Colinet D, da Silva C, Couloux A, Barbe V, Drezen JM,

Volkoff AN. 2015. Recurrent DNA virus domestication leading to different parasite virulence strategies. Sci Adv 1:e1501150. https://doi.org/10.1126/sciadv.1501150.
11. Burke GR, Simmonds TJ, Sharanowski BJ, Geib SM. 2018. Rapid viral symbiogenesis via changes in parasitoid wasp genome architecture. Mol Biol Evol 35:2463–2474. https://doi.org/10.1093/molbev/msy148.
12. Lawrence PO. 2005. Non-poly-DNA viruses, their parasitic wasps, and hosts. J Insect Physiol 51:99–101. https://doi.org/10.1016/j.jinsphys.2004.12.012.
13. Burke GR. 2019. Common themes in three independently derived endogenous nudivirus elements in parasitoid wasps. Curr Opin Insect Sci 32:28–35. https://doi.org/10.1016/j.cois.2018.10.005.
14. Béliveau C, Cohen A, Stewart D, Periquet G, Djoumad A, Kuhn L, Stoltz D, Boyle B, Volkoff A-N, Herniou EA, Drezen J-M, Cusson M. 2015. Genomic and proteomic analyses indicate that banchine and campoplegine polydnaviruses have similar, if not identical, viral ancestors. J Virol 89:8909–8921. https://doi.org/10.1128/JVI.01001-15.
15. Ovruski S, Aluja M, Sivinski J, Wharton R. 2000. 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 Manag Rev 5:81–107. https://doi.org/10.1023/A:1009652431251.
16. Strand MR, Burke GR. 2014. Polydnaviruses: nature's genetic engineers. Annu Rev Virol 1:333–354. https://doi.org/10.1146/annurev-virology-031413-085451.
17. Edson KM, Barlin MR, Vinson SB. 1982. Venom apparatus of braconid wasps: comparative ultrastructure of reservoirs and gland filaments. Toxicon 20:553–562. https://doi.org/10.1016/0041-0101(82)90049-6.
18. Lawrence PO, Akin D. 1990. Virus-like particles from the poison glands of the parasitic wasp *Biosteres longicaudatus* (Hymenoptera: Braconidae). Can J Zool 68:539–546. https://doi.org/10.1139/z90-079.
19. Luo L, Zeng L. 2010. A new rod-shaped virus from parasitic wasp *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae). J Invertebr Pathol 103:165–169. https://doi.org/10.1016/j.jip.2009.08.008.
20. Lawrence PO, Matos LF. 2005. Transmission of the *Diachasmimorpha longicaudata* rhabdovirus (DlRhV) to wasp offspring: an ultrastructural analysis. J Insect Physiol 51:235–241. https://doi.org/10.1016/j.jinsphys.2005.01.002.
21. Simmonds TJ, Carrillo D, Burke GR. 2016. Characterization of a venom gland-associated rhabdovirus in the parasitoid wasp *Diachasmimorpha longicaudata*. J Insect Physiol 91–92:48–55. https://doi.org/10.1016/j.jinsphys.2016.06.009.
22. Lawrence PO. 2005. Morphogenesis and cytopathic effects of the *Diachasmimorpha longicaudata* entomopoxvirus in host haemocytes. J Insect Physiol 51:221–233. https://doi.org/10.1016/j.jinsphys.2004.12.003.
23. Hashimoto Y, Lawrence PO. 2005. Comparative analysis of selected genes from *Diachasmimorpha longicaudata* entomopoxvirus and other poxviruses. J Insect Physiol 51:207–220. https://doi.org/10.1016/j.jinsphys.2004.10.010.
24. Lawrence PO. 2002. Purification and partial characterization of an entomopoxvirus (DlEPV) from a parasitic wasp of tephritid fruit flies. J Insect Sci 2:10. https://doi.org/10.1673/031.002.1001.
25. Lawrence PO, Dillard BE. 2008. A homolog of the vaccinia virus D13L rifampicin resistance gene is in the entomopoxvirus of the parasitic wasp, *Diachasmimorpha longicaudata*. J Insect Sci 8:8. https://doi.org/10.1673/031.008.0801.
26. Mwaengo DM, Lawrence PO. 2003. A putative DNA helicase and novel oligoribonuclease in the Diachasmimorpha longicaudata entomopoxvirus (DlEPV). Arch Virol 148:1431–1444. https://doi.org/10.1007/s00705-002-0975-3.
27. Paladino LZC, Papeschi AG, Cladera JL. 2010. Immature stages of development in the parasitoid wasp, *Diachasmimorpha longicaudata*. J Insect Sci 10:56. https://doi.org/10.1673/031.010.5601.
28. Quicke D. 1997. Parasitic wasps. Chapman & Hall, London, UK.
29. Lawrence PO. 1981. Host vibration - a cue to host location by the parasite, *Biosteres longicaudatus*. Oecologia 48:249–251. https://doi.org/10.1007/BF00347971.
30. Upton C, Slack S, Hunter AL, Ehlers A, Roper RL. 2003. Poxvirus orthologous clusters: toward defining the minimum essential poxvirus genome. J Virol 77:7590–7600. https://doi.org/10.1128/jvi.77.13.7590-7600.2003.
31. Perera S, Li Z, Pavlik L, Arif B. 2010. Entomopoxviruses, p 83–102. *In* Asgari S, Johnson KN (ed), Insect virology. Caister Academic Press, Norfolk, UK.
32. Lawrence PO, Baranowski RM, Greany PD. 1976. Effect of host age on development of *Biosteres* (= *Opius*) *longicaudatus*, a parasitoid of the Caribbean fruit fly, *Anastrepha suspensa*. Fla Entomol 59:33–39. https://doi.org/10.2307/3493166.
33. Roossinck MJ. 2011. The good viruses: viral mutualistic symbioses. Nat Rev Microbiol 9:99–108. https://doi.org/10.1038/nrmicro2491.
34. Moran NA, McCutcheon JP, Nakabachi A. 2008. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet 42:165–190. https://doi.org/10.1146/annurev.genet.41.110306.130119.
35. Douglas AE. 2010. The symbiotic habit. Princeton University Press, Princeton, NJ.
36. Attardo GM, Lohs C, Heddi A, Alam UH, Yildirim S, Aksoy S. 2008. Analysis of milk gland structure and function in *Glossina morsitans*: milk protein production, symbiont populations and fecundity. J Insect Physiol 54:1236–1242. https://doi.org/10.1016/j.jinsphys.2008.06.008.
37. Balmand S, Lohs C, Aksoy S, Heddi A. 2013. Tissue distribution and transmission routes for the tsetse fly endosymbionts. J Invertebr Pathol 112:S116–S122. https://doi.org/10.1016/j.jip.2012.04.002.
38. Gonzalez PI, Montoya P, Perez-Lachaud G, Cancino J, Liedo P. 2007. Superparasitism in mass reared *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae), a parasitoid of fruit flies (Diptera: Tephritidae). Biol Control 40:320–326. https://doi.org/10.1016/j.biocontrol.2006.11.009.
39. Montoya P, Ruiz L, Perez-Lachaud G, Cancino J, Liedo P. 2013. Field superparasitism by *Diachasmimorpha longicaudata* attacking *Anastrepha* spp. larvae on mango fruits. Biol Control 64:160–165. https://doi.org/10.1016/j.biocontrol.2012.10.015.
40. Strand MR. 2012. Polydnavirus gene expression profiling: what we know now, p 139–147. *In* Beckage NE, Drezen J-M (ed), Parasitoid viruses. Academic Press, San Diego, CA.
41. Dupuy C, Gundersen-Rindal DE, Cusson M. 2012. Genomics and replication of polydnaviruses, p 47–61. *In* Beckage NE, Drezen J-M (ed), Parasitoid viruses. Academic Press, San Diego, CA.
42. Strand MR, Burke GR. 2012. Polydnaviruses as symbionts and gene delivery systems. PLoS Pathog 8:e1002757. https://doi.org/10.1371/journal.ppat.1002757.
43. Pennacchio F, Strand MR. 2006. Evolution of developmental strategies in parasitic Hymenoptera. Annu Rev Entomol 51:233–258. https://doi.org/10.1146/annurev.ento.51.110104.151029.
44. Strand M. 2010. Polydnaviruses, p 171–197. *In* Asgari S, Johnson KN (ed), Insect virology. Caister Academic Press, Norfolk, UK.
45. Lawrence PO, Baker FC, Tsai LW, Miller CA, Schooley DA, Geddes LG. 1990. 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 13:53–62. https://doi.org/10.1002/arch.940130106.
46. Palli SR, Ladd TR, Tomkins WL, Shu S, Ramaswamy SB, Tanaka Y, Arif B, Retnakaran A. 2000. *Choristoneura fumiferana* entomopoxvirus prevents metamorphosis and modulates juvenile hormone and ecdysteroid titers. Insect Biochem Mol 30:869–876. https://doi.org/10.1016/S0965-1748(00)00060-6.
47. Nakai M, Shiotsuki T, Kunimi Y. 2004. An entomopoxvirus and a granulovirus use different mechanisms to prevent pupation of *Adoxophyes honmai*. Virus Res 101:185–191. https://doi.org/10.1016/j.virusres.2004.01.003.
48. Nakai M, Kinjo H, Takatsuka J, Shiotsuki T, Kamita SG, Kunimi Y. 2016. Entomopoxvirus infection induces changes in both juvenile hormone and ecdysteroid levels in larval *Mythimna separata*. J Gen Virol 97:225–232. https://doi.org/10.1099/jgv.0.000325.
49. Balgopal MM, Dover BA, Goodman WG, Strand MR. 1996. Parasitism by *Microplitis demolitor* induces alterations in the juvenile hormone titers and juvenile hormone esterase activity of its host, *Pseudoplusia includens*. J Insect Physiol 42:337–345. https://doi.org/10.1016/0022-1910(95)00111-5.
50. Strand MR, Dover BA. 1991. Developmental disruption of *Pseudoplusia includens* and *Heliothis virescens* larvae by the calyx fluid and venom of *Microplitis demolitor*. Arch Insect Biochem Physiol 18:131–145. https://doi.org/10.1002/arch.940180302.
51. Edson KM, Vinson SB, Stoltz DB, Summers MD. 1981. Virus in a parasitoid wasp: suppression of the cellular immune response in the parasitoid's host. Science 211:582–583. https://doi.org/10.1126/science.7455695.
52. Burke GR, Thomas SA, Eum JH, Strand MR. 2013. Mutualistic polydnaviruses share essential replication gene functions with pathogenic ancestors. PLoS Pathog 9:e1003348. https://doi.org/10.1371/journal.ppat.1003348.

53. Lawrence PO. 1979. Immature stages of the Caribbean fruit fly, *Anastrepha suspensa*. Fla Entomol 62:214–219. https://doi.org/10.2307/3494059.
54. Moss B. 2007. Poxviridae: the viruses and their replication, p 2905–2945. *In* Knipe DM, Howley PM (ed), Fields virology, 5th ed, vol 2. Lippincott Williams & Wilkins, Philadelphia, PA.
55. Jehle JA. 2010. Nudiviruses: their biology and genetics, p 153–170. *In* Asgari S, Johnson KN (ed), Insect virology. Caister Academic Press, Norfolk, UK.
56. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012. Primer3-new capabilities and interfaces. Nucleic Acids Res 40:e115. https://doi.org/10.1093/nar/gks596.
57. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N, di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 29:644–652. https://doi.org/10.1038/nbt.1883.
58. Montoya P, Liedo P, Benrey B, Barrera JF, Cancino J, Aluja M. 2000. Functional response and superparasitism by *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae), a parasitoid of fruit flies (Diptera: Tephritidae). Ann Entomol Soc Am 93:47–54. https://doi.org/10.1603/0013-8746(2000)093[0047:FRASBD]2.0.CO;2.