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A viral mutualist employs posthatch transmission for vertical and horizontal spread among parasitoid wasps

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Heritable symbionts display a wide variety of transmission strategies to travel from one insect generation to the next. Parasitoid wasps, one of the most diverse insect groups, maintain several heritable associations with viruses that are beneficial for wasp survival during their development as parasites of other insects. Most of these beneficial viral entities are strictly transmitted through the wasp germline as endogenous viral elements 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 tactics to infect future wasp generations. Here, we demonstrated that transmission of DlEPV is primarily dependent on parasitoid wasps, since viral transmission within fruit fly hosts of the wasps was limited to injection of the virus directly into the larval fly body cavity. Additionally, we uncovered a previously undocumented form of posthatch transmission for a mutualistic virus that entails external acquisition and localization of the virus within the adult wasp venom gland. We showed that this route is extremely effective for vertical and horizontal transmission of the virus within *D. longicaudata* wasps. Furthermore, the beneficial phenotype provided by DlEPV during parasitism was also transmitted with perfect efficiency, indicating an effective mode of symbiont spread to the advantage of infected wasps. These results provide insight into the transmission of beneficial viruses among insects and indicate that viruses can share features with cellular microbes during their evolutionary transitions into symbionts.

parasitoid wasp | symbiosis | poxvirus | transmission | *Diachasmimorpha longicaudata*

Significance

Mutualistic viruses remain a rarity among known animal–microbe symbioses. Yet, several beneficial viruses have been identified within insects called parasitoid wasps. Most of these viral entities are permanent components of wasp genomes. However, a mutualistic poxvirus found within *Diachasmimorpha longicaudata* wasps maintains an independent genome and may therefore behave in ways more similar to cellular microbial symbionts. In this study, we discovered unique properties of viral symbiont transmission, including an evolved dependence on parasitoid wasps for virus spread among fruit fly hosts and a distinct mode of faithful virus transmission among parasitoid wasps. These findings demonstrate that certain symbiont transmission pathways have arisen independently across disparate life forms to play pivotal roles in insect biology and evolution.

Insects are well-known for their widespread and complex associations with microbes (1). Bacterial symbionts remain the predominant focus of current research, although fungal, archaeal, and protozoan symbionts have also been described in insects (2–4). The study of beneficial viruses, in contrast, is limited to isolated examples across the tree of life (5, 6). An increasing number of beneficial viruses have been characterized in insects called parasitoid wasps, in which viruses are important factors for successful parasitism and survival of wasp offspring within host insects (7–9). These viral elements are produced in massive abundance 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 (10, 11). Most of these viruses are transmitted vertically through the germline of wasps as endogenous viral elements (EVEs) within wasp genomes (12). However, a poxvirus produced by the wasp *Diachasmimorpha longicaudata*, known as Diachasmimorpha longicaudata entomopoxvirus (DlEPV), represents a rare example of a true mutualistic viral 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 (13, 14).

An important question concerning the maintenance of viral symbiosis is how DlEPV is transmitted and persists within insect hosts. Because DlEPV retains several characteristics from its pathogenic ancestor, it may employ similar transmission mechanisms to those used by insect poxvirus relatives or other pathogenic viruses of insects. Entomopoxviruses are spread primarily through per os transmission when virus particles are ingested by larval stage host insects and infiltrate through the gut to internal tissues for mass replication (15). Many large DNA viruses utilize per os transmission among insects, while others have evolved unique means for transfer to new hosts. For example, ascoviruses are pathogenic insect viruses that are mechanically transferred to new lepidopteran hosts via inoculation by parasitoid wasp ovipositors (16).

DlEPV may also share modes of transmission with cellular microbial symbionts due to its beneficial function in *D. longicaudata* wasps and its exogenous genome structure. Obligate symbiotic associations, such as the bacterium *Buchnera aphidicola* within pea aphids, often display strict vertical transmission, in which bacteria are incorporated into developing embryos within the female insect (17, 18). Other insect symbionts,

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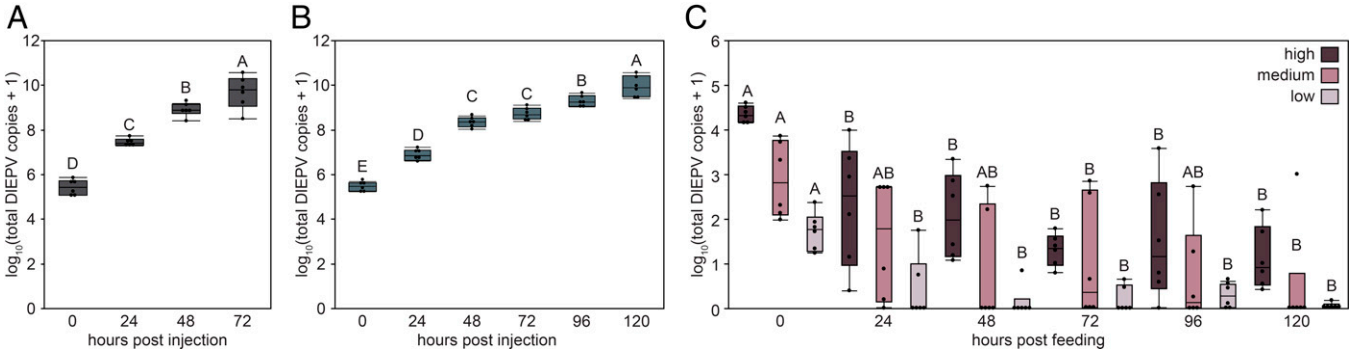


Fig. 1. The mode of DlEPV transmission among *A. suspensa* flies is limited to injection into the hemocoel of larvae. Virus replication was estimated with qPCR measurements of DlEPV copy number in whole flies over time after (*A*) injection of fly larvae with venom gland-propagated DlEPV virions, (*B*) injection of fly larvae with fly-propagated virions, and (*C*) ingestion of virions incorporated into the diet of fly larvae. Boxplots in all panels were generated using $\log_{10}$-transformed copy numbers, each calculated from six biological replicates. Letters above each box plot represent the results of Tukey's honestly significant difference (HSD) tests, in which means within each panel that have a distinct letter are significantly different from one another. Each box plot in *C*, which includes multiple main effects, was analyzed independently for each dose using Tukey HSD tests. High, medium, and low designations in *C* refer to the different viral doses to which fly larvae were subjected.

like those found within hemipterans in the suborder Heteroptera, are acquired after egg-hatching when the insect consumes microbes that the mother has provided near the site of oviposition (19). This form of posthatch symbiont acquisition allows for external vertical transmission, also known as pseudovertical transmission (20). The reproductive manipulator *Arsenophonus* uses a unique form of posthatch transmission within *Nasonia vitripennis* parasitoid 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 (21, 22).

In this study, we explored the transmission capabilities of DlEPV within *D. longicaudata* wasps and *Anastrepha suspensa* fruit fly hosts. We first determined that DlEPV displays a singular transmission strategy among flies, which could represent an evolutionary loss of transmission capacity that constrains DlEPV spread to those routes facilitated by *D. longicaudata* wasps. We also identified an unusual mode of transmission among wasps 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. DlEPV is an uncommon instance of a mutualistic virus that displays this type of posthatch transmission.

Results

DlEPV Is Successfully Transmitted between Fly Hosts via Hypodermic Injection. Previous data have shown that DlEPV can replicate within *A. suspensa* fly hosts (13), and comparative genomic analyses demonstrated that DlEPV likely originated as a pathogen of flies (14). These findings both suggest that DlEPV might maintain ancestral poxvirus transmission strategies within larval stage *A. suspensa* hosts. We therefore first investigated whether DlEPV virions could continuously infect and replicate within fly larvae via microinjection. Briefly, DlEPV virions purified from *D. longicaudata* venom glands were injected into fly larvae and allowed to replicate for 72 h. "Fly-propagated" virions were then isolated from fly hemolymph and injected into new fly larvae, and DlEPV copy number was measured over time with quantitative PCR (qPCR) to determine if the virus continued to replicate within successive fly larval hosts. A dose of $\sim 1 \times 10^5$ copies of venom gland-derived DlEPV was initially injected into second-instar fly larvae, which grew significantly to $>1 \times 10^9$ copies by 72 h postinjection (hpi) ($F_{3,20} = 108.52$, $P < 0.0001$) (Fig. 1*A*). A similar dose of fly-propagated virions was then injected into new third-instar fly larvae, which also resulted in significant DlEPV copy number growth in the 5 d following infection ($F_{5,30} = 198.10$, $P < 0.0001$) (Fig. 1*B*). This demonstrates that infectious DlEPV particles are produced from replication in either wasps or flies, and that intrahemocoelic injection of DlEPV isolated from infected flies is an effective route of viral transmission to new fly hosts.

DlEPV Is Not Transmitted Per Os nor Vertically Among Flies and Viral Transmission between Wounded Fly Larvae Is Rare. We next explored the ability of DlEPV to be transmitted per os among flies. To assess the susceptibility of flies to DlEPV infection via ingestion of virus particles, we mixed purified virions into fly larval diet at three different concentrations, then measured viral abundance with qPCR after feeding to detect whether virus replication had occurred. The different DlEPV-laced diet concentrations were generated from varying numbers of *D. longicaudata* venom glands to produce high-, medium-, and low-ingested doses of DlEPV. After feeding on the three concentrations of virus-contaminated diet for 48 h, fly larvae had ingested $>1 \times 10^4$, 1×10^2, and 1×10^1 copies of DlEPV (Fig. 1*C*). However, the virus failed to increase in copy number following virus ingestion for all three treatments, but rather exhibited a significant decrease in viral abundance over time for all three doses (high dose time effect: $F_{5,30} = 9.64$, $P < 0.0001$; medium dose time effect: $F_{5,30} = 3.21$, $P = 0.0195$; low dose time effect: $F_{5,30} = 14.59$, $P < 0.0001$) (Fig. 1*C*).

Next, we investigated vertical transmission as an additional possible route of virus spread among flies. Adult flies injected with a dose of $>1 \times 10^5$ copies of DlEPV showed no significant increase in viral abundance over several days (*SI Appendix*, Fig. S1). Subsequently, we injected teneral (<24 h posteclosion) female adult flies with a high dose of 1×10^8 copies of virus, and the resulting progeny of virus-injected flies were screened as larvae for the presence of DlEPV using PCR. For the entire 14 d span of egg laying by infected adult females, all sampled larvae reared from eggs were negative for DlEPV, demonstrating that DlEPV was not transmitted vertically.

Finally, we explored whether DlEPV transmission could occur among flies via hemolymph exchanged between two injured fly larvae in physical contact with one another. To test for this route of viral transmission, uninfected fly larvae that had been experimentally wounded with a sterile needle were

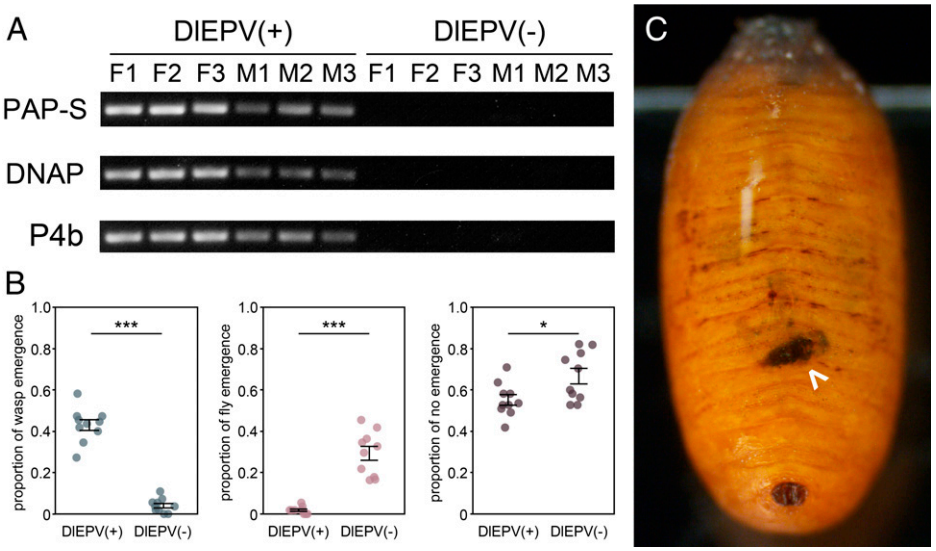


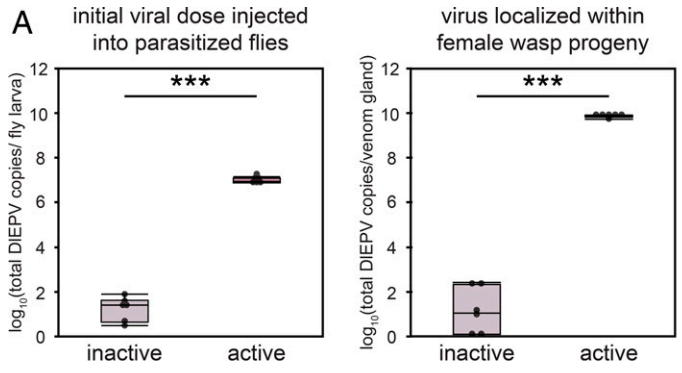
Fig. 2. Establishment of cured *D. longicaudata* wasps highlights reduced survival during parasitism. (*A*) PCR was used to screen (F) female and (M) male wasps from both infected DlEPV(+) and uninfected DlEPV(−) colonies for DlEPV infection. DlEPV genes amplified include the poly(A) polymerase small subunit (PAP-S), the DNA polymerase (DNAP), and the P4b major capsid gene. (*B*) Parasitism assays measured the average emergence rates of (*Left*) wasp progeny, (*Middle*) parasitized flies, and (*Right*) 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. Error bars represent one SE above and below the mean. Statistical significance of *t* tests in *B* is indicated as follows: $*P < 0.05$; $***P < 0.0001$. (*C*) Fly pupa at 72 h postparasitism by a DlEPV(−) wasp. Arrowhead indicates the melanized first-instar wasp larva visible underneath the fly puparium.

directly exposed to hemolymph from DlEPV-infected fly larvae and subsequently screened for DlEPV infection with PCR. All sampled DlEPV-infected "donor" larvae screened positive for the virus prior to hemolymph extraction. DlEPV was successfully transferred from infected donor larvae to 62.5% of wounded "receiver" larvae 1 h after external inoculation. However, only 12.5% of wounded flies were still infected with DlEPV after 5 d, which indicates that hemolymph-based contact spreading of DlEPV is a possible, albeit rare route of viral transmission among flies. Therefore, these cumulative results suggest that DlEPV replication in flies is restricted to the larval stage, and that DlEPV transmission among flies is largely dependent on intrahemocoelic inoculation.

A Cured Line of Wasps Demonstrates the Highly Beneficial Role of DlEPV as a Facultative Symbiont. Given the limited modes of DlEPV transmission observed within flies, we next focused on further understanding viral transmission among *D. longicaudata* wasps by using a line of wasps that had been cured of their DlEPV infection. We used previously established RNA interference-based elimination of DlEPV to found a stable line of uninfected wasps (13). Wasps from both the original infected colony, referred to here as DlEPV(+), as well as the DlEPV(−) colony were screened for the presence of three DlEPV genes with PCR. DlEPV DNA was clearly detected in DlEPV(+) female wasps and to a lesser extent, also in male wasps (Fig. 2*A*), which agree with previous qPCR measurements of DlEPV abundance within our infected colony (13). In contrast, DlEPV was not detected in any females or males from the DlEPV(−) wasp colony, confirming their uninfected status (Fig. 2*A*).

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 used second-generation virus-depleted wasps (13), we found that wasps in our stable 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% (Fig. 2*B*). 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 (Fig. 2*B*). However, fly emergence rates were < 30% on average during DlEPV(−) parasitism, suggesting that other factors introduced within the fly host throughout parasitism, such as venom proteins injected by the wasp mother, larval wasp secretions, or serosal membrane cells from the wasp embryo may also be contributing to fruit fly impairment (23, 24). 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 (Fig. 2*B*). 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 pupal case, or puparium (Fig. 2*C*), suggesting that wasps are killed by the host immune system at this stage when not accompanied by DlEPV. In contrast, healthy first-instar wasp larvae are typically translucent in appearance and are not visible from the fly pupa exterior (25). These results thus provide further support for the highly beneficial yet facultative 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 afterward injected parasitized flies with one oviposition equivalent ($\sim 1 \times 10^7$ viral genome copies) of purified DlEPV from either an unaltered "active" virus stock or an UV-inactivated "inactive" stock (Fig. 3*A*). DlEPV copy number was then measured from the venom glands of female progeny to ascertain whether the virus introduced into DlEPV-injected flies had recolonized the venom gland of DlEPV(−) wasp



B

Replicate trial	Positive no. female progeny/ total no. female progeny	DlEPV Infection Frequency (%)
1	6/6	100
2	2/2	100
3	5/5	100
4	6/6	100
5	3/3	100
6	1/1	100
7	2/2	100

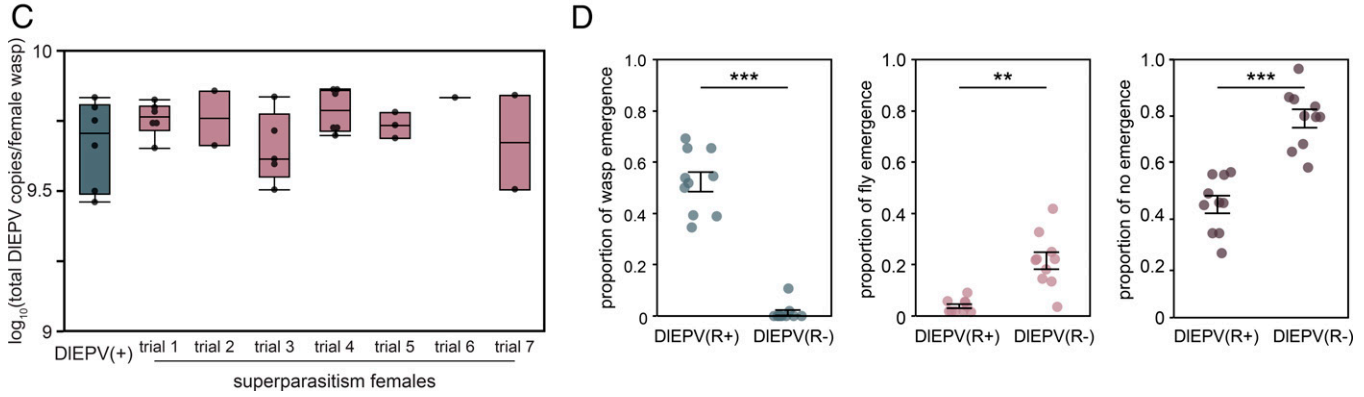


Fig. 3. Introduction of DlEPV during parasitism by DlEPV(−) wasps elicits a full recovery of viral load and parasitism success in adult progeny. (*A*) qPCR was used to measure the abundance of DlEPV (*Left*) initially injected into flies that had been parasitized by DlEPV(−) wasps immediately prior to injection, and (*Right*) in the venom glands of resulting female wasp progeny after eclosion. Venom glands were pooled in triplicate for each biological replicate. Boxplots were generated using $\log_{10}$-transformed viral copy numbers per specimen from six biological replicate samples. Statistical significance of *t* tests in both panels is as follows: ***$P < 0.0001$. (*B*) Results from seven replicate trials in which DlEPV(−) female wasps developed in the same hosts as DlEPV(+) wasps. DlEPV infection frequencies for female progeny after superparasitism are reported. (*C*) qPCR was used to estimate viral abundance in female progeny that developed during superparasitism (pink plots) compared to normal DlEPV(+) females (blue plot). Box plots were generated with copy numbers from all biological replicates per trial, as indicated in *B*, while 6 biological replicates were used for the DlEPV(+) control treatment. (*D*) Parasitism assay results depicting the emergence proportions for (*Left*) wasp progeny, (*Middle*) parasitized flies, and (*Right*) no emergence from DlEPV(−) wasp mothers that had reacquired viral infection, labeled DlEPV(R+), compared to those wasps that remained uninfected, or DlEPV(R−). Data and statistical analysis are as follows: **$P < 0.001$; ***$P < 0.0001$.

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 (Fig. 3*A*). 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 (13), which signifies that a complete reversal of infection status can occur in a single generation.

We next tested whether DlEPV(−) wasps could naturally reacquire the virus during superparasitism with DlEPV(+) wasps by utilizing the haplodiploid nature of parasitoid wasp sex determination, in which unmated female wasps will produce only haploid male eggs, while mated female wasps can produce either haploid male or diploid female eggs (26). We allowed mated DlEPV(−) females to oviposit within the same hosts as unmated DlEPV(+) wasps in seven replicate trials, such that any female progeny from these superparasitism events would be from the DlEPV(−) background. We then evaluated individual female progeny by dissecting the venom gland, noting whether it had blue iridescence [a morphological indicator of virus infection (13)], screening whole wasp tissue for the virus with PCR, and measuring 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 when they developed under superparasitism conditions with DlEPV(+) progeny (Fig. 3*B*). In addition, average viral abundance was not significantly different when female adults from the DlEPV(+) colony were compared with those from superparasitism trials ($F_{7,23} = 0.98$, $P = 0.4676$) (Fig. 3*C*). Thus, either manual or natural inoculation of fly hosts with DlEPV caused uninfected wasp progeny to fully reacquire the viral infection.

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 previously to measure whether active DlEPV-treated wasp progeny that had regained their venom gland viral population, now referred to as DlEPV(R+), had improved parasitism success compared to inactive DlEPV-treated wasps that largely remained 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 (Fig. 3*D*). 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% (Fig. 3*D*). Fly emergence and no emergence rates

were also similar in DlEPV(R+) and DlEPV(R−) treatments to the original DlEPV(+) and DlEPV(−) colonies, respectively (Fig. 3*D*). These findings show that the beneficial effects of DlEPV during parasitism are fully reinstated in the same generation as the virus is reacquired.

DlEPV Is Transmitted via Cuticle Contamination of Developing Wasps. We further explored the mechanism(s) by which DlEPV is transmitted among wasps by testing the hypothesis that DlEPV virions located on the cuticle of wasps developing within a virus-infected fly host are later incorporated into the venom gland of female adults. This hypothesis was prompted due to the high abundance of virions in parasitized fly tissue to which developing DlEPV(+) wasps are exposed and the ectodermal origin of the venom gland, which is formed from the invagination of the cuticle (26, 27). We dissected third-instar DlEPV(+) wasp larvae from within fly host puparia, surface-sterilized the wasp larvae via submersion in a 5% bleach solution, and housed the surface-sterilized wasps in individual empty wells of sterile 96-well plates for the remainder of their development. We then checked the infection status of female wasps as adults by noting dissected venom gland iridescence and screening each whole wasp for DlEPV using PCR. As a control treatment, we dissected third-instar DlEPV(+) wasp larvae and placed them directly into 96-well plates for continued development outside of their host puparia. DlEPV(+) wasp larvae that were bleach-treated contained an average 1×10^9 copies of DlEPV, which was on the same order of magnitude as control DlEPV(+) wasps (*SI Appendix*, Fig. S2*A*). Nevertheless, surface sterilization of DlEPV(+) wasp larvae led to a loss of infection for over half of females as adults: only 46.2% (12/26) of female adults remained infected with DlEPV, while 100% (37/37) of control DlEPV(+) wasps were still infected after developing outside of the puparium (Fig. 4 and *SI Appendix*, Table S1).

We next performed a complementary experiment, in which we dissected third-instar DlEPV(−) wasp larvae from their puparia, bathed them in active DlEPV stock solution to coat their cuticles with virions, and screened female adults for DlEPV to determine whether cuticle contamination resulted in reinfection of adult wasps. We bathed separate DlEPV(−) wasp larvae in inactive virus stock and placed other DlEPV(−) larvae into individual wells without any treatment, both as negative controls. Several millions of DlEPV copies were detected via qPCR on DlEPV(−) wasp larvae following submersion in purified virus (*SI Appendix*, Fig. S2*A*). Subsequently, we found that 46.7% (14/30) of DlEPV(−) females that were surface-contaminated with active DlEPV as larvae became reinfected with the virus upon adulthood, and 0% of those DlEPV(−) female wasps coated with inactive virus (0/34) or those without any treatment (0/36) were reinfected with DlEPV as adults (Fig. 4 and *SI Appendix*, Table S1). Furthermore, we found that DlEPV copy number did not significantly differ between control DlEPV(+) and reinfected "virus-bathed" DlEPV(−) adult wasps (*SI Appendix*, Fig. S2*B*). These combined results indicate that external DlEPV virions located on the wasp cuticle are capable of eventually infecting the venom gland as part of DlEPV transmission to future generations of female wasps.

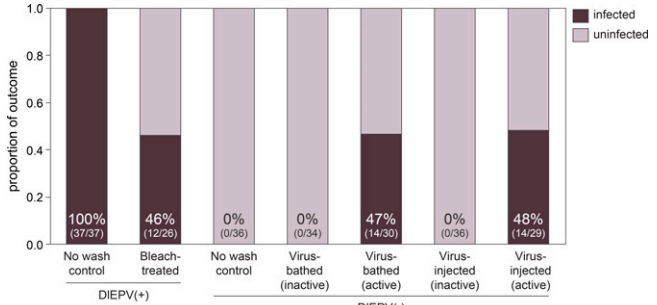


Fig. 4. Manipulation of DlEPV virions in *D. longicaudata* larvae causes changes in adult infection status. Bars indicate the proportions of adult female wasps that were infected with DlEPV after various treatments as third-instar larvae. First, infection frequency of DlEPV(+) females that were not treated, labeled "No wash control," is compared to those that were surface sterilized with bleach as larvae, labeled "Bleach-treated." Second, infection frequency of DlEPV(−) females that were not treated, labeled "No wash control," is compared to those that were bathed in virus, labeled "Virus-bathed (inactive)" and "Virus-bathed (active)," and those that were injected with virus, labeled "Virus-injected (inactive)" and "Virus-injected (active)."

Injection of Virus into DlEPV(−) Wasp Larvae Also Causes Reinfection of Adult Females. An additional component to DlEPV transmission in wasps could involve internal migration of virus particles through the hemolymph to the venom gland once it is formed during pupal development. We tested the hypothesis by injecting DlEPV(−) wasp larvae with DlEPV and screening female adults for the virus. UV-inactivated virus was once again used as a negative control via injection into separate DlEPV(−) wasp larvae. Each wasp larva was initially injected with an average of $>1 \times 10^6$ copies of DlEPV (*SI Appendix*, Fig. S2*A*). Upon adulthood, 48.3% (14/29) of female adults injected with active DlEPV as larvae were reinfected with virus in their venom gland, compared to none (0/36) of the inactive virus-injected wasps that were reinfected (Fig. 4 and *SI Appendix*, Table S1). Viral abundance measurements showed that reinfected female adults also contained a full viral load of DlEPV (*SI Appendix*, Fig. S2*B*). These results suggest that DlEPV transmission within wasps may also include an internal component, in which virions present within wasp hemolymph can eventually colonize the venom gland.

Discussion

DlEPV represents an anomaly compared to other beneficial viral elements inherited by parasitoid wasps, in that it is not integrated within the *D. longicaudata* genome and maintains replicative autonomy, while supplying a considerable advantage to developing wasps (13, 14). These characteristics indicate that DlEPV is a true mutualistic symbiont that retains more features from its pathogenic ancestor than parasitoid EVEs. Therefore, while we expect this virus to share traits with other beneficial viral elements due to its similar transition from an insect pathogen into a heritable parasitoid mutualist, we might also expect some aspects of the DlEPV system to resemble other insect-microbe symbioses due to its relatively preserved ancestral qualities. We determined here that similar to parasitoid EVEs, DlEPV is generally dependent on wasps for transmission among fruit fly hosts. Interestingly, we also found that DlEPV displays a unique form of transmission among wasps that is more akin to other microbial symbionts. Taken together, our findings have revealed DlEPV to be an intermediate point along the continuum of symbiogenesis between viral insect pathogens and fully integrated parasitoid EVEs.

DlEPV Transmission in Flies Is Primarily Dependent on Parasitoid-Mediated Strategies. Injection of DlEPV directly into the hemocoel of fly larvae was the only method of continuous viral propagation in flies that we observed in this study. Isolation of fly-propagated DlEPV particles followed by injection into

uninfected fly larvae caused a similar growth in viral copy number over time compared to flies injected with wasp-propagated virus. However, neither fly larvae that ingested DlEPV nor fly offspring of virus-infected mothers exhibited viral infection. These results suggest that DlEPV has lost the potential for per os infectivity and cannot be vertically transmitted among flies, the former being a commonly observed strategy of entomopoxvirus transmission (15).

An additional route of horizontal DlEPV transmission explored in this study involves the transfer of virus particles through direct contact of hemolymph from mutually wounded fly larvae. Under conducive laboratory settings, we found that most uninfected larvae internalized DlEPV when wounded and exposed to hemolymph from an infected fly. Nevertheless, only a small percentage of these flies maintained a persistent infection days after DlEPV exposure. This mode of transmission has been documented for *Wolbachia* symbionts in a gregarious woodlouse species (28), and DlEPV spread could occur in a similar manner due to the aggregation behavior observed for some tephritid fruit fly larvae, as well as *Drosophila* species (29, 30). Hemolymph-based contact spreading could therefore cause fly larvae developing within the same fruit environment to become infected with DlEPV. However, the frequency of DlEPV transmission via wounding is likely much lower under natural conditions compared to parasitoid-mediated transmission. Furthermore, any transmission that occurs via wounding is unlikely to spread throughout a population of flies in the absence of parasitoids due to a lack of larval movement between fruits. It is also improbable that DlEPV-infected adult flies could transmit the virus to fly larvae or conversely from larvae to adults, due to the physical separation of these two different life stages.

Therefore, it appears that the predominant route of transmission for DlEPV within flies is via hypodermic injection of the fly larva, which would most likely be achieved in a natural scenario via the ovipositor of a probing parasitoid wasp. Prior work has shown that DlEPV localization within larval flies primarily occurs in the hemocytes, and therefore virus released into the hemolymph may be amenable to transport to new flies on a wasp ovipositor (13). DlEPV thus has this mode of transmission in common with ascoviruses, which are considerably more infectious when administered to new hosts via injection than by per os infection (31). Iridoviruses, which are close relatives of ascoviruses, can also be transmitted by parasitoids and favor injection over per os modes of transmission (32–34). Therefore, parasitoid-mediated transmission is not particularly uncommon for insect viruses and has likely evolved independently multiple times, although DlEPV is the only known insect poxvirus that utilizes this as a primary transmission strategy. Furthermore, our results demonstrate that despite maintaining the larval-stage specificity of most entomopoxviruses (15), DlEPV has dramatically shifted its transmission dynamics compared to its relatives. This shift supports a dependence on *D. longicaudata* for DlEPV transmission that may have contributed to the stability of this mutualism over evolutionary time.

The Wasp Venom Gland Population and Beneficial Phenotype of DlEPV Can Be Reestablished Through Posthatch 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 oviposition-manipulating virus *Leptopilina boulardi* filamentous virus (LbFV) might be similarly infectious, as it can be transmitted to uninfected *L. boulardi* wasps through superparasitism events. However, LbFV infection and the resulting transfer of behavioral manipulation is gradual among uninfected wasps (35–37). DlEPV, in comparison, exhibited an immediate and absolute infection frequency within 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, the average 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.

DlEPV Is Capable of Both Vertical and Horizontal Transmission Among Wasps. As demonstrated by the virus transmission route uncovered here, DlEPV is capable of both vertical and horizontal transmission strategies among *D. longicaudata* wasps. Pseudovertical transmission is supported through this route when a wasp mother oviposits an egg within a fly host and her progeny are contaminated by virus-infected fly tissue, and horizontal transmission is supported through superparasitism events involving uninfected wasps developing within the same host as infected wasps. This posthatch mode of transmission adds to previous findings that suggest virus particles are transmitted transovarially within wasp eggs (13). These combined results raise an intriguing question regarding the relative importance of internal, transovarial transmission compared to external, pseudovertical and horizontal transmission of DlEPV within wasps. Here, our results involving the surface sterilization of DlEPV(+) larvae suggest that external transmission is the predominant route of DlEPV spread. The majority of resulting female wasps that were bleach-treated as larvae lost their DlEPV infection, indicating that the small amount of virus present within the ovaries of most developing wasps may not always be sufficient to allow for transovarial transmission. Moreover, DlEPV(+) wasp larvae still contained several million DlEPV copies immediately following surface-sterilization (*SI Appendix*, Fig S2*A*), which mostly consisted of ingested virus particles within the larval wasp gut (13). Therefore, ingested DlEPV particles did not migrate to the venom gland of adult wasps in most cases, suggesting that per os transmission among wasps feeding on DlEPV-infected fly tissue is also not a primary mode of viral spread in this system.

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 (38, 39). 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 (40–42). 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.

The Precise Mode of DlEPV Posthatch Transmission Involves External and Internal Routes. While we have demonstrated in this study that DlEPV can be acquired by *D. longicaudata* wasps after hatching, the exact route that DlEPV traverses to eventually colonize the venom gland is still unknown. A common path of symbiont posthatch transmission involves the ingestion and internal migration of symbionts to the symbiont-housing organ during development. However, our results here show that DlEPV utilizes a different pathway to recolonize the *D. longicaudata* venom gland, which occurs by means of surface virions that exist on the cuticle of wasps as they develop within the virus-infected host remains and are later incorporated into the venom gland. Surface sterilization of DlEPV(+) wasps before venom gland formation caused over half of resulting adult females to lose their viral infection. Inversely, adding virions to the cuticle of DlEPV(−) wasps produced roughly half of adult female wasps that had regained a full viral infection. The absence of virus replication in the venom gland of surface-sterilized DlEPV(+) wasps combined with the reconstitution of virus replication in the venom gland of surface-contaminated DlEPV(−) wasps supports that DlEPV transmission is externally acquired during the formation of the venom gland. Further investigation of this phenomenon using microscopy to follow DlEPV internalization to the venom gland would help fully elucidate the precise mechanism of this particular transmission route.

A possible complication to this external, cuticle-based model for DlEPV transmission among wasps is our data demonstrating that injection of virus into DlEPV(−) wasp larvae also caused approximately half of resulting female adults to regain a full viral infection. This finding suggests that part of the viral pathway to the adult venom gland involves internal migration of virus particles. Alternatively, this internal route may represent an altogether separate transmission strategy also used by DlEPV. Many questions therefore remain regarding the mechanisms by which DlEPV is sequestered from the external environment, migrates to the venom gland, and is stimulated for virus replication.

Methods and Materials

Insect Colonies. *A. suspensa* flies and DlEPV(+) *D. longicaudata* wasps were reared as reported previously (43). The DlEPV(−) wasp colony was initially established in November 2019 by injecting female wasp pupae from the DlEPV(+) colony with 1 μg of a double-stranded RNA (dsRNA) mixture targeting the DlEPV RNA polymerase 147 kDa large subunit (RPO147, DLEV067), DNA polymerase (DNAP, DLEV168), and major capsid protein P4b (DLEV147), as in (13). 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.

Virus Isolation and Infectivity in Flies via Microinjection. DlEPV virions were isolated from DlEPV(+) wasp venom gland tissue through filter purification as in (13) to produce active and UV-inactivated virus stocks for downstream experiments. To assess the hypodermic infectivity of DlEPV in flies, second-instar fly larvae were injected with 0.5 μL of diluted virus stock, then placed into fresh diet for continued larval development. Larvae were collected daily for 72 hpi to measure the initial increase in viral abundance. After 72 h, virus-injected larvae had reached the late third-instar stage, in which they crawl out of their diet substrate to pupate. We collected larvae at this stage by placing larval diet tubs over water, which immobilized larvae once they jumped from the diet. To isolate fly-propagated DlEPV particles, individual larvae were bled of their hemolymph by making a longitudinal incision along the ventral side of the larva in a 50-μL droplet of 1 × phosphate-buffered saline (PBS). A total of 12 larvae were bled in this manner into two combined droplets, for a total volume of 100 μL. Fly hemocytes were then pelleted through centrifugation of the pooled hemolymph at 1,000 × *g* for 3 min at 4 °C, and the supernatant containing extracellular virus particles was passed through a 0.45-μm filter. Separate, uninfected third-instar fly larvae were then each injected with 1 μL of the plasma filtrate and collected daily to measure viral abundance over time. DNA isolation by phenol:chloroform extraction and qPCR were performed on each whole-body fly sample, and viral abundance was estimated by quantifying the absolute copy number of the DlEPV poly(A) polymerase small subunit (PAP-S) gene (DLEV167), as previously described (13).

Per Os Virus Infectivity in Flies. To generate varying concentrations of virus-contaminated fly larval diet, filter-purified virus was prepared as above and equivalent volumes of virus stock solution for 10, 20, and 200 venom glands were each diluted in 1 × PBS to a final volume of 4 mL. The three virus preparations were each mixed into 1 oz of HCl-free fly larval diet and given to ∼50 second-instar fly larvae. After 48 h of feeding on the virus-laden diet, flies (now late third-instar larvae) were surface sterilized through submersion of individual larvae in 200 μL 5% bleach, 2% sarkosyl for 10 min, followed by submersion in 200 μL 1 × PBS, and then larvae were either sampled immediately or placed into moistened vermiculite to continue development. Whole-body flies were collected daily, and viral abundance was measured as above.

Viral Abundance and Vertical Transmission within Adult Flies. To assess DlEPV replicative ability in adult flies, teneral female adults were each injected with 1 μL diluted virus stock solution, and whole-body flies were sampled daily to measure DlEPV abundance for the following 72 h. To investigate whether adult flies could vertically transmit DlEPV to offspring, a cohort of 60 teneral females were injected each with 1 μL virus stock and returned to a cage with uninfected adult males. Female flies began to lay eggs after 5 d and continued to their death at 19 d postinjection. Eggs were collected at 2- to 6-d intervals and reared with standard conditions until larvae reached the late third-instar stage. Larvae ($n = 15$) from eggs sampled on days 5 and 19 postinjection were collected from diet, rinsed with distilled water, and screened for DlEPV infection with PCR following phenol:chloroform DNA extraction, as described above. Standard PCR employed gene-specific primer sequences for the PAP-S gene (13) with the following thermocycling conditions: 1 cycle of 95 °C for 2 min, 35 cycles of 95 °C for 20 s, 60 °C for 20 s, and 65 °C for 30 s, and a final extension at 65 °C for 7 min. PCR products were then loaded on 1% agarose gels and subjected to electrophoresis for 25 min at 120 V to visualize product bands.

Viral Transmission via Larval Fly Wounding. Second-instar fly larvae were presented to DlEPV(+) female wasps for 2 h to allow for oviposition and DlEPV inoculation, then placed into fresh larval fly diet for continued development and DlEPV replication. After 48 h, parasitized third-instar larvae and age-matched nonparasitized larvae were surface sterilized in 5% bleach, 2% sarkosyl for 5 min to remove cuticular opportunistic pathogens. Three microliters of hemolymph was bled from each parasitized donor larva and pipetted directly onto a nonparasitized receiver larva that had been wounded moments before with a glass capillary needle. Receiver larvae were exposed to hemolymph for ∼20 s, then briefly rinsed in distilled water, and placed into ventilated 1-oz cups filled with 1% agarose gel for 1 h to recover. Separate wounded larvae were exposed to hemolymph from nonparasitized larvae as a control. After the recovery period, larvae were surface-sterilized once more as described above for the per os infectivity assay to remove lingering external DlEPV particles. Receiver larvae were either sampled immediately or allowed to pupate in vermiculite and mature for 5 d before sampling. Whole-body donor and receiver fly samples were screened for DlEPV with PCR targeting the PAP-S gene as described above. Eight biological replicates were analyzed for both infected and uninfected treatments at each sampled timepoint, with the exception of the 5 d uninfected control timepoint, in which only six replicates were analyzed. No flies at any timepoint in the uninfected control treatment screened positive for DlEPV.

PCR Screening for DlEPV in Wasp Colonies. DNA was isolated from whole-body female and male adult wasps of both DlEPV(+) and DlEPV(−) colonies using the same protocol as was used with the fly samples. DNA from each sample was eluted in 30 μL water and diluted 1:10 prior to PCR screening. PCR was performed as described previously for fly samples.

Parasitism Success Assays. Assays measuring the parasitism success of DlEPV(+)/DlEPV(−) wasps and DlEPV(R+)/DlEPV(R−) wasps were performed as previously described (13). Briefly, *A. suspensa* second-instar larvae were presented to groups of six female *D. longicaudata* wasps of one treatment for 4 h of oviposition. Afterward, flies were placed back into fresh fly diet to continue larval development. Resulting fly pupae were examined 2 d later and only those containing a single oviposition scar were kept for observation. At 4-wk postparasitism, 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.

Viral Transmission via Parasitized Fly Injections. The ability of wasps to become reinfected with DlEPV after manual reintroduction of virus during parasitism was investigated through injection of filter-purified virus into flies parasitized by DlEPV(−) wasps. Third-instar fly larvae were offered to DlEPV(−) wasps for 2 h, and immediately afterward, 1 μL virus stock from active or inactive treatment were injected 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. Unmated 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. Unmated 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 six 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, ∼17 d later. Upon emergence, female progeny were surface sterilized by vortex mixing each wasp in 1 mL 5% bleach, 2% sarkosyl for 1 min, followed by three rounds of vortex mixing in 1 mL water. Each wasp was then dissected in 1 × PBS to note its venom gland coloration, and all dissected tissues were subsequently combined into the same tube to obtain a whole-body sample. Wasp samples were next subjected to PCR screening for DlEPV using the methods described before, and samples that displayed blue venom gland morphology and/or PCR amplification of the viral PAP-S gene were scored as positive for DlEPV infection. Of note, two samples across all experiments with clear venom gland morphology screened positive for DlEPV via PCR, although qPCR copy numbers for both outliers were >2 orders of magnitude less than other positive samples from the same treatment. The overall correlation between blue venom gland observations and positive DlEPV PCR results thus supports venom gland iridescence as a largely faithful indicator of viral infection.

DlEPV(+) Wasp Surface Sterilization Assay. DlEPV(+) wasp larvae were dissected from their host fly puparia at 7 d postparasitism (dpp), at which time larvae have finished consuming their fruit fly hosts and are nearing the transition to the pupal stage (25). Wasp larvae were then individually submerged in wells of a sterile 96-well plate that each contained 150 μL 5% bleach, 2% sarkosyl. Plates containing larvae in bleach were set on a slow rocker for 10 min, and larvae were afterward transferred into individual wells of two successive plates that each contained 150 μL 1 × PBS. Each larva was then placed in its own empty well of a humidified 96-well plate to continue the remainder of its development until adulthood. Since developing wasps were devoid of their host puparia, adult wasp eclosion was estimated by using the voided meconium as an indication of adulthood, which is a behavior that normally occurs immediately after eclosion in opiine wasps (26). Upon adulthood, each female wasp was removed from its individual well, and its venom gland was dissected to note the coloration or lack thereof prior to collecting each whole-body wasp sample. Samples were then subjected to DNA extraction and PCR screening for DlEPV as described above.

DlEPV(−) Wasp Cuticle Contamination Assay. At 7 dpp, host fly puparia that contained third-instar DlEPV(−) wasp larvae were first surface-sterilized by swirling in 5% bleach, 2% sarkosyl for 5 min prior to dissecting wasp larvae out to prevent opportunistic pathogens from killing unprotected developing wasps. Each larva was then submerged in a droplet of 100 μL purified DlEPV stock for 1 min, then allowed to air-dry on the same Petri dish for 1 min before being placed into individual empty wells of a sterile, humidified 96-well plate. Female adults were surface sterilized by vortex mixing in 5% bleach, 2% sarkosyl following eclosion to remove any remaining external virions. Each wasp was then dissected to note venom gland morphology and collected as whole-body samples for DNA extraction and PCR screening as described above.

DlEPV(−) Wasp Viral Injection Assay. Third-instar DlEPV(−) wasp larvae were also used for purified virus injections, although larvae were allowed to remain within host puparia throughout development following injection. At 7 dpp, each larva was injected with 0.5 µL purified virus stock of either active or inactive treatment. Female wasps were dissected after eclosion to note venom gland morphology and collect for PCR screening of DlEPV.

Statistical Analyses. All statistical analyses were conducted using JMP Pro-14 software. Mean copy numbers obtained from qPCR were $\log_{10}$-transformed prior to statistical analysis. Statistical differences in average copy number between two treatments were calculated with *t* tests assuming equal variance, between three or more treatments with one-way ANOVA, and between treatments of multiple effects with two-way ANOVA. Statistically significant differences between average emergence rates between DlEPV(+) and DlEPV(−) or DlEPV(R+) and DlEPV(R−) treatments in parasitism success assays were analyzed with *t* tests assuming equal variance.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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