AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

VIRUS-CELL INTERACTIONS

Culex tarsalis Is a Competent Host of the Insect-Specific Alphavirus Eilat Virus (EILV)

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ABSTRACT Eilat virus (EILV) is an insect-specific alphavirus that has the potential to be developed into a tool to combat mosquito-borne pathogens. However, its mosquito host range and transmission routes are not well understood. Here, we fill this gap by investigating EILV's host competence and tissue tropism in five mosquito species: *Aedes aegypti*, *Culex tarsalis*, *Anopheles gambiae*, *Anopheles stephensi*, and *Anopheles albimanus*. Of the tested species, *C. tarsalis was* the most competent host for EILV. The virus was found in *C. tarsalis* ovaries, but no vertical or venereal transmission was observed. *Culex tarsalis* also transmitted EILV via saliva, suggesting the potential for horizontal transmission between an unknown vertebrate or invertebrate host. We found that reptile (turtle and snake) cell lines were not competent for EILV infection. We tested a potential invertebrate host (*Manduca sexta* caterpillars) but found they were not susceptible to EILV infection. Together, our results suggest that EILV could be developed as a tool to target pathogenic viruses that use *Culex tarsalis* as a vector. Our work sheds light on the infection and transmission dynamics of a poorly understood insect-specific virus and reveals it may infect a broader range of mosquito species than previously recognized.

IMPORTANCE The recent discovery of insect-specific alphaviruses presents opportunities both to study the biology of virus host range and to develop them into tools against pathogenic arboviruses. Here, we characterize the host range and transmission of Eilat virus in five mosquito species. We find that *Culex tarsalis*—a vector of harmful human pathogens, including West Nile virus—is a competent host of Eilat virus. However, how this virus is transmitted between mosquitoes remains unclear. We find that Eilat virus infects the tissues necessary for both vertical and horizontal transmission—a crucial step in discerning how Eilat virus maintains itself in nature.

KEYWORDS Eilat virus, insect-specific virus, *Alphavirus*, *Culex tarsalis*

The small, spherical, enveloped, positive-sense RNA viruses in the genus *Alphavirus* (family *Togaviridae*) are primarily mosquito-borne and include important human pathogens such as Mayaro, O'nyong-nyong, chikungunya, and Ross River viruses (1). The 11- to 12-kb alphavirus RNA genome has two open reading frames (ORFs): The 5′ end of the genome encodes four nonstructural proteins (nsP1 to 4), and the 3′ end encodes five structural proteins (sPs; capsid, E3, E2, 6K, and E1) expressed by a subgenomic promoter (1) (Fig. S1). Alphaviruses typically have a broad host range spanning vertebrates such as humans, nonhuman primates, horses, birds, reptiles, and amphibians, as well as invertebrates such as mosquitoes, ticks, and lice (2). Horizontal transmission between mosquitoes and vertebrates is how alphaviruses typically maintain themselves in nature (2). However, several insect-specific alphaviruses that cannot infect vertebrate cells have been recently discovered (3–6). Though their host range and transmission route(s) remain poorly described, a better understanding of these host-restricted viruses may lead to new insights into virus

Editor Mark T. Heise, University of North Carolina at Chapel Hill

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The authors declare no conflict of interest.

Received 21 December 2022
Accepted 13 March 2023
Published 26 April 2023

biology and, ultimately, to the development of new tools for curbing the spread of mosquito-borne pathogens.

Eilat virus (EILV) is an insect-specific alphavirus originally isolated from *Anopheles coustani* mosquitoes collected during an arbovirus survey in the Negev Desert of Israel (3). Phylogenetically, EILV clusters with the mosquito-borne clade of the *Alphavirus* genus, basal to the western equine encephalitis complex (3). Nasar et al. were the first to characterize EILV and found that it infects and replicates in the insect cell lines C6/36 and C7/10 (*Aedes albopictus*), CT (*Culex tarsalis*), and PP-9 (*Phlebotomus papatasi*), but not in mammalian, avian, or amphibian cell lines (3)—indicating it is an insect-specific virus (ISV). Similarly, EILV could not infect newborn mice, a model for alphavirus infections (7). EILV host range restriction was found to occur at both the attachment/entry and the viral genome replication levels in vertebrates (7).

A broad range of mosquito species act as vectors for different alphaviruses, including members of the genera *Aedes*, *Anopheles*, and *Culex* (2, 8). In previous work, four mosquito species were found to be susceptible to EILV infection to various degrees (9). *Aedes aegypti* was the most susceptible following oral challenge, while *A. albopictus*, *Anopheles gambiae*, and *Culex quinquefasciatus* were only susceptible to EILV at the highest dose tested (10^9 virus particles capable of forming plaques per mL [PFU/mL]) (8). In orally infected *Ae. aegypti*, *An. gambiae*, and *C. quinquefasciatus* (but not *Ae. albopictus*), EILV was not able to disseminate beyond the midgut (8). Nevertheless, these findings suggested that EILV has a restricted host range, as all but one of the examined species (*Ae. aegypti*) were refractory to infection at titers typical for other alphaviruses (9, 10).

ISVs such as *Culex* flaviviruses (CxFV) are thought to be adapted to a single host system, within which they are transmitted vertically from mothers to offspring (11, 12). Vertical transmission routes used by ISVs include transovarial (viral infection of germ line tissue in mosquito ovaries) and transovular transmission (viral infection of mosquito eggs as they pass the oviduct) (11–13). In contrast, horizontal transmission can occur when a virus infects the mosquito salivary glands and is subsequently passed to a new host by salivation during feeding (2). Thus, knowledge of tissue tropism can shed light on viral transmission routes. In the case of EILV, Nasar et al. found that that the midguts of four mosquito species were infected following intrathoracic injection with the virus (8). EILV was found in the salivary glands of *Ae. aegypti*, *An. gambiae*, and *C. quinquefasciatus* but was not detected in the ovaries of the tested species, suggesting the potential for horizontal but not vertical transmission.

The absence of EILV in the ovaries is unexpected for an insect-specific virus (ISV), as it calls into question how this virus is transmitted between mosquitoes. However, EILV was detected in both adult and larval *Culex pipiens* in Morocco, consistent with the hypothesis that EILV uses transovarial or transovular transmission routes (14), typical of an ISV, though environmental transmission is an alternative explanation. The failure to detect EILV in mosquito ovaries in a laboratory setting thus far indicates a need for more research into its transmission route(s).

Alternately, the presence of EILV in the salivary glands suggests horizontal transmission to an unknown host such as an uncharacterized vertebrate or perhaps another invertebrate. Reptiles have not yet been assessed for their competence to host EILV. Additionally, George et al. showed that *Anopheles stephensi* is attracted to and can successfully feed on the laboratory model caterpillars *Manduca sexta* and *Heliothis subflexa* (15). This makes caterpillars such as *Manduca sexta* and *Heliothis subflexa* ideal invertebrate models to test for a possible role in the transmission of EILV or other ISVs.

To address these questions and gaps, here, we investigate (i) the ability of *Aedes aegypti* (positive control), *Culex tarsalis*, *Anopheles gambiae* (negative control), *Anopheles stephensi*, and *Anopheles albimanus* (together representing all three major genera of vector mosquitoes) to become infected, disseminate, and transmit EILV, (ii) the tissue tropism and transmission route of EILV in these species, and (iii) the susceptibility of reptile cell lines and *Manduca sexta* to EILV infection.

TABLE 1 Infection (IR), dissemination (DIR), and transmission (TR) rates and transmission efficiency (TE) of five mosquito species orally challenged with EILV-eGFP[a]

	Data from 7 dpi				Data from 14 dpi			
Mosquito species	IR (%) (n_I/n_T)	DIR (%) (n_L/n_I)	TR (%) (n_S/n_L)	TE (%) (n_S/n_T)	IR (%) (n_I/n_T)	DIR (%) (n_L/n_I)	TR (%) (n_S/n_L)	TE (%) (n_S/n_T)
Ae. aegypti	56.86 (29/51)	65.5 (19/29)	0 (0/19)	0 (0/51)	58.8 (30/51)	90 (27/30)	0 (0/27)	0 (0/51)
C. tarsalis (YOLO)	75 (33/44)	24.2 (8/33)	50 (4/8)	9.1 (4/44)	88.2 (30/34)	96.6 (29/30)	21 (6/29)	17.6 (6/34)
An. gambiae	0 (0/50)	0 (0/0)	0 (0/0)	0 (0/50)	0 (0/50)	0 (0/0)	0 (0/0)	0 (0/50)
An. stephensi	0 (0/50)	0 (0/0)	0 (0/0)	0 (0/50)	0 (0/50)	0 (0/0)	0 (0/0)	0 (0/50)
An. albimanus	0 (0/38)	0 (0/0)	0 (0/0)	0 (0/50)	0 (0/45)	0 (0/0)	0 (0/0)	0 (0/45)

[a]n_I, number of mosquitoes infected; n_T, total number of mosquitoes tested; n_L, number of mosquitoes with EILV-positive legs; n_S, number of mosquitoes with EILV-positive saliva.

RESULTS

***Culex tarsalis* is a competent host of orally acquired EILV.** To determine the host competence of each species, 102 *Ae. aegypti* (Rockefeller strain), 78 *C. tarsalis* (YOLO strain), 100 *An. gambiae* (Keele strain), 100 *An. stephensi* (Liston strain), and 83 *An. albimanus* (STECLA strain) mosquitoes were orally challenged with 10^7 virus particles capable of forming fluorescent foci per mL (FFU/mL) of EILV-enhanced green fluorescent protein (eGFP) (Table 1). The infection rate (IR) was defined as the proportion of infected mosquitoes among the total number of engorged mosquitoes, the dissemination rate (DIR) as the proportion of infected mosquitoes with virus-positive legs, the transmission rate (TR) as the proportion of mosquitoes with virus-positive saliva among those with virus-positive legs, and the transmission efficiency (TE) as the proportion of mosquitoes with virus-positive saliva among the total number of mosquitoes engorged.

We found that EILV infected two species, *Ae. aegypti* and *C. tarsalis*, with infection rates (IRs) in the range of 57 to 88% at both 7 and 14 days postinfection (dpi). In contrast, *An. gambiae*, *An. stephensi*, *and An. albimanus* were refractory to oral infection with EILV. Of the two susceptible species, *C. tarsalis* was more likely to become infected. Specifically, the IR of *C. tarsalis* at 14 dpi was significantly greater than that of *Ae. aegypti* (Fisher's exact test, $P \leq 0.01$), though at 7 dpi the species did not differ (Fisher's exact test, $P > 0.05$). Within each species, IRs did not significantly change over time (i.e., 7 dpi versus 14 dpi; Table 1; Fisher's exact test, $P > 0.05$ for both).

EILV infections disseminated beyond the midgut at both time points in both infected species, but the dissemination rate (DIR) of *Ae. aegypti* was significantly higher (Fisher's exact test, $P \leq 0.01$) than that of *C. tarsalis* at 7 dpi. However, this difference disappeared by 14 dpi ($P > 0.05$) due to increased dissemination in *C. tarsalis*. Only *C. tarsalis* had EILV-positive saliva, with the transmission efficiency (TE) rising over time from 9.1% (7 dpi) to 17.6% (14 dpi), while the transmission rate (TR) dropped over time from 50% to 21% due to the increase in the number of disseminated infections at the later time point (Table 1). The mean EILV titers in positive saliva samples at 7 dpi and 14 dpi were 5.8 and 4.5 FFU/mosquito, respectively.

At the titer level, *Ae. aegypti* and *C. tarsalis* (YOLO) body and leg samples did not differ at 7 dpi (Fig. 1; Mann-Whitney U test, $P > 0.05$ for both comparisons). However, by 14 dpi, the EILV titers in *C. tarsalis* body and leg samples were significantly greater (Fig. 1; $P \leq 0.0001$ for both) than those of *Ae. aegypti*. Together, our results show that *C. tarsalis* is a competent transmitting host for EILV via the oral route of infection.

Genetically diverse *C. tarsalis* strains are susceptible to EILV infection and may transmit it via saliva. Having established that a lab strain of *C. tarsalis* (YOLO) is a competent host for EILV, we next asked if this susceptibility is strain specific (i.e., limited to YOLO) or, rather, widespread across diverse colonies of this species. We therefore assessed the ability of EILV to infect an additional lab colony of this species (KNWR strain), as well as *C. tarsalis* recently captured from the wild in California.

We orally challenged 73 KNWR and 63 wild *C. tarsalis* with EILV-eGFP and tested body, leg, and saliva samples for EILV at 7 and 14 dpi to determine the IR, DIR, TR, and TE of both strains and compare these values to those of YOLO (Table 2). We found that the KNWR colony had similar susceptibility to EILV infection as YOLO. Specifically, their

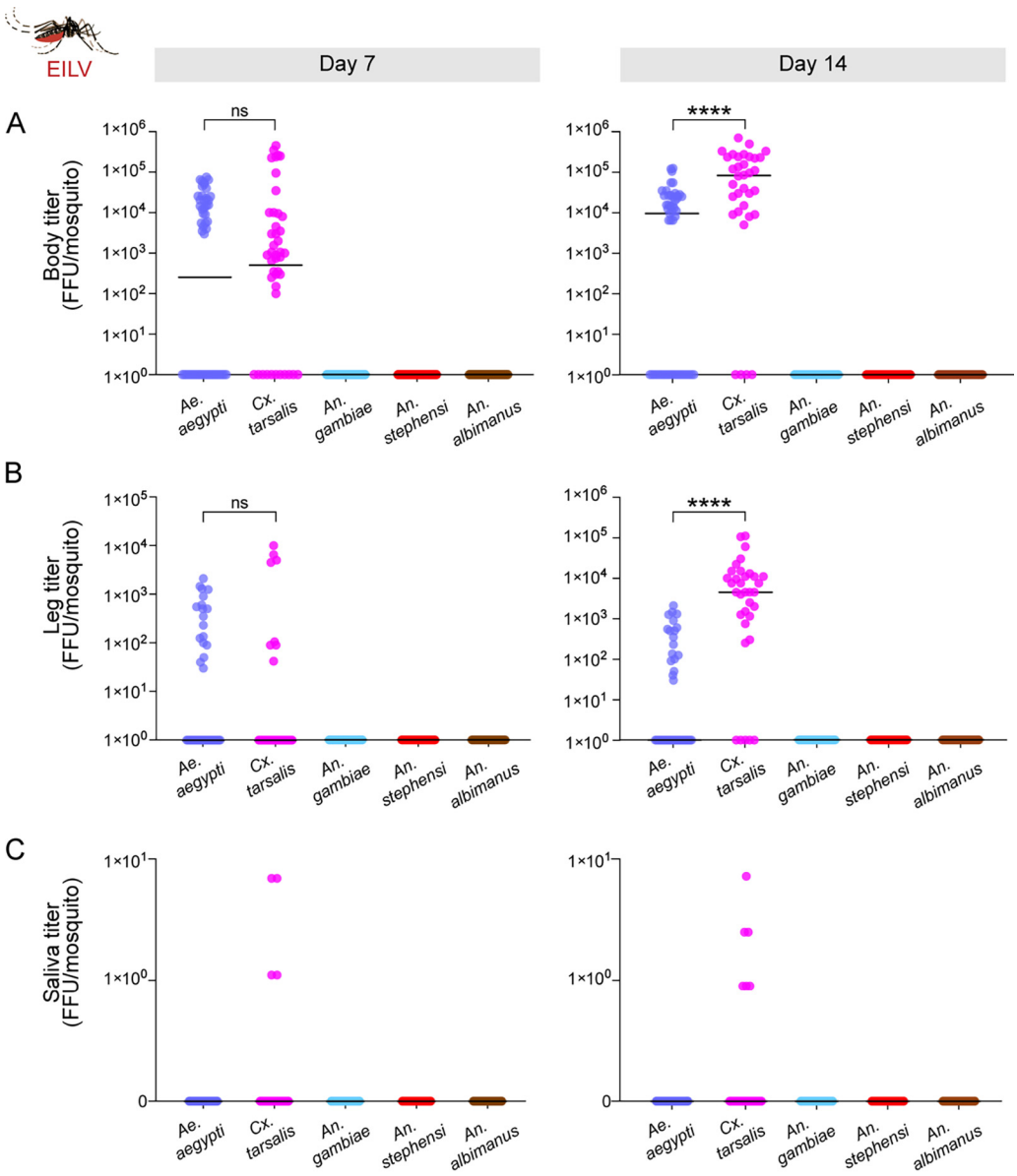

FIG 1 Viral titers of body, leg, and saliva samples from five mosquito species orally challenged with infectious EILV-eGFP. (A to C) Titers are plotted at 7 and 14 dpi for (A) body, (B) leg, and (C) saliva samples collected from mosquitoes orally challenged with EILV-eGFP (10^7 FFU/mL). Each point represents a single mosquito sample, while horizontal bars depict the group medians. Significance was evaluated using Mann-Whitney U tests. ****, $P < 0.0001$.

IRs did not differ at 7 or 14 dpi (Fisher's exact test, $P > 0.05$ for both). In contrast, wild *C. tarsalis* were less susceptible to EILV infection, with IRs significantly lower than those of YOLO across time (Fisher's exact test, 7 dpi and 14 dpi, both $P \leq 0.0001$). We found that differences in dissemination were limited to late infections—no differences in DIR were found at 7 dpi ($P > 0.05$ for both strain comparisons), but by 14 dpi, both KNWR and wild *C. tarsalis* had lower rates than YOLO (both $P \leq 0.05$).

EILV was found in the saliva of all assayed *C. tarsalis* strains. The TR and TE of KNWR and YOLO at 7 dpi and 14 dpi did not differ (Fisher's exact test, all $P > 0.05$). Similarly, the TR and TE of wild *C. tarsalis* and YOLO were statistically indistinguishable at 7 dpi (both $P > 0.05$), though wild mosquitoes had a lower TE (but not TR) at 14 dpi than YOLO ($P \leq 0.05$).

There were additional differences in EILV host competence at the viral titer level. The titers in the body and leg samples of KNWR did not differ from those of YOLO at 7 dpi (Mann-Whitney U test, both $P > 0.05$) but were lower at 14 dpi ($P \leq 0.01$ for

TABLE 2 Infection (IR), dissemination (DIR), and transmission (TR) rates and transmission efficiency (TE) of *C. tarsalis* mosquitoes orally challenged with EILV-eGFP[a]

	Data from 7 dpi				Data from 14 dpi			
***C. tarsalis* strain**	**IR (%) (n_I/n_T)**	**DIR (%) (n_L/n_I)**	**TR (%) (n_S/n_L)**	**TE (%) (n_S/n_T)**	**IR (%) (n_I/n_T)**	**DIR (%) (n_L/n_I)**	**TR (%) (n_S/n_L)**	**TE (%) (n_S/n_T)**
C. tarsalis (YOLO)	75 (33/44)	24.2 (8/33)	50 (4/8)	9.1 (4/44)	88.2 (30/34)	96.6 (29/30)	21 (6/29)	17.6 (6/34)
C. tarsalis (KNWR)	76.3% (29/38)	27.5 (8/29)	37.5 (3/8)	7.9 (3/38)	68.6 (24/35)	71 (17/24)	35.3 (6/17)	17.1 (6/35)
Wild *C. tarsalis*	22 (7/31)	57 (4/7)	25 (1/4)	3.2 (1/31)	25 (8/32)	62.5 (5/8)	0 (0/5)	0 (0/32)

[a]n_I, number of mosquitoes infected; n_T, total number of mosquitoes tested; n_L, number of mosquitoes with EILV-positive legs; n_S, number of mosquitoes with EILV-positive saliva.

both) (Fig. 2A and B). The leg and body titers of wild *C. tarsalis* were significantly lower than those of YOLO in most comparisons (see Fig. 2 for full results). EILV titers in saliva samples did not differ between any *C. tarsalis* strains at either time point (all $P > 0.05$), though EILV was absent in the saliva of wild *C. tarsalis* at 14 dpi. Together, these results demonstrate that genetically diverse *C. tarsalis* mosquitoes are susceptible to EILV via the oral route of infection, though there was variation among strains with regard to EILV infection dynamics—with *C. tarsalis* (YOLO) being the most susceptible and wild *C. tarsalis* the least susceptible to EILV infection.

Midgut infection barriers block EILV infections in *Anopheles*. By injecting virus directly into the thorax, we next asked if EILV host competence is broadened when any tissue-specific barriers to infection (e.g., midgut barriers) are bypassed. We therefore examined the presence of EILV in bodies at 7 and 14 dpi following intrathoracic (IT) injections in 74 *Ae. aegypti*, 80 *C. tarsalis* (YOLO), 66 *An. gambiae*, 76 *An. stephensi*, and 73 *An. albimanus* (Table 3) mosquitoes. Whereas only two species were susceptible to oral infections, all five mosquito species were susceptible to EILV infection when challenged intrathoracically, implying the existence of a strong midgut infection barrier to EILV infection in the tested anophelines. Following IT injections, the IR for *Ae. aegypti*, *C.* tarsalis, and *An. gambiae* was 100% at both time points, while *An. stephensi* had a slightly lower rate of 92.7% and 94.3% at 7 and 14 dpi, respectively (Table 3). *An. albimanus* had the lowest infection rates—94.7% and 85.7% at 7 and 14 dpi, respectively (Table 3). EILV IR did not differ by species or time point (Fisher's exact test, all $P > 0.05$). However, EILV titers of *C. tarsalis* (YOLO) body samples at 7 and 14 dpi were significantly higher than those of the other tested species (Fig. 3A), consistent with this species being the most vulnerable to EILV infection.

Tissue-specific barriers block EILV from infecting salivary glands in *Aedes* and *Anopheles*. By bypassing the midgut barriers to infection by injecting virus directly into the thorax, EILV had access to the salivary glands of all tested mosquito species, allowing us to test whether EILV can enter their saliva. We examined saliva samples from the five species following intrathoracic (IT) injections for the presence of EILV. As with oral infections, EILV was only found in the saliva of *C. tarsalis* following IT challenge, where it was prevalent at both 7 and 14 dpi (Fig. 3B and Table 3; TE of 62.2% and 35% at 7 and 14 dpi, respectively). The mean titers of virus-positive saliva samples at 7 and 14 dpi were 8.8 and 7.3 FFU/mosquito, respectively (Fig. 3B). Overall, our results show that *C. tarsalis* has limited barriers to EILV infection compared to the other mosquito species in our study and was the only transmission-competent host for EILV following IT infection.

EILV is present in the ovaries and salivary glands of orally infected *C. tarsalis* mosquitoes. Following an infectious bloodmeal, we used fluorescence microscopy to examine the tissue tropism of EILV-eGFP across the body of 80 *Ae. aegypti* (Rockefeller strain), 65 *C. tarsalis* (YOLO strain), 72 *C. tarsalis* (KNWR strain), 63 wild *C. tarsalis*, 78 *An. gambiae* (Keele strain), 80 *An. stephensi* (Liston strain), and 74 *An. albimanus* (STECLA strain) mosquitoes at 7 and 14 dpi. The tissue tropism of EILV informs where EILV may interact with pathogenic arboviruses within mosquitoes and thereby determine what EILV-based methods for vector control hold the most promise. All examined tissues (i.e., midgut, salivary glands, and ovaries) of orally challenged *An. gambiae*, *An. albimanus*, and *An. stephensi* were negative for eGFP expression (Fig. 4), indictive of the

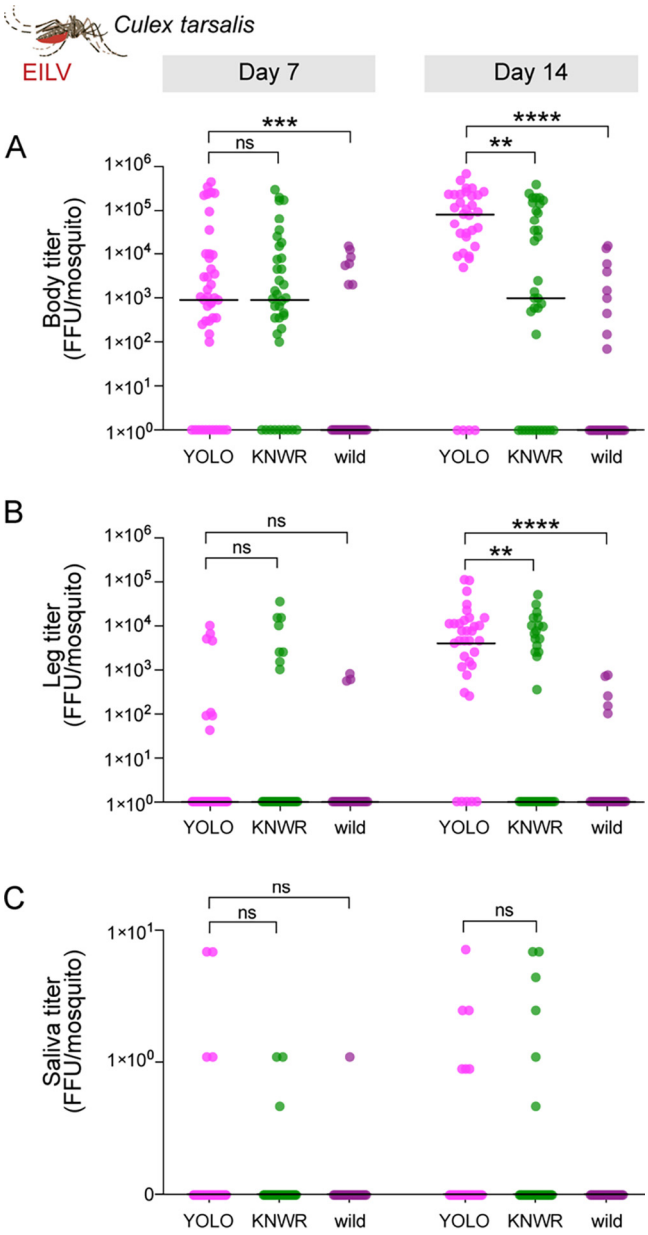

FIG 2 Viral titers of body, leg, and saliva samples from three *C. tarsalis* strains orally challenged with EILV-eGFP. (A to C) Titers are plotted at 7 and 14 dpi for (A) body, (B) leg, and (C) saliva samples from mosquitoes orally challenged with EILV-eGFP (10^7 FFU/mL). Each point represents a single mosquito sample, while horizontal bars depict the group medians. Note that data for *C. tarsalis* (YOLO) are replotted from Fig. 1. Statistical significance was evaluated using Mann-Whitney U tests. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

absence of EILV infection in these mosquito tissues. However, eGFP expression was detected in the midgut of 25 YOLO, 27 KNWR, 6 wild *C. tarsalis*, and 26 *Ae. aegypti* mosquitoes at 7 dpi (Fig. 4). Of these infected mosquitoes, the salivary glands of 3 YOLO, 3 KNWR, 2 wild *C. tarsalis*, and 5 *Ae. aegypti* were eGFP positive at 7 dpi (Fig. 4). Similarly, the midguts of 28 YOLO, 25 KNWR, 10 wild *C. tarsalis*, and 20 *Ae. aegypti* mosquitoes were observed to be EILV infected at 14 dpi (Fig. 4). The salivary glands of 5 YOLO, 4 KNWR, 1 wild *C. tarsalis*, and 3 *Ae. aegypti* mosquitoes were also EILV positive at 14 dpi (Fig. 4). Expression of eGFP in the ovary was only observed in two of the *C. tarsalis* strains— 4 YOLO and 4 KNWR at 7 dpi and 5 YOLO and 4 KNWR at 14 dpi (Fig. 4). Expression of eGFP in the ovaries was not observed in wild *C. tarsalis* or *Ae. aegypti* at either time point (Fig. 4).

TABLE 3 Infection rates (IR) and transmission efficiency (TE) of intrathoracically injected mosquitoes[a]

	Data from 7 dpi		Data from 14 dpi	
Mosquito species	IR (%) (n_I/n_T)	TE (%) (n_S/n_T)	IR (%) (n_I/n_T)	TE (%) (n_S/n_T)
Ae. aegypti	100 (38/38)	0 (0/38)	100 (36/36)	0 (0/36)
C. tarsalis (YOLO)	100 (37/37)	62.2 (23/37)	100 (43/43)	53.4 (23/43)
An. gambiae	100 (33/33)	0 (0/33)	100 (33/33)	0 (0/33)
An. stephensi	92.7 (38/41)	0 (0/41)	94.3 (33/35)	0 (0/35)
An. albimanus	94.7 (36/38)	0 (0/38)	85.7 (30/35)	0 (0/35)

[a]n_I, number of mosquitoes infected; n_T, total number of mosquitoes tested; n_S, number of mosquitoes with EILV-positive saliva.

EILV is absent in the ovaries of *C. tarsalis* but present in the ovaries *of An. gambiae* post-IT injection. We additionally examined EILV tissue tropism at 7 and 14 dpi in 84 *Ae. aegypti* (Rockefeller strain), 61 *C. tarsalis* (YOLO strain), 78 *An. gambiae* (Keele strain), 80 *An. stephensi* (Liston strain), and 67 *An. albimanus* (STECLA strain) mosquitoes injected with the virus. Strong eGFP expression—indicating EILV infection—was detected in the midguts of all *C. tarsalis* (YOLO) and *Ae. aegypti* mosquitoes tested at both 7 and 14 dpi (Fig. 5). The midguts of IT-injected mosquitoes, 38 *An. gambiae*, 41 *An. stephensi*, and 29 *An. albimanus*, showed limited eGFP expression at 7 dpi (Fig. 5). Similarly, limited eGFP expression was detected in the midguts of 40 *An. gambiae*, 34 *An. stephensi*, and 33 *An. albimanus* mosquitoes at 14 dpi (Fig. 5). EGFP was also observed in the salivary glands of 19 *C. tarsalis* and 24 *Ae. aegypti* mosquitoes at 7 dpi

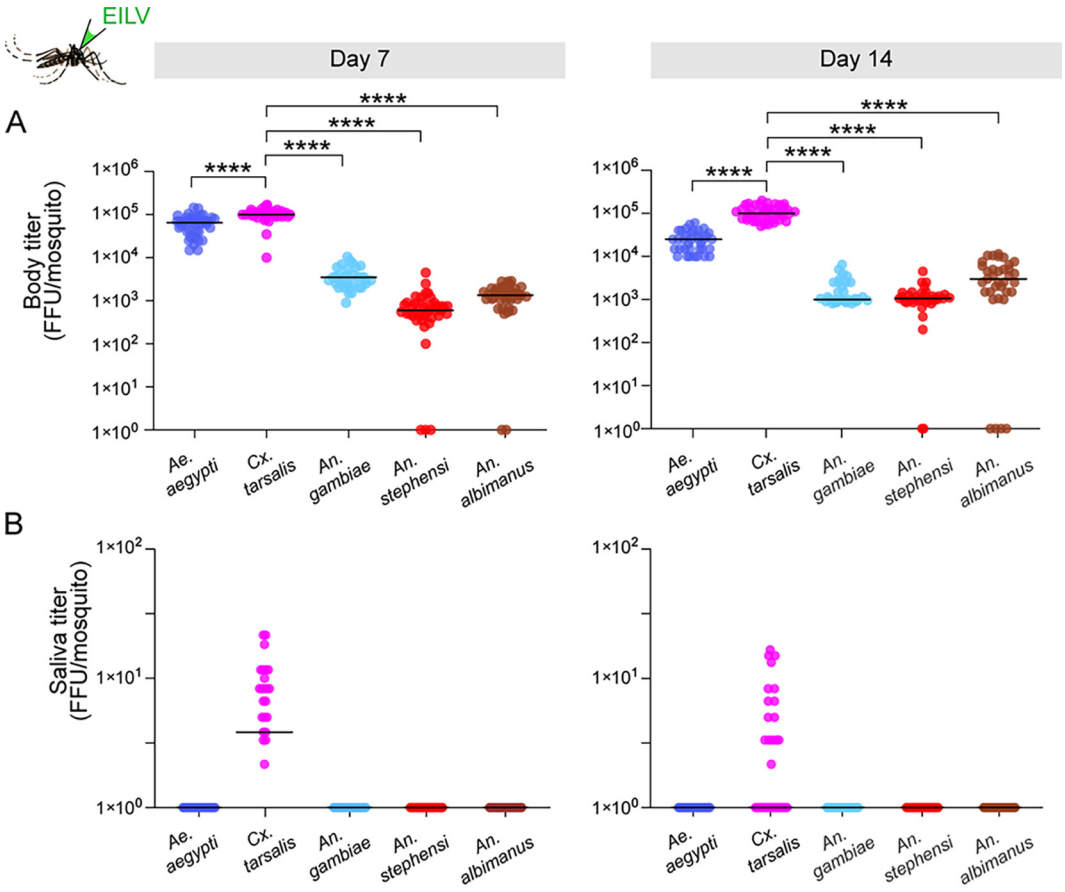

FIG 3 Viral titers of body, leg, and saliva samples from five mosquito species intrathoracically injected with EILV-eGFP. (A and B) Titers are plotted at 7 and 14 dpi for (A) body and (B) saliva samples following IT injection with EILV-eGFP (10^7 FFU/mL). Each point represents a single mosquito sample, and horizontal bars depict group medians. Statistical significance was evaluated using the Mann-Whitney U test. ****, $P < 0.0001$.

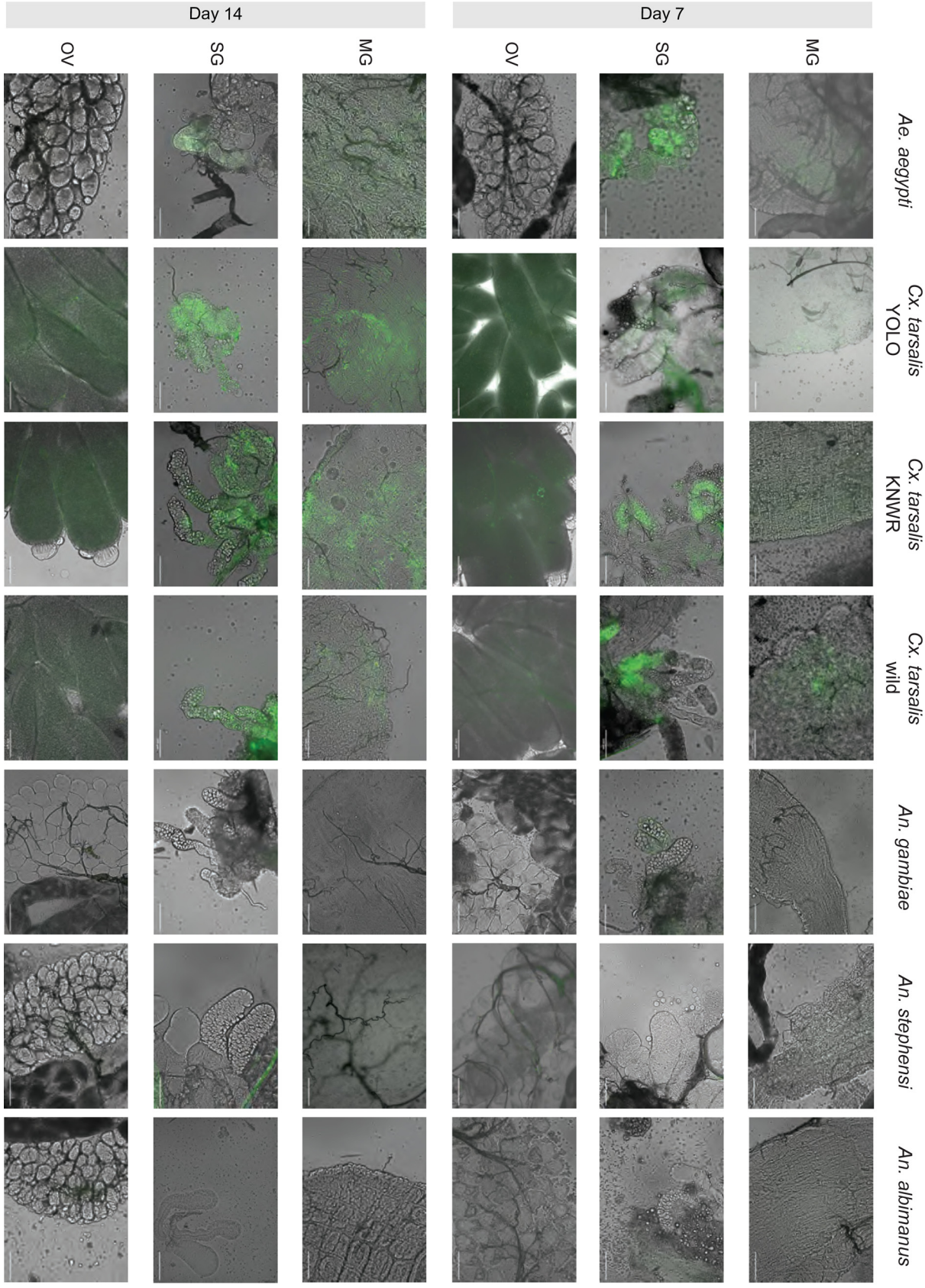

FIG 4 EILV tissue tropism at 7 and 14 dpi in orally challenged mosquitoes. Representative images show eGFP fluorescence (or its absence) in midguts (MG), salivary glands (SG), and ovaries (OV) of mosquitoes fed an infectious bloodmeal containing EILV-eGFP (10^7 FFU/mL). The bright-field and FITC images have been merged. All scale bars equal 100 μm.

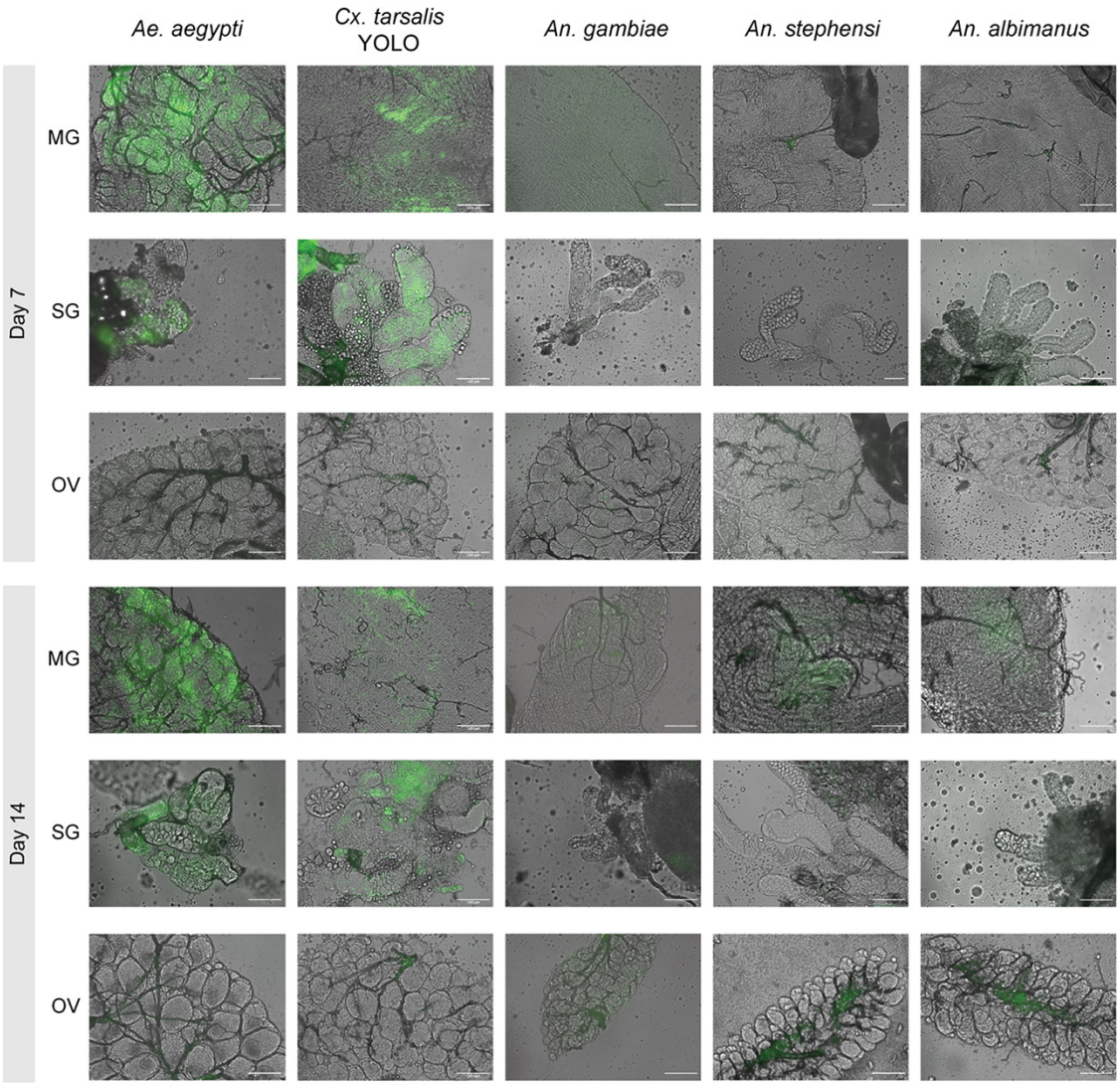

FIG 5 EILV tissue tropism at 7 and 14 dpi in IT-injected mosquitoes. Representative images show eGFP fluorescence (or its absence) in midguts (MG), salivary glands (SG), and ovaries (OV) of mosquitoes injected with EILV-eGFP (10^7 FFU/mL). The bright-field and FITC images have been merged. All scale bars equal 100 μm.

and 17 *C. tarsalis* and 25 *Ae. aegypti* mosquitoes at 14 dpi (Fig. 5). However, eGFP was not observed in the salivary glands of any of the anophelines at either time point (Fig. 5). Surprisingly, the ovaries of IT-injected *An. gambiae*, two at 7 dpi and three at 14 dpi, were positive for EILV, but the ovaries of the other four species were negative for EILV at 7 and 14 dpi (Fig. 5).

EILV was not transmitted venereally or vertically in *C. tarsalis*. Given the ability of EILV to infect ovaries of *C. tarsalis* (YOLO) following oral infection, we next evaluated whether this virus could be transmitted vertically from females to offspring, and also if EILV could be spread from females to males during mating. Parental *C. tarsalis* female mosquitoes (n = 15) were orally challenged with EILV-eGFP and then later fed two non-infectious bloodmeals to trigger gonotrophic cycles. After their final oviposition, parental females were dissected and screened for EILV by examining midgut tissue for eGFP fluorescence. We found that 80% of parental females remained infected at the midgut level (Table 4). Initially uninfected parental males (n = 45) were harvested postmating and screened for EILV by fluorescence microscopy, focus-forming assay (FFA), and reverse transcriptase PCR (RT-PCR), and none were found to harbor EILV, indicating that no venereal transmission occurred in our assays (Table 4; see Fig. S2B in the

TABLE 4 Presence of EILV in parental and F1 *C. tarsalis* (YOLO) to detect vertical and venereal transmission

EILV infection	Data for:			
	Parental female *C. tarsalis* (YOLO)	Parental male *C. tarsalis* (YOLO)	Female *C. tarsalis* (YOLO) from ER2	Female *C. tarsalis* (YOLO) from ER3
No. infected	12	0	0	0
No. uninfected	3	45	55	35
IR (%)	80	0	0	0

supplemental material; 95% binomial confidence interval, 0.0 to 7.9% venereal transmission efficiency). We next searched for instances of vertical transmission among offspring of infected females by microscopy, FFA, and RT-PCR. The midguts of adult female offspring that emerged from egg raft 2 (ER2; $n = 55$) and ER3 ($n = 35$) were screened for EILV by examining them for eGFP fluorescence. No eGFP was detected in any of the screened offspring midguts, suggesting that no vertical transmission occurred (Fig. S2A). FFAs were performed on pooled homogenized female offspring mosquito samples, but no FFUs were detected (Table 4). Similarly, pooled samples from ER2 and ER3 were also negative for EILV RNA by RT-PCR (Fig. S2B), further confirming that EILV was not transmitted vertically (95% binomial confidence interval, 0.0 to 4.1% vertical transmission efficiency).

Reptile cell lines are refractory to EILV infection. Mammalian, avian, and amphibian cell lines were previously shown to be refractory to EILV infection, but reptile cell lines have not been investigated. As mosquitoes can and do feed on reptiles, we investigated the hypothesis that reptiles may be a vertebrate host for EILV. We attempted to infect two reptile cell lines—TH-1, subline B1 (derived from the box turtle *Terrapene carolina*) and VH 2 cells (derived from Russel's viper, *Daboia russelii*)—along with C6/36 cells (positive control) with EILV-eGFP (10^7 FFU/mL) and screened for EILV infection at 24, 72, and 120 h postinfection under a fluorescence microscope. No eGFP expression was noted in either cell line, suggesting that reptile cells are not competent for EILV infection and/or replication.

***Manduca sexta* caterpillars are not susceptible to EILV infection.** Because EILV was found in mosquito saliva and because mosquitoes are known to bite caterpillars (15), we next tested caterpillars of the laboratory model *Manduca sexta* for EILV host competence. Caterpillars were injected with EILV-eGFP ($n = 10$) or 1× phosphate-buffered saline (PBS) ($n = 10$) and screened for EILV infection at 7 dpi by examining abdominal tissue near the injection site under a fluorescence microscope. No eGFP expression was noted in the injected caterpillars (Fig. S3). Additionally, FFAs were performed on homogenized injected *Manduca sexta* samples and controls, and no FFUs were found. Our results suggest that EILV is unable to infect the invertebrate *Manduca sexta*.

DISCUSSION

EILV, an insect-specific alphavirus unable infect vertebrates, has the potential to be used as a tool against pathogenic viruses in mosquito vectors (3, 7, 16). For example, EILV is known to limit the replication of some pathogenic viruses, such as chikungunya virus, via superinfection exclusion *in vitro* and *in vivo* (16). EILV also has the potential to be developed into a tool for paratransgenesis of antiviral agents to vector mosquitoes, as EILV, like other alphaviruses, tolerates the insertion and expression of foreign genes from its genome in both cells and mosquitoes (3, 17). The goal of this study was to better understand the host range and transmission route(s) of EILV. Here, we report that the anophelines *An. gambiae*, *An. albimanus*, and *An. stephensi* were not susceptible to EILV by oral infection but were susceptible when the virus was injected intrathoracically. *Ae. aegypti*, on the other hand, was susceptible to EILV by both the oral and IT infection routes in our study. These results comport with the findings of a previous study on *An. gambiae* and *Ae. aegypti* (8). In a new finding, we identify *C. tarsalis* as a competent host for EILV.

Culex tarsalis (YOLO) was the most competent host for EILV in our study, irrespective of infection route. When challenged orally or IT, *C. tarsalis* showed higher infection prevalence than other species, and it also frequently presented with higher viral loads (i.e., viral titer). *Culex tarsalis* was also the only mosquito species in our study to transmit EILV via saliva, where the virus was found following oral and IT infection. Only a handful of other insect-specific alphaviruses have been found, and aside from EILV, each was isolated from within the genus *Culex* (4–6). EILV too has been known to infect *C. pipiens* in the wild (12). However, *C. quinquefasciatus*, a closely related species (18), has low competence for EILV (8).

Although most ISVs are thought to have a narrow host range or even be adapted to a single principal host (8), EILV does not match this framework, as it infects multiple mosquito taxa. Mosquito species reported to have host competence for EILV now include *An. coustani* (3), *C. pipiens* (12), *C. tarsalis*, and *Ae. aegypti* (8), and horizontal transmission through invertebrate hosts or via shared habitats could potentially sustain a multihost ISV. Such a virus would differ from the ISVs characterized to date, though some, e.g., *Culex* flavivirus, whose principal host is *Culex pipiens*, can infect closely related species within a single genus (11, 19). However, further work is needed to determine the mosquito host use of EILV in nature.

We found that the susceptibility of *C. tarsalis* to EILV was shared by diverse genetic strains of this species. Specifically, KNWR, another laboratory-adapted *C. tarsalis* strain, and wild *C. tarsalis* from Yolo County, CA, USA (lab reared from egg and larval stage for one life cycle) both became infected following oral exposure to EILV. However, there were differences among the three tested *C. tarsalis* strains. For example, the EILV DIRs of KNWR and wild *C. tarsalis* were significantly lower than those of YOLO at 14 days following oral infection. Congruent with our findings, the susceptibility of mosquitoes to alphaviruses by oral infection is known to vary between strains of the same mosquito species (20–22). Most strikingly, wild *C. tarsalis* mosquitoes had significantly lower IRs across time, with EILV present in the saliva of wild mosquitoes less often, even when they did become infected. One possible explanation is that the laboratory-adapted colonies have less genetic variation than their wild counterparts, increasing their susceptibility to viruses (20, 23–25). Additionally, wild *C. tarsalis* and lab-adapted colonies may have different microbiomes that are known to influence the viral susceptibility and vectorial capacity of these mosquitoes (26, 27).

Our characterization of tissue tropism implies the presence of barriers to EILV infection that differ among species. We found that the anophelines *An. gambiae*, *An. albimanus*, and *An. stephensi* were not susceptible to EILV by oral infection but were susceptible when the virus was injected into the thorax, indicating the presence of a midgut infection barrier against EILV in those species (28). In *Ae. aegypti*, we did not detect EILV in saliva despite the salivary gland tissue being infected, suggesting a salivary gland escape barrier against EILV (28). Overall, these findings agree with previous work (9), though more studies are needed verify these barriers.

Interestingly, we observed the presence of EILV in the ovaries of IT-infected *An. gambiae* at 7 and 14 dpi—a tissue tropism not observed in the previous EILV host range study (8). This difference in ovarian susceptibility could stem from the different *An. gambiae* strains that were tested (8). Our study used the Keele strain, which was developed by the balanced interbreeding of four *An. gambiae sensu stricto* strains, one of which is the G3 strain used in the previous study (29). Thus, there was genetic overlap in the strains tested. However, the Keele strain has significantly higher allelic diversity, which could underlie the observed disparity in EILV tissue tropism, as genetic diversity can confer increased resistance to infections (30, 31).

Because many insect-specific viruses are thought to be adapted to a single host system, they likely depend on vertical transmission (and, to a lesser extent, venereal transmission) to maintain themselves in host populations (11, 12, 32–36). Moreover, the efficiency of vertical transmission of insect-specific viruses seems to be higher (32) than that of viruses that spread by horizontal transmission (37–39). For example, an insect-

specific flavivirus in *C. pipiens* (CxFV) was found to vertically transmit at 100% efficiency via transovarial transmission (TOT) (32). Consistent with the hypothesis that it transmits vertically, EILV has been detected in *C. pipiens* larvae in the wild (14). Moreover, the presence of EILV in the ovaries of *C. tarsalis* (YOLO) and *C. tarsalis* (KNWR) in the present study is preliminary evidence of TOT, though the virus was not found in the ovaries of wild *C. tarsalis* following oral infection. This absence could indicate a barrier to EILV infection similar to the midgut escape barrier—though one that has been lost in the tested laboratory-adapted strains (40). Further experiments are needed to verify and characterize this variation in ovarian tropism and to determine if EILV can be transmitted vertically under different conditions than those tested here.

Strikingly, no EILV was detected in progeny of infected *C. tarsalis* females in our vertical transmission study, indicating that vertical transmission is rare to nonexistent. Moreover, no venereal transmission from infected females to their male partners was noted. This may indicate that vertical and venereal transmission of EILV is rare or that *C. tarsalis* (YOLO) is not a native host for EILV and vertical transmission is restricted to a more specialized (unknown) host. Another possibility is that persistently EILV-infected populations may be more efficient at vertical transmission due to physiological changes caused by persistent natural infection. There is some empirical evidence for the latter hypothesis: CxFV (an ISV) was transmitted vertically by naturally infected *C. pipiens* but not by IT-infected naive *C. pipiens* (32).

In *C. tarsalis* (YOLO), EILV tissue tropism did not differ between oral and IT inoculation routes, with the exception that no EILV was found in IT-infected *C. tarsalis* (YOLO) ovaries. This suggests that blood-feeding is necessary for EILV to infect the ovaries. Mosquito ovaries undergo oogenesis post-blood feeding, during which the ovaries expand as nutrients enter to form the yolk in a process called vitellogenesis (41). These morphological changes may be used by EILV to infect the ovaries. Similarly, plant viruses are known to use vitellogenesis to infect the ovaries of white flies, and in mosquitoes, protein and DNA cargo have been transported into the ovaries using vitellogenin (42, 43). However, more research is required to determine the mechanism of infection of the ovaries by EILV in *C. tarsalis* (YOLO).

EILV is thought to have evolved into a single-host virus from a dual-host ancestor, like the insect-specific flaviviruses that cluster with dual-host viruses (3, 7, 44). Phylogenetically, EILV is most closely related to alphaviruses that infect both mosquitoes and vertebrates (3), but EILV lost its ability to infect vertebrates. The presence of EILV in the saliva of *C. tarsalis* (YOLO), *C. tarsalis* (KNWR), and wild *C. tarsalis* may be a remnant of its past ability to transmit from mosquitoes to vertebrates horizontally (3, 7, 8). Alternatively, EILV may still be a dual- or multihost virus, horizontally transmitted between mosquitoes and another vertebrate or invertebrate host. Our data suggest that reptile cells are not competent for EILV infection, suggesting that reptiles are not a vertebrate host for the virus. Recent research has shown that mosquitoes not only feed on vertebrates but can also feed on invertebrates such as worms, leeches, and caterpillars (15, 45). These diverse food sources increase the possible host ranges of arboviruses. While we find that EILV infection is not supported by *Manduca sexta* larvae, another invertebrate may be susceptible to EILV. Further research could explore the role of diverse invertebrates in the maintenance of arboviruses in nature.

Our study adds to an emerging picture that EILV may have nonstandard characteristics for an insect-specific virus. Ostensibly, it can infect multiple mosquito species, including in the genera *Anopheles* (9), *Aedes* (9), and—as we demonstrate here—*Culex* (12). However, its transmission route(s) remains elusive. There is limited evidence for transovarial transmission, the dominant route for better-characterized ISVs, but EILV was also found in salivary glands and was secreted in saliva—revealing the potential for horizontal transmission. Notably, we find EILV replicating in *C. tarsalis* ovary tissue, the first report of EILV in the ovary of any species. Because it efficiently infects *Culex tarsalis*—and grew in all examined tissues—EILV may prove a useful tool to combat pathogenic viruses transmitted by this mosquito, such as West Nile virus and Western equine encephalitis.

MATERIALS AND METHODS

Cells and cell culture. The *An. albopictus* mosquito cell line C6/36 was propagated at 28°C with no CO_2 in complete RPMI medium, which comprised Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Gibco/Thermo Fisher Scientific), penicillin (100 U/mL) (Gibco/Thermo Fisher Scientific), streptomycin (100 μg/mL) (Gibco/Thermo Fisher Scientific), and 2% (vol/vol) tryptose phosphate broth (Sigma-Aldrich).

TH-1, subline B1 (*Terrapene carolina*) and VH 2 (*Daboia russelii*) cells were acquired from ATCC and propagated in complete minimum essential medium (MEM) (Gibco/Thermo Fisher Scientific) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Gibco/Thermo Fisher Scientific), penicillin (100 U/mL) (Gibco/Thermo Fisher Scientific), and streptomycin (100 μg/mL) (Gibco/Thermo Fisher Scientific). TH-1, subline B1 cells were maintained at 28°C with no CO_2, while VH 2 cells were maintained at 30°C with 5% CO_2.

Viral cDNA clone and virus rescue. We used an EILV (strain EO329) cDNA clone with an enhanced green fluorescent protein inserted in the hypervariable region of non-structural protein 3; (nsp3) of the EILV genome (EILV-eGFP) for all experiments. The addition of eGFP into the HVD has been shown to have no measurable effects on the virus (46). The EILV-eGFP cDNA clone was obtained from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch.

EILV-eGFP was rescued as previously described with some modifications (7). The cDNA clone (10 μg) was linearized using NotI (New England BioLabs [NEB], Ipswich, MA, USA) and then purified and concentrated using the DNA Clean and Concentrator-25 kit (Zymo Research, Irvine, CA, USA). The linearized cDNA was transcribed using a MEGAscript SP6 transcription kit (Invitrogen/Thermo Fisher Scientific) with the addition of an m7G(5′)ppp(5′)G RNA cap (NEB). Transcription was carried out at 42°C for 2 h (h) on a thermocycler. The transcribed RNA was then purified using a MEGAclear transcription clean-up kit (Invitrogen/Thermo Fisher Scientific) and stored at −80°C.

Before transfection, C6/36 cells were seeded in a T75 flask at a density of 6×10^7 cells and incubated overnight at 28°C with no CO_2 to achieve ~70 to 80% confluence. Transfection of EILV-eGFP RNA was performed using the TransIT-mRNA transfection kit (Mirus Bio, Madison, WI, USA). We verified EILV-eGFP infection in transfected cells by examining them for eGFP fluorescence using a Zeiss Axiovert S 100 instrument equipped with a Fourier-transform infrared spectroscopy (FTIR) filter. To collect infectious virus, cell supernatant was harvested 5 days posttransfection and centrifuged for 10 min at 3,000 $\times$ *g* at 4°C in a swinging bucket rotor centrifuge to remove cell debris. The supernatant was then aliquoted and stored at −80°C. EILV-eGFP viral titer was quantified by focus-forming assays (described below).

To inoculate reptile cell lines, 12-well tissue culture plates were seeded with 1×10^7 TH-1, subline B1, or VH 2 cells per well and incubated overnight at 28°C with no CO_2 and 30°C with 5% CO_2, respectively, to achieve ~70 to 80% confluence. Then, 12-well tissue culture plates were also seeded with 1×10^7 C6/36 cells per well and incubated overnight at 28°C with no CO_2 to serve as a positive control for EILV infection. The cells were washed with medium containing no FBS and inoculated with 0.5 mL of EILV-eGFP (10^7 FFU/mL) and incubated under their respective growth conditions for 1 h. Then, the virus was removed and replaced with complete medium. We scored EILV-eGFP infection in the reptile cells by examining them for fluorescence at 24, 72, and 120 h postinfection using a Zeiss Axiovert S 100 instrument equipped with a Fourier-transform infrared spectroscopy (FTIR) filter.

Mosquitoes and mosquito rearing. We examined competence for EILV infection in five mosquito species: *Aedes aegypti*, *Culex tarsalis*, *Anopheles gambiae*, *An. stephensi*, and *An. albimanus*. A positive-control species for EILV infection (8), *Ae. aegypti* (Rockefeller strain), was provided by Johns Hopkins University (Baltimore, MD, USA). *Anopheles gambiae* (Keele strain) was obtained from The National Institutes of Health (Bethesda, MD, USA) and served as a negative control (8). *Anopheles albimanus* (STELCA strain) was obtained from BEI Resources (Manassas, VA, USA). *Anopheles stephensi* (Liston strain) was acquired from Johns Hopkins University. We tested three strains of *C. tarsalis*: YOLO strain (from BEI Resources), KNWR strain (from Christopher Barker's laboratory, UC Davis School of Veterinary Medicine, Davis, CA, USA) and wild *Culex tarsalis* collected in the field (from the Sacramento-Yolo Mosquito and Vector Control District, Elk Grove, CA, USA).

All mosquitoes were reared and maintained at the Millennium Sciences Complex (Pennsylvania State University, University Park, PA, USA). *Culex tarsalis* (YOLO and KNWR strains) larvae were reared in 30 by 30 by 30-cm cages and maintained at 25°C ± 1°C with a 16:8-h light:dark diurnal cycle and 80% relative humidity. The other mosquitoes were reared in 30 by 30 by 30-cm cages in a walk-in environmental chamber maintained at 27°C ± 1°C with a 12:12-h light:dark diurnal cycle and 80% relative humidity. Wild *Culex tarsalis* eggs collected in the field were reared for one generation in the laboratory. Gravid *Culex tarsalis* female mosquitoes were collected from the Sacramento-Yolo area by the Sacramento-Yolo Mosquito and Vector Control District during early September 2021. The first clutch of eggs from these mosquitoes hatched on 17 September 2021, and the second, on 21 September 2021. Wild mosquitoes were not prescreened for any viruses naturally occurring in the Sacramento-Yolo area. *Aedes aegypti*, *An. albimanus*, and *C. tarsalis* (YOLO and KNWR strains) larvae were fed Koi pellets (Tetra, Melle, Germany). *Anopheles gambiae and C. tarsalis* (wild) larvae were fed TetraMin (Tetra). A slurry made with TetraMin and baker's yeast (1:2 by volume) was used to feed *An. stephensi* larvae. All adult mosquitoes were provided with 10% sucrose solution on cotton balls *ad libitum*.

Mosquito infections. For both infection routes, each mosquito species was challenged separately using aliquots of the same virus stock. For each species, we challenged two replicate batches of mosquitoes. Half of the mosquitoes from each batch were then sampled at each of the two time points (i.e., 7 dpi and 14 dpi).

Oral infections. Adult female mosquitoes (3 to 5 days postemergence) were sugar-starved for 24 h and then fed an infectious blood meal comprising 1:1 anonymous human blood (BioIVT, Westbury, NY, USA) and 10^7 FFU/mL EILV-eGFP at 37°C using a water-jacketed membrane feeder. A virus dose of 10^7 FFU/mL was chosen for our experiments, as it is the 50% infectious dose for *Aedes aegypti* (8). Mosquitoes were then cold-anesthetized, and fully engorged mosquitoes were counted and placed in cardboard cup cages until processing.

Intrathoracic infections. Adult female mosquitoes (3 to 5 days postemergence) were cold-anesthetized and placed on a glass slide on top of a chill block maintained at 4°C. Under an Olympus SZX7 stereo microscope, each mosquito was then injected in the thorax with 100 nL of EILV-eGFP virus (107 FFU/mL) at a rate of 100 nL/s using a Nanoject III device (Drummond Scientific Company, Broomall, PA, USA). Injected mosquitoes were counted and placed in a sealed cardboard cup cage until processing.

Imaging. Mosquitoes challenged with EILV-eGFP were dissected on days 7 and 14 dpi. For each time point, the midgut, ovaries, and salivary glands of each mosquito were dissected into 50 μL of 1$\times$ PBS on a glass slide. A coverslip was placed over the mosquito tissues for imaging. EILV infection status in organs was determined by examining tissue for eGFP fluorescence using an Olympus BX41 inverted microscope equipped with a fluorescein isothiocyanate (FITC) filter. The organs were also imaged under bright-field illumination.

Host competence assays. The host competence of each mosquito species for EILV at 7 and 14 dpi was determined as previously described (47–49). Briefly, challenged mosquitoes were anesthetized using triethylamine (Sigma-Aldrich, St. Louis, MO, USA) and forced to salivate into a capillary glass tube containing FBS and 50% sucrose mixed at a ratio of 1:1 for 30 min. After 30 min, the saliva was pushed into a 2-mL microcentrifuge tube containing 100 μL mosquito dilutant (1$\times$ PBS mixed with 20% FBS, 100 μg/mL of streptomycin, 100 units/mL of penicillin, 50 μg/mL of gentamicin [Gibco/Thermo Fisher Scientific], and 2.5 μg/mL of amphotericin B [Gibco/Thermo Fisher Scientific]). Next, bodies and legs were collected and placed separately into 2-mL microcentrifuge tubes, each containing 300 μL mosquito dilutant and a 4.5-mm zinc-plated steel bead (Daisy Outdoor Products, Rogers, AR, USA). Samples were briefly stored on ice, and then leg and body samples were homogenized using a TissueLyser II (Qiagen, Hilden, Germany) at 30 Hz for 2 min. Homogenized samples were centrifuged at 6,000 $\times$ *g* at 4°C for 5 min using a benchtop microcentrifuge. All samples were stored at $-$80°C until quantified by FFA (described below). From FFA counts, the IR was calculated as the proportion of infected mosquitoes among the total number of engorged mosquitoes, the DIR as the proportion of infected mosquitoes with virus-positive legs, the TR as the proportion of mosquitoes with virus-positive saliva among those with virus-positive legs, and the TE as the proportion of mosquitoes with virus-positive saliva among the total number of mosquitoes engorged.

Focus-forming assays (FFA). We determined infection status and quantified EILV-eGFP titers in samples using focus-forming assays. C6/36 cells were seeded in 96-well plates at a density of 1 $\times$ 10^5 cells/well and incubated at 28°C with no CO_2 overnight. Complete RPMI medium was then removed, and serially diluted (10° to 10^{-4}) mosquito samples in serum-free RPMI medium were then added (30 μL) in duplicate to the prepared cells. Saliva samples were not diluted. The cells were incubated at 28°C with no CO_2 for 1 h. The samples were then removed, and cells were covered with 100 μL of RPMI containing 0.8% methylcellulose (Sigma-Aldrich). The infected cells were incubated at 28°C without CO_2 for 48 h. The infected C6/36 cells were fixed using 50 μL of 4% formaldehyde (Sigma-Aldrich) in 1$\times$ PBS for 30 min at room temperature. Fixed cells were washed two times with 100 μL of 1$\times$ PBS, and finally, 50 μL of 1$\times$ PBS was added to the cells (to prevent drying), and fluorescent (i.e., EILV-eGFP) foci were counted using an FITC filter-equipped Olympus BX41 inverted microscope. The limit of detection for EILV FFA as described above is $\leq$1 FFU/mosquito.

Vertical transmission assays. Vertical transmission assays were carried out as previously described (17) with some modifications. We collected eggs from virus-challenged females at three time points to evaluate vertical transmission of EILV. Adult female *C. tarsalis* mosquitoes (3 to 5 days postemergence) were fed an artificial infectious blood meal supplemented with EILV-eGFP (10^7 FFU/mL) to induce EILV infection. Blood-fed mosquitoes were cold-anesthetized, and fully engorged females were counted and sorted into sealed cardboard cups. EILV-eGFP-fed females were then fed noninfectious blood meals at both 7 and 14 dpi. Oviposition containers, consisting of a wide-mouth plastic cup filled halfway with deionized (DI) water, were placed inside the cages 4 days after each feeding and were collected 5 days after each feeding (i.e., mosquitoes had ~24 h of access). The egg rafts from the three gonotrophic cycles were labeled ER1, ER2, and ER3. ER1 was discarded, as EILV does not disseminate to the ovaries of *C. tarsalis* 4 to 5 dpi. Meanwhile, ER2 and ER3 were hatched and reared to the adult stage. Midguts were dissected from parental female and male mosquitoes as well as from adult offspring (3 days postemergence) reared from ER2 and ER3 eggs. Midguts were evaluated for EILV-eGFP infection by fluorescence microscopy as described above. The carcasses were then collected in pools of five in a 2-mL microcentrifuge tube containing 500 μL of mosquito diluent and one 4.5-mm zinc-plated steel bead. Carcass samples of parental males and offspring included midguts, which were returned postimaging. The samples were homogenized using a TissueLyser II device (Qiagen) at 30 Hz for 2 min and then centrifuged at 6,000 $\times$ *g* at 4°C for 5 min using a benchtop microcentrifuge. All samples were quantified by both FFA, as described above, as well as by RT-PCR.

RT-PCR to detect EILV infection. We used RT-PCR (in addition to FFAs) to test for the presence of EILV in venereal and vertical transmission assays. RNA was extracted from test and control mosquito samples using the Direct-zol RNA miniprep kit (Zymo Research). The positive and negative controls for the RT-PCR were RNA extracted from an FFA-confirmed EILV-eGFP-positive mosquito and an FFA-confirmed EILV-eGFP-negative mosquito (EILV-eGFP challenged), respectively. The extracted RNA samples

were quantified using a NanoDrop ND-1000 (NanoDrop Technologies/Thermo Fisher Scientific), and then the template was amplified using the OneStep RT-PCR kit (Qiagen) with the EILV-specific primers 5′-CGA CGA TGA CCG GAG AAG AG-3′ and reverse primer 5′-AAG ACT CGG TCT GCC TGC-3′. Amplicons were analyzed using gel electrophoresis.

***Manduca sexta* rearing and infection.** We tested EILV host competence in the laboratory model *Manduca sexta*. Early-stage (L2 to L3) *Manduca sexta* larvae were acquired from Rudolf Schilder at Pennsylvania State University (University Park, PA, USA). The larvae were reared at room temperature in 30 by 30 by 30-cm plastic containers with air holes and fed an artificial diet (Frontier Agricultural Sciences, Newark, DE, USA) *ad libitum*.

Larvae were anesthetized on ice, and the injection site was swabbed with 70% ethanol. Larvae were then injected with either 10 μL of 1×10^7 FFU/mL EILV-eGFP or 1× phosphate-buffered saline (PBS; Gibco/Thermo Fisher Scientific; negative control) between the abdominal prolegs using sterile insulin syringes. Injected treatment and control *Manduca sexta* larvae were sacrificed 7 days postinjection at −80°C for 10 min. We initially screened larvae for EILV infection by dissecting the tissue near the injection site and examining it for eGFP fluorescence (indicating expression of EILV-eGFP) using an Olympus BX41 inverted microscope equipped with an FITC filter. Postimaging, samples were homogenized using a motorized homogenizer and pestle, followed by centrifugation for 10 min at 3,000 × *g* at 4°C in a swinging bucket rotor centrifuge. All samples were stored at −80°C until processing. EILV-eGFP viral titers were measured by FFA as described above.

Statistical analysis. Fisher's exact tests were used to evaluate differences in the infection rate (IR), dissemination rate (DIR), transmission rate (TR), and transmission efficiency (TE) between mosquito species orally and IT infected with EILV-eGFP. The EILV viral titers of body, leg, and saliva samples were compared using Mann-Whitney U tests. All statistical tests were run using GraphPad Prism version 9.0.4. We used the binconf() function from the R package Hmisc to calculate binomial confidence intervals.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 4.8 MB.

ACKNOWLEDGMENTS

We acknowledge Amelia Romo for her assistance with mosquito rearing, Sarah Wheeler from Sacramento-Yolo Mosquito and Vector Control District, Elk Grove, CA, USA, for providing us with *C. tarsalis* eggs from wild-caught gravid female *C. tarsalis* mosquitoes, and Rudolf Schilder at Pennsylvania State University (University Park, PA, USA) for supplying us with *Manduca sexta* larvae and technical assistance rearing them.

This study was supported by NIH/NIAID grants R01AI128201 and R01AI150251, NSF/BIO grant 1645331, USDA Hatch Project 4769, a grant from the Pennsylvania Department of Health using Tobacco Settlement Funds, and funds from the Dorothy Foehr Huck and J. Lloyd Huck endowment to J.L.R.

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