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Abstract
This study determined the transovarial transmission (TOT) potential and tissue tropisms of Culex flavivirus (CxFV), an insect-specific flavivirus, in *Culex pipiens* (L.). Several hundred mosquito egg rafts were collected in the field, transferred to the insectaries, reared to the fourth larval instar, and identified using morphological characteristics. *Cx. pipiens* were reared to adults, allowed to oviposit in individual containers, and tested for CxFV RNA by reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequencing. Eighteen CxFV RNA-positive females were identified from 26 females that oviposited viable egg rafts. Thirty F_1 adults from each positive female were individually tested by RT-PCR for CxFV RNA. Viral RNA was detected in 526 of 540 progeny, and thus, the filial infection rate was 97.4%. Because all 18 positive females produced infected offspring, the TOT prevalence was 100%. These data indicated that efficient TOT of CxFV occurs in nature. To define the tissue tropisms of CxFV, different tissues (salivary glands, ovaries, testes, head, fat bodies, and midguts) were removed from the remainder of the F_1 and tested by RT-PCR for CxFV RNA. Viral RNA was detected in all tissues. Additionally, uninfected laboratory-colonized *Cx. pipiens* were infected with CxFV by needle inoculation, and ovaries were collected at 4, 6, 8, and 12 d postinoculation and tested for CxFV RNA by RT-PCR. Viral RNA was detected at all time points, demonstrating that CxFV infects the ovaries as early as 4 d postinoculation. Surprisingly, however, we were unable to demonstrate transovarial transmission despite the presence of viral RNA in the ovaries. Nevertheless, the experiments performed with field-infected *Cx. pipiens* demonstrate that TOT is an efficient mechanism by which CxFV is maintained in mosquitoes in nature.

Keywords
Veterinary Microbiology and Preventive Medicine, Flavivirus, Culex flavivirus, Culex pipiens, Transvarial transmission, Tissue tropisms

Comments
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Evidence of Efficient Transovarial Transmission of Culex Flavivirus by *Culex pipiens* (Diptera: Culicidae)

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ABSTRACT This study determined the transovarial transmission (TOT) potential and tissue tropisms of Culex flavivirus (CxFV), an insect-specific flavivirus, in *Culex pipiens* (L.). Several hundred mosquito egg rafts were collected in the field, transferred to the insectaries, reared to the fourth larval instar, and identified using morphological characteristics. *Cx. pipiens* were reared to adults, allowed to oviposit in individual containers, and tested for CxFV RNA by reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequencing. Eighteen CxFV RNA-positive females were identified from 26 females that oviposited viable egg rafts. Thirty F_1 adults from each positive female were individually tested by RT-PCR for CxFV RNA. Viral RNA was detected in 526 of 540 progeny, and thus, the filial infection rate was 97.4%. Because all 18 positive females produced infected offspring, the TOT prevalence was 100%. These data indicated that efficient TOT of CxFV occurs in nature. To define the tissue tropisms of CxFV, different tissues (salivary glands, ovaries, testes, head, fat bodies, and midguts) were removed from the remainder of the F_1 and tested by RT-PCR for CxFV RNA. Viral RNA was detected in all tissues. Additionally, uninfected laboratory-colonized *Cx. pipiens* were infected with CxFV by needle inoculation, and ovaries were collected at 4, 6, 8, and 12 d postinoculation and tested for CxFV RNA by RT-PCR. Viral RNA was detected at all time points, demonstrating that CxFV infects the ovaries as early as 4 d postinoculation. Surprisingly, however, we were unable to demonstrate transovarial transmission despite the presence of viral RNA in the ovaries. Nevertheless, the experiments performed with field-infected *Cx. pipiens* demonstrate that TOT is an efficient mechanism by which CxFV is maintained in mosquitoes in nature.

KEY WORDS flavivirus, Culex flavivirus, *Culex pipiens*, transovarial transmission, tissue tropisms

The majority of viruses in the genus *Flavivirus* are transmitted horizontally between vertebrate hosts and hematophagous arthropods, such as mosquitoes and ticks (ICTV 2005). Viruses in this group include dengue virus (DENV), yellow fever virus, Japanese encephalitis virus, and West Nile virus (WNV), all of which are human pathogens of global importance. Other viruses in this genus are considered to be vertebrate specific, because they have a vertebrate host, but no known arthropod vector. Finally, another group of flaviviruses has been isolated strictly from Diptera (mosquitoes and sandflies), has no apparent vertebrate host, and therefore is considered to be insect specific (Hoshino et al. 2007, Moureau et al. 2009, Sanchez-Seco et al. 2009). Nine insect-specific flaviviruses have been isolated from mosquitoes: Culex flavivirus (CxFV) (Hoshino et al. 2007), cell fusing agent virus (Stollar and Thomas 1975, Cook et al. 2006, Kihara et al. 2007), Kamiti River virus (KRV) (Crabtree et al. 2003, Sang et al. 2003), Quang Binh virus (Crabtree et al. 2009), Aedes flavivirus (Hoshino et al. 2009), Nakiwogo virus (Cook et al. 2009), Lammi virus (Huhtamo et al. 2009), Nounane virus (Junglen et al. 2009), and Calbertado virus (Tyler et al., in press).

CxFV has a wide geographic distribution, having been isolated from *Culex* spp. mosquitoes in Asia (Hoshino et al. 2007), Guatemala (Morales-Betoulle et al. 2008), Mexico (Farfan-Ale et al. 2009, 2010; Saiyasombat et al. 2010), Trinidad (Kim et al. 2009), the United States (Blitvich et al. 2009, Kim et al. 2009), and Uganda (Cook et al. 2009). In Mexico, CxFV was detected in similar proportions of male and female *Culex quinquefasciatus;* the CxFV minimal infection rates, expressed as the number of positive mosquito pools per 1,000 mosquitoes tested, were 7.2 and 8.3, respectively (Farfan-Ale et al. 2010). These data indicate that CxFV is maintained in nature by vertical transmission, consistent with its vertebrate replication-incompetent phenotype. The isolation of KRV from immature *Aedes macintoshi* (Marks) provides

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further evidence that vertical transmission of insect-specific flaviviruses occurs in mosquitoes in nature (Sang et al. 2003).

One mechanism of vertical transmission is transovarial transmission (TOT), defined as the process by which progeny of infected females are directly infected in the egg stage within the ovary before release and subsequent insemination. Transovum transmission, in contrast, entails the infection of the egg as it moves down the oviduct. Vertical transmission is believed to be inefficient in mosquitoes infected with arthropod-borne flaviviruses because of low direct infection rates of the ovarian tissue and the requirement for transovum infection during a limited window of time. Alternatively, bunyaviruses with high vertical infection efficiencies frequently exhibit ovariole or follicle infection and undergo TOT. In a classic study, Tesh (1980) compared the vertical infection rates of WNV and DENV to that of a bunyavirus, San Angelo virus, in *Aedes albopictus* (Skuse). Progeny infection rates exceeded 13% for San Angelo virus, but were always <1% for WNV and DENV, even after selection. TOT is also inefficient in mosquitoes infected with yellow fever virus, Japanese encephalitis virus, St. Louis encephalitis virus, and Murray Valley encephalitis virus (Rosen et al. 1978, Aitken et al. 1979, Beaty et al. 1980, Kay and Carley 1980, Francy et al. 1981, Hardy et al. 1984). Mosquitoes are very permissive to flavivirus replication, and virus antigen is abundant in the ovarian sheath and oviducts, but not in ovarioles or follicles of infected vectors (Rosen 1988, Turell 1988). Clearly, although rarely, flaviviruses are vertically transmitted, and there are occasional reports of isolation of DENV and other flaviviruses from a small proportion of field-collected larvae and male adult mosquitoes. This is frequently called TOT, but is a misnomer. Mosquito eggs typically become infected with flaviviruses during insemination (transovum infection) as the egg is moving through the heavily infected common oviduct (Rosen 1988). During this time the micropyle is open, and sperm and fluids can enter the egg for fertilization. Resulting filial transovum infection (FI) rates are very low (<1%) (Aitken et al. 1979, Beaty et al. 1980) especially as compared with the >80% FI rates associated with TOT of La Crosse virus (*Bunyaviridae*) in *Aedes triseriatus* (Say) (Beaty and Bishop 1988, Woodring et al. 1998, Hughes et al. 2006).

As a result of the paucity of data on the mechanism(s) by which insect-specific flaviviruses are maintained in nature, the current study investigated the ability of CxFV to be transovarially transmitted by *Culex pipiens.* Because CxFV has been detected in similar proportions of female and male mosquitoes in the field (Farfan-Ale et al. 2010) and lacks the capacity to replicate in vertebrates (Hoshino et al. 2007), we tested the hypothesis that efficient TOT of CxFV occurs in the mosquito host.

Materials and Methods

Field-Collected Mosquitoes. Mosquito egg rafts were collected at study sites in three counties (Polk, Roosevelt, and Story) in the state of Iowa from September through October 2009 and from July through October 2010. Collections were made using gravid traps containing hay infusion (Lee and Rowley 2000). Mosquitoes were transported to the insectaries at Iowa State University (ISU), reared to the fourth larval stage, and identified using morphological characteristics. *Cx. pipiens* were retained; all other species were discarded. Larvae and pupae were reared in polypropylene plastic trays containing tap water supplemented with a slurry of Tetramin. Adult mosquitoes were maintained on a 10% sucrose solution at 27°C and 80% RH with a light-dark photocycle of 16:8 h.

Laboratory-Colonized Mosquitoes. *Cx. pipiens* (ISU strain) were originally collected as egg rafts at various sites in Iowa in 2002 and have been maintained continuously in the insectaries at ISU using the conditions described above. Mosquitoes from this colony are periodically tested for the presence of flavivirus RNA by reverse transcription-polymerase chain reaction (RT-PCR) and continually test negative.

Virus and Titers. CxFV (strain CxFV-Iowa07) was isolated from a pool of *Cx. pipiens* collected in Iowa in 2007 (Blitvich et al. 2009). Because CxFV does not plaque or cause extensive cytopathic effect in mosquito cell culture (Hoshino et al. 2007, Blitvich et al. 2009), the titer of the virus stock was measured by quantitative RT-PCR (qRT-PCR). To correlate qRT-PCR RNA copy determinations with infectivity, 6-well plates of confluent *Ae. albopictus* (C6/36) cells were inoculated with a 10-fold dilution series of CxFV and incubated at 28°C for 9 d. Cells and supernatants were harvested, after which total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and subjected to qRT-PCR, as described below. Reed-Muench calculations were employed to estimate infectious units (infectious dose 50%) and to determine specific infectivity of the input virus. The qRT-PCR assay was performed using primers specific to a 207-nucleotide region of the CxFV envelope gene (CxFV-E-forward, 5′-TGA ATT GCT CGC TGA TTG TC-3′ and CxFV-E-reverse, 5′-TTA TAC CCC TCT CCG CAA TG-3′). Amplification standards were prepared from RNA transcripts produced from a plasmid generated to contain the first 2,567 nucleotides of the CxFV genome downstream of a T7 RNA polymerase promoter. In vitro transcriptions were performed using an AmpliScribe T7 transcription kit (Epicenter Biotechnologies, Madison, WI). Viral RNA was DNase treated, extracted using TRIzol reagent, and quantified using a spectrophotometer. RNA transcripts were diluted to 10^{10} copies/μl, and 10-fold serial dilutions were used to construct standard curves. Viral RNA was quantified using the Quantitect SYBR Green One-Step RT-PCR kit (Qiagen, Valencia, CA) on a Bio-Rad iCycler iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). Reactions were performed in duplicate and consisted of 10 μl of Quantitect SYBR Green

RT-PCR Master Mix, 0.2 μl Quantitect RT Mix, 1 μl each of forward and reverse primers (10 μM), 10.8 μl of nuclease-free water, and 50 ng of template RNA. Nontemplate samples and RNA from uninfected C6/36 cells were included as controls. The thermal profile consisted of reverse transcription at 50°C for 30 min, reverse transcriptase inactivation/denaturation at 95°C for 15 min, and 40 cycles of PCR at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Dissociation analysis was conducted to detect nonspecific amplicons and primer dimers. To avoid the incorporation of nonspecific fluorescence in quantitative measurements, the temperatures at which fluorescence detection was acquired were adjusted to quantify specific products only. Fluorescence profiles from the standard curves were used to directly estimate initial RNA copy numbers of viral genomes in the samples.

TOT Experiments With Field-Collected Mosquitoes. Field-collected *Cx. pipiens* were reared to adults, placed in a single cage for 7 d to facilitate mating, and then allowed to feed on a quail (*Colinus virginianus*) (Institutional Animal Care and Use Committee Protocol 12-2-5400-Z) to initiate egg development. Before feeding on the quail, mosquitoes were starved by replacing the sucrose solution with water at 24-h preblood meal and by removing the water at 6-h preblood meal. Engorged females were transferred to individual cartons containing hay infusion water for oviposition. The resulting egg rafts were transferred to individual polypropylene plastic trays containing tap water supplemented with Tetramin. F_1 progeny were removed at the pupal stage and placed into cartons. All F_0 that produced viable eggs were individually tested by RT-PCR and nucleotide sequencing using CxFV-specific primers and a 3730x1 DNA sequencer (Applied Biosystems, Foster City, CA). Thirty F_1 adult progeny (15 females and 15 males) from each CxFV RNA-positive F_0 female were collected on the day of emergence and stored at −80°C before being individually tested for CxFV RNA by RT-PCR. The remaining progeny were collected at 8–10 d postemergence and used for the tissue tropism experiments.

TOT Experiments With Laboratory-Colonized Mosquitoes. Adult female *Cx. pipiens* (4–5 d postemergence) were cold anesthetized and injected with CxFV by intrathoracic inoculation into the cervical membrane using a fine needle. Each mosquito received an estimated 1.6×10^5 50% tissue culture-infective dose of CxFV equivalents as determined by qRT-PCR. Inoculated females were placed in a cage with uninfected adult males at a ratio of 1:2 to facilitate mating. Mosquitoes were starved as outlined above and, at 8 d postinoculation, allowed to feed on a quail. Engorged females were transferred to individual oviposition cartons. Egg rafts were transferred to individual cartons, and mosquitoes were reared to adults, as described above. All F_0 females that produced viable eggs were individually tested for CxFV RNA by RT-PCR to confirm that they were infected with CxFV. F_1 progeny were collected on the day of emergence and stored at −80°C until tested by RT-PCR using CxFV-specific primers.

RT-PCR. Total RNA was isolated from whole mosquitoes (either individually or in groups of five) and from mosquito organs (salivary glands, ovaries, testes, head, fat bodies, and midguts) previously ground in TRIzol reagent (Invitrogen) using a mortar and pestle on ice, as described by the manufacturer. Numbers of organs pooled and used in each reaction, denoted in parentheses, are as follows: salivary glands (100), ovaries (100), testes (90), head (5), fat bodies (40), and midguts (90). Total RNA was amplified by RT-PCR using CxFV-specific primers (CxFV-NS5-forward, 5′-TTG ACT CCA ACG CCT C-3′ and CxFV-NS5-reverse, 5′-ACC TTG AGT TCG AAG CG-3′) that target a 446-nucleotide region of the CxFV NS5 gene. Actin-specific primers were included as positive RT-PCR and normalization controls (Staley et al. 2010). Complementary DNAs were generated using Superscript III reverse transcriptase (Invitrogen), and PCRs were performed using *Taq* polymerase (Invitrogen). RT-PCR products were examined by 1% agarose gel electrophoresis and visualized with ethidium bromide staining.

Results

To determine whether CxFV is transovarially transmitted by mosquitoes in the field, several hundred mosquito egg rafts were collected at study sites in Iowa, transferred to the insectaries, reared to the fourth larval stage, and identified using morphological characteristics. *Cx. pipiens* were reared to adults, allowed to mate, and offered a blood meal to facilitate egg development. A total of 162 female *Cx. pipiens* produced egg rafts, of which 26 (16%) hatched. Female mosquitoes that produced viable eggs were individually tested for CxFV RNA by RT-PCR and nucleotide sequencing. Eighteen CxFV RNA-positive mosquitoes were identified. BLAST analysis of the resulting sequences revealed that all had ≥99% nucleotide identity to the homologous region of CxFV-Iowa07, the prototype CxFV strain from Iowa (Blitvich et al. 2009).

Thirty F_1 adults (15 females and 15 males) from each CxFV RNA-positive F_0 were collected on the day of emergence and individually assayed by RT-PCR using CxFV-specific primers. Actin-specific primers were included as positive controls. CxFV RNA was detected in 526 of 540 progeny, and thus, the overall estimated FI rate (defined as the percentage of infected F_1 progeny from the CxFV RNA-positive females) was 97.4% (Table 1). All 18 CxFV RNA-positive females produced infected progeny, and therefore, the estimated TOT rate (defined as the percentage of CxFV RNA-positive females that transmitted virus to at least one of their progeny) was 100%. There was no significant difference in the overall proportion of CxFV RNA-positive female and male F_1 (98.5 and 96.3%, respectively; $P = 0.1042$, χ^2 test). There was, however, a significant (albeit modest) difference in the proportion of infected offspring produced by each CxFV-infected F_0 female, with values ranging from 86.7 to 100% ($P = 0.047$, df = 17, χ^2 test).

Table 1. Culex flavivirus filial infection rates in the F_1 progeny of field-collected CxFV RNA-positive *Cx. pipiens*

Identification no. F_0	Egg raft collection date	No. CxFV RNA-positive F_1 adults		
		Female	Male	Total
RC9	09/2009	15/15	14/15	29/30
PC31	09/2009	15/15	14/15	29/30
PC39	09/2009	14/15	15/15	29/30
PC96	09/2009	15/15	14/15	29/30
H1	07/2010	15/15	14/15	29/30
H4	07/2010	15/15	15/15	30/30
H6	07/2010	15/15	15/15	30/30
H7	07/2010	14/15	12/15	26/30
H11	07/2010	13/15	14/15	27/30
H18	07/2010	15/15	14/15	29/30
H25	07/2010	15/15	14/15	29/30
H27	07/2010	15/15	15/15	30/30
H32	07/2010	15/15	15/15	30/30
H42	07/2010	15/15	15/15	30/30
H56	07/2010	15/15	15/15	30/30
H61	07/2010	15/15	15/15	30/30
H79	07/2010	15/15	15/15	30/30
I15	08/2010	15/15	15/15	30/30
Total		266/270 (98.5%)	260/270 (96.3%)	526/540 (97.4%)

One additional F_0 female yielded a faint band of the expected size when tested by RT-PCR for the presence of CxFV RNA. Thirty F_1 from this mosquito were then tested by RT-PCR using CxFV-specific primers. Of these, nine mosquitoes yielded a strong band of the correct size, 14 yielded a faint positive band, and seven were negative. As a result of the ambiguous nature of these data, they were not included in Table 1 or used to calculate the overall TOT and FI rates. Had these data been included, they would have had a negligible affect on our findings; the overall FI rate would have been 96.3%, and the TOT rate would have remained at 100%.

To define the tissue tropism of CxFV, select tissues (salivary glands, ovaries, testes, head, fat bodies, and midguts) were removed from the remainder of the F_1 progeny produced from the field-collected *Cx. pipiens* and tested for CxFV RNA by RT-PCR. Actin-specific primers were included as positive controls. CxFV and actin RNA were detected in all tissues (Fig. 1). CxFV and/or actin RNA were not detected in the fat bodies and heads when >3 μg of total RNA was used in the reverse-transcription reactions (data not shown), but were detected when lower quantities of total RNA were used. These data suggest that fat bodies and heads contain dose-dependent inhibitory factors for the enzymatic activity of reverse transcriptase and/or *Taq* polymerase.

To further investigate the TOT potential of CxFV, laboratory-colonized adult female *Cx. pipiens* were infected with CxFV by needle inoculation and transferred to a cage with uninfected adult males. Eight days later, mosquitoes were offered a blood meal, and engorged females were transferred to individual oviposition cartons. RT-PCR analysis confirmed that all 30 F_0 females that produced viable egg rafts were positive for CxFV RNA. A total of 950 F_1 progeny from the CxFV RNA-positive F_0 mosquitoes was tested either individually or in pools of five by RT-PCR using CxFV and actin-specific primers. All F_1 progeny were negative for CxFV RNA, but positive for actin RNA (data not shown). To establish whether these mosquitoes were refractory to TOT because the virus was unable to disseminate to their ovaries, a second cohort of laboratory-colonized adult female *Cx. pipiens* was infected with CxFV by needle inoculation and held for 4, 6, 8, or 12 d. Ovaries were removed and tested by RT-PCR using CxFV-specific primers. CxFV RNA was detected in all samples (Fig. 2), demonstrating that CxFV can disseminate to the ovaries within 4 d when administered by the needle route.

Additional experiments were performed using fourth laboratory-generation mosquitoes derived from one of the eight uninfected, field-collected *Cx. pipiens* that oviposited viable egg rafts. Briefly, 100 F_4 adult females were infected with CxFV by needle

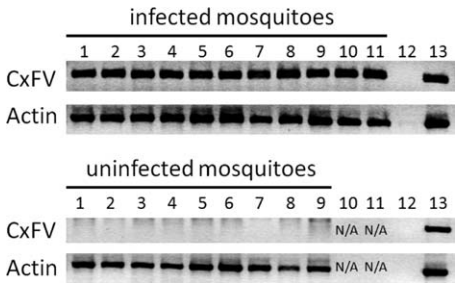

Fig. 1. Tissue tropism of CxFV in *Cx. pipiens.* Total RNA was extracted from female and male whole bodies (lanes 1 and 2), female and male midguts (lanes 3 and 4), female and male fat bodies (lanes 5 and 6), female and male heads (lanes 7 and 8), ovaries (lane 9), testes (lane 10), and female salivary glands (lane 11), and assayed by RT-PCR using CxFV and actin-specific primers. Negative and positive control RT-PCRs were included in lanes 12 and 13, respectively. These experiments were performed using F_1 adults from field-collected CxFV RNA-positive *Cx. pipiens* (top panel) and uninfected laboratory-colonized adult mosquitoes (bottom panel), although reproductive organs were not dissected from the latter (denoted as N/A).

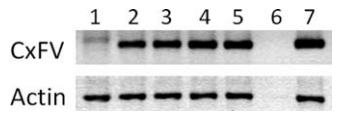

Fig. 2. Time-course analysis of CxFV dissemination to the ovaries of infected *Cx. pipiens.* Laboratory-colonized mosquitoes were infected with CxFV by needle inoculation and held for 4, 6, 8, or 12 d (lanes 2–5, respectively). Ovaries were dissected, and total RNA was extracted and assayed using RT-PCR using CxFV and actin-specific primers. Ovaries from uninfected laboratory-colonized mosquitoes were also tested (lane 1). Negative and positive control RT-PCRs were included (lanes 6 and 7).

inoculation. Eleven of these mosquitoes produced viable egg rafts. Subsequent analysis of the parental mosquitoes by RT-PCR using CxFV-specific primers revealed that nine produced strong positive bands, whereas two produced weak positive bands (data not shown). A subset of adult progeny, from the nine adults that yielded strong positive bands, was assayed in pools of five for CxFV RNA. Two F_4 (denoted as K2 and K9) generated CxFV RNA-positive progeny. Five of the 10 pools derived from K2 were positive, as were all 10 pools from K9. All 63 progeny from the remaining seven F_4 were negative for CxFV RNA. Thus, the extremely high TOT rate observed in the initial studies was not duplicated with the laboratory-colonized needle-inoculated mosquitoes, as the TOT rate for CxFV by this cohort of mosquitoes was 22.2%.

Discussion

The current study provides evidence that efficient transovarial transmission of CxFV by *Cx. pipiens* occurs in the field: the FI and TOT rates for CxFV in naturally infected adult female mosquitoes were 97.4 and 100%, respectively. These values are considerably greater than the <1% FI, and vertical infection rates typically reported in mosquitoes infected with flaviviruses that possess the capacity to replicate in both vertebrates and mosquitoes (Rosen et al. 1978, Aitken et al. 1979, Beaty et al. 1980, Kay and Carley 1980, Tesh 1980, Francy et al. 1981, Hardy et al. 1984). The detection of CxFV RNA in the ovaries of infected *Cx. pipiens* is not surprising in context of the above CxFV TOT data because successful dissemination of the virus to the ovarioles and follicles is required for TOT to occur. Flaviviruses that cycle between vertebrates and mosquitoes, however, rarely disseminate to the ovaries of mosquitoes, consistent with the inefficient rate at which they are vertically transmitted by their arthropod vectors (Turell 1988, Girard et al. 2004, Zhang et al. 2010). Although we have assumed that our findings provide evidence of efficient TOT, it is possible (albeit unlikely) that the high infection rate was instead because of another form of vertical transmission, such as transovum transmission. To provide more conclusive evidence that CxFV is maintained in nature by efficient TOT, future experiments should investigate whether CxFV persistently infects the germline tissues in the ovaries. The efficiency by which *Cx. pipiens* vertically transmits CxFV does not preclude the possibility that the virus is also amplified in the field by other modes of transmission. Indeed, horizontal (i.e., venereal) and/or mechanical (i.e., per os) transmission of CxFV could also occur in nature. In this regard, larval and adult *Aedes aegypti* (L.) are susceptible to KRV infection per os (Lutomiah et al. 2007). Future studies should be performed to identify other routes used by CxFV to infect mosquitoes.

Lutomiah et al. (2007) recently demonstrated vertical transmission of KRV in laboratory-colonized *Ae. aegypti.* In these experiments, female mosquitoes were exposed to KRV by artificial blood meal, subjected to single-pair mating, and allowed to oviposit. Thirteen KRV-infected F_0 females were identified. The FI rate in the F_1 produced by these infected mosquitoes after the second and third ovarian cycles was 3.9%. The TOT rate was not reported. One likely explanation for the dramatically lower FI rate in the above study as compared with the FI rate of 97.4% reported in this work is that there is no direct evidence to indicate the *Ae. aegypti* is a natural host of KRV. This virus has only been isolated from *Ae. macintoshi* in the field (Sang et al. 2003), and vertical transmission is presumably more efficient in the natural mosquito host. The lower FI rate could also be attributed to the different method used to assay the F_1 (e.g., virus isolation) or because laboratory-colonized mosquitoes were used. The aforementioned CxFV and KRV studies were performed with *Aedes* spp. mosquitoes from different subgenera, and therefore, the contrasting FI rates could also be the result of host differences.

Although our study demonstrated efficient TOT of CxFV by naturally infected *Cx. pipiens*, this virus was not detected in the F_1 of any laboratory-colonized mosquitoes infected by needle inoculation. One explanation for the different TOT rates between the naturally and experimentally infected mosquitoes could be that the latter mosquitoes are refractory or less susceptible to TOT as a consequence of their long-term maintenance under laboratory conditions. This could explain the intermediate TOT rate (22.2%) reported in the experiments performed with mosquitoes maintained in the laboratory for only four generations. Alternatively, a subset of mosquitoes, including the majority of the mosquitoes used to establish the short-term (fourth generation) and long-term laboratory colonies, could naturally possess an ovarian escape barrier that renders them refractory to TOT. Another explanation is that mosquitoes with lifelong infections (i.e., vertically infected mosquitoes) may be more susceptible to TOT than mosquitoes infected as adults. For instance, vertical infections could cause long-term pathological manifestations in the ovaries during development that increase susceptibility to efficient vertical passage.

Mosquitoes inoculated with CxFV via the needle route could also be refractory or less susceptible to TOT by virtue of the artificial means by which they were infected. However, this is unlikely given that efficient TOT has been reported in studies performed using mosquitoes infected with bunyaviruses by nee-

dle inoculation (Tesh 1980, Turell et al. 1982, Chandler et al. 1990). It is also important to note that administration of CxFV via the needle route does not appear to inhibit viral dissemination to the ovaries, as demonstrated by the detection of CxFV RNA in these tissues as early as 4 d postinoculation. However, it is possible, albeit unlikely, that the RT-PCR results were because of trace amounts of neighboring infected tissue removed with the ovaries rather than successful viral dissemination to the ovaries. Detailed immunohistochemistry studies of needle-inoculated versus F_1 vertically infected mosquitoes using CxFV-specific antibodies will need to be conducted to assess this theory further. The number of gonotrophic cycles completed by mosquitoes can greatly influence their ability to transovarially transmit virus (Miller et al. 1979, Francy et al. 1981, Anderson et al. 2008). For example, La Crosse virus FI rates of 0, 43, and 58% were reported in *Ae. triseriatus* after the first, second, and third ovarian cycles, respectively (Miller et al. 1979). However, this does not explain the differential TOT rates reported in this work because the field and laboratory mosquitoes received equal numbers of blood meals. Nevertheless, it is certainly feasible that TOT of CxFV could have occurred with the laboratory mosquitoes had they been provided with more than one blood meal.

The tissue tropism experiments revealed the presence of CxFV RNA in all of the mosquito organs examined, suggesting that CxFV establishes a systemic infection in the mosquito host. The detection of CxFV RNA in the salivary glands of infected mosquitoes is intriguing because, as a result of the inability of this virus to infect vertebrates, establishment of a salivary gland infection does not appear necessary for the virus to persist in nature. These findings imply that the viral genetic determinants needed for vertebrate-mosquito flaviviruses to disseminate to the salivary glands of their mosquito vectors have been maintained by viruses in the insect-specific lineage. Recently, Kent et al. (2010) demonstrated that CxFV is not secreted into the saliva of infected *Cx. quinquefasciatus* Say. These data, together with our findings, could indicate that CxFV replicates poorly in the salivary glands of infected *Culex* spp. mosquitoes, thereby resulting in viral titers that do not support efficient secretion into the saliva. Another explanation is that *Culex* spp. mosquitoes possess a salivary escape barrier that inhibits the secretion of CxFV into the saliva. Interestingly, however, CxFV was present in the saliva of mosquitoes coinfected with CxFV and WNV (Kent et al. 2010), which implies that, under certain conditions, the potential salivary escape barrier can be overcome. Kent et al. (2010) also reported significantly higher WNV transmission rates in mosquitoes infected with both viruses as compared with mosquitoes infected with WNV alone. The potential for exacerbated interactions between WNV and CxFV has been further demonstrated by a study performed in Illinois in which WNV-positive *Cx. pipiens* pools were four times more likely to be infected with CxFV than WNV-negative pools from the same area, and 40% of individual WNV-infected mosquito pools were also CxFV positive (Newman et al. 2011). Thus, despite the apparent inability of CxFV to replicate in vertebrates, this virus could have an indirect negative impact on human and animal health by enhancing transmission of coinfecting viruses, thereby highlighting the important need to further understand the mechanisms by which CxFV is maintained in mosquito populations.

In summary, we provide evidence that efficient TOT of CxFV occurs in naturally infected *Cx. pipiens.* It is likely that other insect-specific flaviviruses use the same strategy to persist in nature, but additional research will be required to directly address this issue. Future studies should also investigate whether other forms of transmission are used by CxFV to persist in nature.

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