



## Transmission dynamics of an insect-specific flavivirus in a naturally infected *Culex pipiens* laboratory colony and effects of co-infection on vector competence for West Nile virus

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### ABSTRACT

We established a laboratory colony of *Culex pipiens* mosquitoes from eggs collected in Colorado and discovered that mosquitoes in the colony are naturally infected with *Culex flavivirus* (CxFV), an insect-specific flavivirus. In this study we examined transmission dynamics of CxFV and effects of persistent CxFV infection on vector competence for West Nile virus (WNV). We found that vertical transmission is the primary mechanism for persistence of CxFV in *Cx. pipiens*, with venereal transmission potentially playing a minor role. Vector competence experiments indicated possible early suppression of WNV replication by persistent CxFV infection in *Cx. pipiens*. This is the first description of insect-specific flavivirus transmission dynamics in a naturally infected mosquito colony and the observation of delayed dissemination of superinfecting WNV suggests that the presence of CxFV may impact the intensity of enzootic transmission of WNV and the risk of human exposure to this important pathogen.

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### Introduction

The genus *Flavivirus* (family *Flaviviridae*) comprises over 70 single-stranded, positive-sense RNA viruses, most of which are transmitted to vertebrates by arthropod vectors. Flaviviruses have a genome of approximately 11 kb that contains a single open reading frame encoding a polyprotein that is co- and post-translationally processed to produce three structural proteins and seven nonstructural proteins. Many flaviviruses are human and veterinary pathogens, causing considerable morbidity and mortality associated with febrile illness, hemorrhagic fevers, and encephalitides. Among flaviviruses causing human disease are West Nile virus (WNV), dengue virus, and Japanese encephalitis virus. The flaviviruses have been divided into three main ecological groups: mosquito-borne, tick-borne, and no-known-vector (Heinz et al., 2000). The mosquito-borne flaviviruses have been further subdivided based on the main vector genus, with the *Culex*-borne viruses often associated with encephalitic disease in humans and the *Aedes*-borne viruses with hemorrhagic disease (Gaunt et al., 2001). Another group, termed insect-specific flaviviruses, consists of viruses that replicate only in invertebrate

cells, in contrast to arthropod-borne (arbo)viruses that replicate in both vertebrate and arthropod cells (Cook and Holmes, 2006), and are antigenically unrelated to other flaviviruses (Gritsun and Gould, 2006). Recent isolations of insect-specific flaviviruses in numerous mosquito species suggest that these viruses are widespread in nature.

Cell fusing agent virus (CFAV) was the first insect-specific flavivirus characterized (Stollar and Thomas, 1975). It was isolated from an *Aedes aegypti* mosquito cell culture line and has recently been isolated from field-caught mosquitoes in Puerto Rico (Cook et al., 2006). Other viruses tentatively placed in the insect-specific flavivirus group include Kamiti River virus (KRV) (Crabtree et al., 2003; Sang et al., 2003), *Culex flavivirus* (CxFV) (Hoshino et al., 2007), Quang Binh virus (Crabtree et al., 2009), *Aedes flavivirus* (Hoshino et al., 2009), Nounané virus (Junglen et al., 2009), Lammi virus (Huhtamo et al., 2009), Nakiwogo virus (Cook et al., 2009), and Calbertado virus (Bolling et al., 2011; Tyler et al., 2011).

We isolated both *Culex flavivirus* and Calbertado virus from *Culex* mosquitoes collected during field studies conducted in northern Colorado from 2006 to 2007. Viral RNA sequences were detected in male as well as female mosquito pools. After the detection of these two insect-specific flaviviruses in local mosquitoes, all laboratory mosquito colonies at the Arthropod-borne and Infectious Diseases Laboratory (AIDL), Colorado State University, were tested with universal flavivirus primers by standard RT-PCR. A *Culex pipiens* laboratory colony established from egg raft collections in Fort Collins in 2005

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was found to be persistently infected with *Culex flavivirus*. In order to investigate the transmission dynamics of CxFV in the naturally infected laboratory colony, egg rafts, individual larvae, and individual adults were tested for CxFV RNA and horizontal transmission among larvae, venereal transmission and casual contact transmission among adults were examined. We compared the above-mentioned CxFV-persistently infected *Cx. pipiens* colony established from Fort Collins, CO collections (CPCO) with a *Cx. pipiens* colony established from mosquitoes collected in Iowa in 2002 (CPIA), which were not infected with CxFV.

Data are lacking on how insect-specific flaviviruses are maintained in nature and what effects they may have on arbovirus transmission. The goals of this study were to investigate how CxFV is maintained and transmitted in a naturally infected *Cx. pipiens* laboratory colony and whether persistent infection with CxFV alters vector competence of *Cx. pipiens* for WNV. The circulation of insect-specific flaviviruses in nature raises questions regarding possible interactions with arthropod-borne flaviviruses in vector populations (Crabtree et al., 2003). To explore the dynamics of co-infection with a persistent insect-specific flavivirus and an exogenously-acquired arthropod-borne flavivirus, we conducted studies in cultured C6/36 mosquito cells infected with CxFV and WNV. To further investigate potential interactions, we performed vector competence experiments by infecting the CPCO mosquitoes with WNV to determine the effects of persistent infection with CxFV on WNV infection, dissemination, and transmission.

## Results

### *Culex flavivirus* maintenance in *Cx. pipiens* populations

#### Vertical transmission

*Culex flavivirus* RNA was detected in egg rafts and individual larvae, adult males, and adult females from the CPCO mosquito colony, suggesting natural vertical transmission. Viral titers, determined as CxFV RNA genome equivalents, were variable within and among life stages (Fig. 1). There appeared to be a bimodal distribution, with many of the specimens containing low to moderate titers and a few from each group with very high titers. The titers for infected specimens ranged from 1.12 to 8.95 log<sub>10</sub> RNA copies per individual. The infection rates for each life stage were 100% (13/13) for egg rafts, 83% (15/18) for larvae, 96% (25/26) for adult males, 85% (22/26) for adult females, and 75% (18/24) for blood fed females.

#### Horizontal transmission among larvae

Three pans, each containing 15 first instar larvae from the CxFV-infected CPCO colony and 15 first instar larvae from the non-infected CPIA colony, were reared to fourth instar stage and tested for the presence of CxFV RNA by qRT-PCR. In pan 1, 11 individual larvae were CxFV-positive; in pan 2, 12 larvae were CxFV-positive; and in pan 3, 15 larvae were CxFV-positive. All larvae were alive when they were removed from the rearing pans for testing. Based on this experiment performed in triplicate, combining CxFV-infected and uninfected larvae did not result in virus transmission. To further investigate the possibility of horizontal CxFV transmission between larvae, water was collected from CPCO colony pans containing approximately 100 larvae per pan. RNA was extracted from 1 ml aliquots of water from six larval pans and all tested negative for CxFV RNA by qRT-PCR. These data further suggest that horizontal transmission among larvae is unlikely to contribute to the maintenance of CxFV in natural populations.

#### Venereal transmission

Newly eclosed CPCO male mosquitoes (n = 40) and CPIA female mosquitoes (n = 42) were combined in a single cage. After 20 days, all females were found to be inseminated. Mosquitoes were then

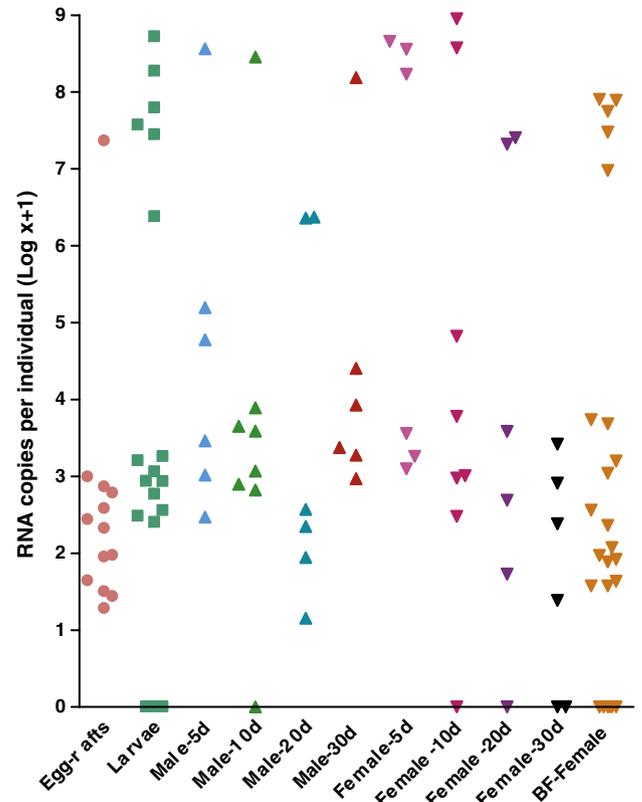


Fig. 1. *Culex flavivirus* titers, shown as log<sub>10</sub> RNA copies per individual, for different life stages of *Culex pipiens* (Colorado colony, naturally infected with CxFV). Titers were determined by qRT-PCR, using CxFV-specific primers. Specimens were tested individually, except for eggs, which were tested as rafts (BF = blood-fed).

tested individually for CxFV RNA by qRT-PCR and one CPIA female (2.4%) was found to be infected. The reverse experiment was also performed with CPCO female mosquitoes (n = 39) and CPIA males (n = 38) combined in a single cage. Again, all females were found to be inseminated after 20 days. Mosquitoes were processed individually for CxFV RNA by qRT-PCR and 2 CPIA males (5.3%) tested positive. Based on these observations, it appears that venereal transmission may play a minor role in CxFV maintenance.

#### Contact transmission

Male mosquitoes from the CPCO (n = 44) and CPIA (n = 40) colonies were combined in a single cage for 20 days. Female mosquitoes from the CPCO (n = 41) and CPIA (n = 29) colonies were also combined into a single cage for 20 days. Total RNA was extracted from individual mosquitoes and tested by qRT-PCR with CxFV primers. Twenty-eight males tested positive for CxFV RNA from the male contact transmission cage and 28 females tested positive for CxFV RNA from the female contact transmission cage. The number of CxFV-positive mosquitoes in each cage did not exceed the number of CPCO colony mosquitoes added, suggesting that contact transmission did not take place between adults of the same sex, based on our detection methods.

#### Vector competence studies

##### Viral interference in cell culture

*Aedes albopictus* (C6/36) cells were infected with CxFV at a MOI of 0.1 RNA copy/well and challenged 48 h later with WNV at two different MOIs: 0.1 and 0.01 pfu/well. *Culex flavivirus* and WNV titers in growth medium were determined by qRT-PCR and plaque assay in

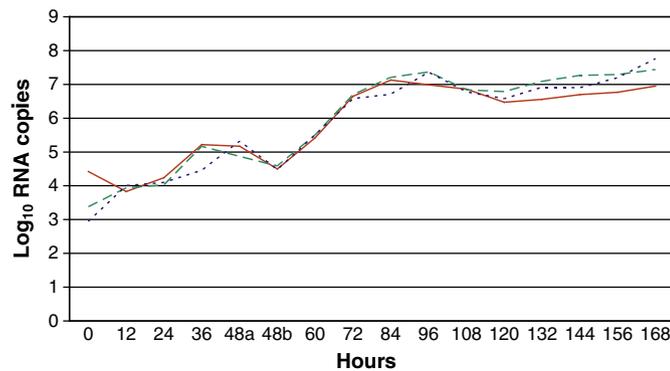
Vero cells, respectively. *Culex flavivirus* growth curves in cells co-infected 48 h later with WNV were similar to the CxFV growth curve in cells not co-infected with WNV (Fig. 2). The dip seen in the growth curves at the 48 h time point resulted from all growth media being removed and replaced (from both challenged and control cultures) at the time of WNV challenge. *Culex flavivirus* titers, expressed as RNA genome equivalents (ge), appeared to plateau around 7 log ge/ml by 84 h post infection. No significant differences were seen between CxFV co-infection growth rates and virus growth rates in cultures that were not co-infected with WNV.

West Nile virus growth curves in C6/36 cells infected 48 h earlier with CxFV (Fig. 3), were similar to the WNV growth curves in cells not co-infected with CxFV up to 84 h post-infection, but by 108 h post-infection the WNV titers in co-infected cultures were ~1 log lower than for cultures infected with WNV only. However, by 168 h post-infection, when the experiment was terminated, WNV titers in co-infected and solely-infected cultures were equivalent. Overall, WNV growth rates in cell cultures co-infected with CxFV were significantly lower compared to cultures infected with WNV alone (WNV MOI 0.01,  $p < 0.001$  and WNV MOI 0.1,  $p = 0.042$ ). Viral titers were also compared at each time-point using Wilcoxon rank-sum test with significant differences seen from as early as 60 h post-infection until 156 h post-infection (Fig. 3).

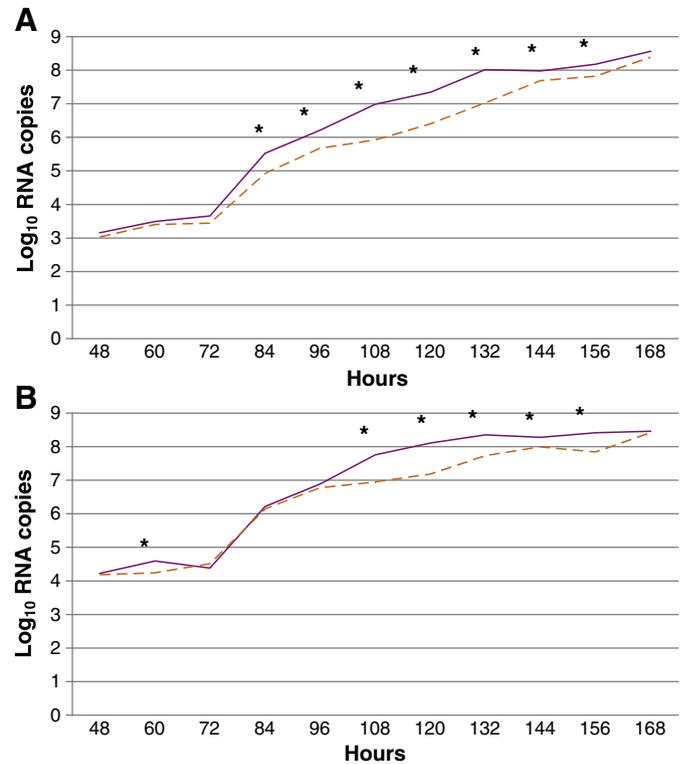
#### Viral interference in mosquitoes

West Nile virus infection rates in *Cx. pipiens* were compared between laboratory colonies CPCO (Colorado), which is persistently infected with CxFV, and CPIA (Iowa), which is not infected with CxFV. For the first experiment, a WNV infectious bloodmeal was administered to mosquitoes and after 14 days post-infection (dpi), 24 females from each colony were processed to determine infection rates. All 24 CPCO females were WNV RNA positive and 22/24 of the CPIA females were WNV RNA positive by qRT-PCR (Table 1). The WNV titers of the positive mosquitoes, determined as genome RNA genome equivalents and  $\log_{10}$  transformed, were compared between the two colonies (CPCO:  $6.58 \pm 0.43$ , CPIA:  $6.77 \pm 0.30$ ) and were not significantly different (Table 2) ( $p = 0.75$ , Student's two-tailed  $t$ -test).

For the second experiment, mosquitoes from each colony were processed to determine presence of WNV RNA at 7 and 14 dpi. Saliva from each female was tested to estimate transmission, and bodies



**Fig. 2.** *Culex flavivirus* growth curves in the absence or presence of West Nile virus superinfection. C6/36 cell cultures were infected with *Culex flavivirus* at a dose of 0.1 RNA copy/well and 48 h later with WNV at doses of 0.1 or 0.01 pfu/well. CxFV RNA in cell culture medium was titrated by qRT-PCR with CxFV-specific primers. *Culex flavivirus* growth curves in cells challenged with WNV were compared to those in cells infected with CxFV alone using linear regression and were not significantly different (48a = 48 h titer, pre-WNV challenge and 48b = 48 h titer, post-WNV challenge and medium change). Solid line, CxFV alone; dashed line, CxFV + WNV MOI = 0.1; dotted line, CxFV + WNV MOI = 0.01.



**Fig. 3.** West Nile virus growth curves in C6/36 cells in the absence or presence of co-infection with *Culex flavivirus*. C6/36 cells were infected with CxFV at a dose of 0.1 RNA copy/well and 48 h later were challenged with WNV at doses of 0.01 pfu/well (A) and 0.1 pfu/well (B). West Nile virus titers in cell culture medium were determined by plaque assay in Vero cells. West Nile virus growth curves in cells co-infected with CxFV were compared to growth curves in cells infected with WNV alone using linear regression and were significantly different (WNV MOI 0.01,  $p < 0.001$  and WNV MOI 0.1,  $p = 0.042$ ). Titters were also compared at each time-point using the Wilcoxon rank-sum test. Solid lines, WNV alone, MOI = 0.01 (A), MOI = 0.1 (B); dashed lines, WNV + CxFV. \*Statistically significant at  $\alpha \leq 0.05$ .

(thorax and abdomen) and legs, heads and wings (together) were tested to determine infection and dissemination rates, respectively. At 7 dpi, the WNV dissemination rate for CPIA (94%) was significantly higher ( $p = 0.04$ , Fisher's Exact test) compared to CPCO (72%). The infection rate for CPIA (97%) was also higher than the infection rate for CPCO (86%), but this difference was not significant ( $p = 0.18$ ). The estimated transmission rates were the same (3%) for females from CPCO versus CPIA at 7 dpi. At 14 dpi, there were no statistically significant differences in infection, dissemination, or transmission rates detected between the two colonies. The infection rate was slightly higher for CPIA (79%) compared to CPCO (71%). Dissemination rates were the same at 67% and the transmission rate for CPCO (21%) was higher than for CPIA (13%).

West Nile virus RNA titers for positive specimen samples were compared between colonies. At 7 dpi, the average titer for CPCO bodies (thorax and abdomen) was  $6.72 \log_{10}$  WNV RNA ge per mosquito, which was higher than the average titer for CPIA with  $5.94 \log_{10}$  ge per mosquito. The difference between CPIA and CPCO WNV body titers was, however, not significantly different ( $p = 0.08$ , Student's two-tailed  $t$ -test). The WNV RNA titers for dissemination in CPIA samples (legs, head, and wings) were significantly higher than CPCO dissemination ( $p = 0.01$ , Student's two-tailed  $t$ -test). The average titers were 3.35 and  $2.29 \log_{10}$  WNV RNA ge per mosquito for CPIA and CPCO, respectively. At 14 dpi, overall, WNV titers were higher in CPCO mosquitoes compared to CPIA mosquitoes, but these differences were not statistically significant (Table 2).

**Table 1**

Infection, dissemination, and transmission rates for West Nile virus in *Culex pipiens* mosquitoes. CPCO (*Culex pipiens*-Colorado, naturally infected with *Culex* flavivirus) and CPIA (*Culex pipiens*-Iowa, not infected with *Culex* flavivirus) colonies were administered a West Nile virus infectious blood meal. Infection (abdomen and thorax), dissemination (legs, head, and wings), and transmission (saliva) rates for WNV were determined by qRT-PCR and compared between the colonies by Fisher's Exact test.

	Experiment 1		Experiment 2			Experiment 2		
	14 dpi		7 dpi			14 dpi		
	Infection		Infection	Dissemination	Transmission	Infection	Dissemination	Transmission
CPCO	100% (24/24)		86% (25/29)	72% (21/29)	3% (1/29)	71% (17/24)	67% (16/24)	21% (5/24)
CPIA	92% (22/24)		97% (32/33)	94% (31/33)	3% (1/33)	79% (19/24)	67% (16/24)	13% (3/24)
p-value	0.49		0.18	0.04 <sup>a</sup>	1.00	0.74	1.00	0.70

<sup>a</sup> Statistically significant at  $\alpha \leq 0.05$ .

**Table 2**

West Nile virus titers (mean log<sub>10</sub> RNA genome equivalents per mosquito  $\pm$  standard error) in *Culex pipiens* mosquitoes. CPCO (*Culex pipiens*-Colorado, naturally infected with *Culex* flavivirus) and CPIA (*Culex pipiens*-Iowa, not infected with *Culex* flavivirus) colonies were administered a West Nile virus infectious blood meal. Bodies (abdomen and thorax), legs (with head and wings), and saliva samples were tested for WNV RNA and titers were compared between the colonies by the Student's two-tailed *t*-test.

	Experiment 1		Experiment 2			Experiment 2		
	14 dpi		7 dpi			14 dpi		
	Bodies		Bodies	Legs	Saliva	Bodies	Legs	Saliva
CPCO	6.58 $\pm$ 0.43		6.72 $\pm$ 0.28	2.29 $\pm$ 0.25	0.22 $\pm$ 0.00 <sup>a</sup>	7.18 $\pm$ 0.54	5.24 $\pm$ 0.62	2.98 $\pm$ 0.27
CPIA	6.77 $\pm$ 0.30		5.94 $\pm$ 0.32	3.35 $\pm$ 0.25	0.29 $\pm$ 0.00 <sup>a</sup>	6.20 $\pm$ 0.51	4.52 $\pm$ 0.56	2.53 $\pm$ 0.17
p-value	0.75		0.08	0.01 <sup>b</sup>	n/a	0.19	0.40	0.29

<sup>a</sup> One saliva sample from each colony was WNV-positive, so these titers were not compared.

<sup>b</sup> Statistically significant at  $\alpha \leq 0.05$ .

## Discussion

It is important to understand the dynamics of vector-borne pathogen systems in nature in order to make efficient use of arbovirus surveillance and vector control resources. The isolation and description of numerous insect-specific flaviviruses in recent years has prompted interest in how these viruses may interact with pathogenic arboviruses (which replicate in both mosquitoes and vertebrates) in vector mosquitoes.

### *Culex flavivirus* maintenance in *Cx. pipiens* populations

We detected CxFV RNA in a majority of egg rafts and individual larvae, adult males and adult females from a *Cx. pipiens* colony established from egg rafts collected in Fort Collins, Colorado, in 2005. Detection of insect-specific flavivirus RNA in all life stages, including adult mosquitoes of both sexes, suggested vertical transmission as an important mechanism of viral maintenance in nature, as has been shown for other insect-only flaviviruses (Cook et al., 2006). Similarly, KRV, another insect-specific flavivirus, was first isolated from *Aedes macintoshi* larvae and pupae collected from flooded dambos in Kenya (Sang et al., 2003). Additional studies of oral exposure to KRV of laboratory reared *A. aegypti* mosquitoes indicated that vertical transmission can occur (Lutomiah et al., 2007). *Culex* flavivirus, first isolated during a field survey of mosquito-borne viruses in Japan, was detected in both adult males and females (Hoshino et al., 2007). *Aedes* flavivirus, another insect-specific flavivirus isolated by the same researchers in Japan, was also found in male and female adult mosquitoes (Hoshino et al., 2009). These data and our findings support the idea that vertical transmission plays an important role in the maintenance of insect-specific flaviviruses in nature.

*Culex* flavivirus RNA titers in individual CPCO colony mosquitoes revealed a bimodal pattern (Fig. 1), with the majority of RNA copy numbers in individuals ranging between 2 and 4 logs and several individuals within each life stage reaching as high as 9 log ge.

### Horizontal transmission among larvae

Transmission experiments were conducted to investigate viral maintenance mechanisms other than vertical transmission. The potential for horizontal transmission among larvae was examined by combining first instar larvae from CPCO (CxFV-positive) and CPIA (CxFV-negative) colonies and rearing to fourth instar larvae in a shared pan. Although they could not be differentiated, we tested larvae individually for CxFV RNA by qRT-PCR and found no evidence of CxFV transmission between infected and uninfected larvae. Additionally, CxFV RNA was not detected in water samples from CPCO colony rearing pans each containing approximately 100 larvae in 500 ml. These findings suggest that although larvae from the CPCO colony are infected with CxFV, larval horizontal transmission does not play a role in viral maintenance. This was not unexpected, as flaviviruses are unlikely to be stable in water.

### Venereal and contact transmission

Adult mosquitoes from CPCO and CPIA colonies were allowed to mate in order to investigate the possibility of venereal transmission. One out of 42 (2.4%) CPIA females tested positive for CxFV RNA by qRT-PCR after mating with CPCO males. Venereal transmission of arthropod-borne flaviviruses from male to female *Culex* spp. mosquitoes has been documented (Nayar et al., 1986; Shroyer, 1990), but occurs at a low frequency. Surprisingly, 2 out of 38 (5.3%) CPIA males were positive for CxFV after mating with CPCO females. There are no previous reports of venereal transmission of flaviviruses from female to male mosquitoes. Studies with dengue virus in *A. albopictus* (Rosen, 1987) showed that experimentally infected females did not transmit their infection sexually to males. It is possible that the CPIA males from our experiments became infected with CxFV by other means than sexual transmission, such as sharing food sources with CxFV-positive females. To test this possibility, we also initiated contact transmission studies, where CPCO males were combined with CPIA males and CPCO females were combined with CPIA

females. The results of these experiments suggest that CxFV transmission did not occur between same-sex mosquitoes while sharing cage space and food sources. These data support our findings that transmission from CxFV-positive females to uninfected males was venereal. Further studies are warranted to confirm these observations.

#### *Viral interference in cell culture*

We investigated possible interactions between CxFV and WNV during co-infection in mosquito cell cultures and adult female mosquitoes. Overall, WNV growth curves in C6/36 cells co-infected with CxFV were significantly lower than WNV growth curves in single-virus infected cells (Fig. 3). Differences in WNV titers were seen between 84 and 156 h post infection, which may represent interference between CxFV and WNV during this early time period. By 168 h post infection, WNV titers in all C6/36 cell cultures had reached approximately  $8.5 \log_{10}$  pfu/ml, regardless of co-infection status. Interestingly, differences seen at earlier time-points were reflected in our vector competence study. Similar experiments in C6/36 cells co-infected with CxFV (Izabal strain) and WNV also resulted in lower WNV titers compared to WNV alone, but the difference was not statistically significant (Kent et al., 2010). Additional experiments are needed to further explore possible mechanisms of apparent CxFV interference with WNV growth in cell cultures. Within-host interaction among arboviruses varies depending on the biological system being investigated (Pepin et al., 2008). Some systems demonstrate superinfection exclusion, where a cell infected with one virus cannot be productively infected with the same or a closely-related virus. For example, *A. albopictus* cells persistently infected with Sindbis virus were refractory to infection with homologous strains of Sindbis and other heterologous alphaviruses (Eaton, 1979; Karpf et al., 1997). Competitive suppression has been documented between dengue virus serotypes 2 and 4, where replication of both viruses was suppressed in superinfected C6/36 cells (Pepin et al., 2008). Experiments with C6/36 cells persistently infected with *A. albopictus* densovirus suggested that viral suppression was occurring, as DENV2 titers and infected cell numbers were significantly lower in superinfected cell cultures compared to naïve cell cultures (Burivong et al., 2004). It would be informative to perform future studies with CxFV in a *Culex* cell line, as opposed to C6/36 (*A. albopictus*) cells, as the virus appears to be exclusively associated with *Culex* species mosquitoes in nature (Blitvich et al., 2009; Cook et al., 2009; Farfan-Ale et al., 2009; Hoshino et al., 2007; Kim et al., 2009; Morales-Betoulle et al., 2008) and C6/36 cells have a defective innate immune response to flavivirus infections (Scott et al., 2010; Brackney et al., 2010). It should be noted that *in vitro* co-infection experiments are inherently different from *in vivo* experiments, as tissues of whole organisms (mosquitoes) have different innate immune responses to viral infections from some cultured mosquito cells (Scott et al., 2010), which may result in variable data outcomes.

#### *Viral interference in mosquitoes*

Vector competence for WNV was examined in the two *Cx. pipiens* laboratory colonies, CPCO (*Cx. pipiens*-Colorado, CxFV-positive) and CPIA (*Cx. pipiens*-Iowa, CxFV-negative). Mosquitoes were administered a WNV infectious bloodmeal and infection, dissemination, and transmission rates were compared at 7 and 14 dpi (Table 1). A significant difference was detected between the two colonies in the WNV dissemination rate at 7 dpi with the rate in CPIA being significantly higher than in CPCO. West Nile virus RNA levels were also significantly different between the two colonies at 7 dpi (Table 2), with mean titers for CPIA being higher than for CPCO. To further examine possible interactions between CxFV and WNV in *Cx. pipiens*, CxFV and WNV titers ( $\log_{10}$  RNA copies per mosquito) were analyzed by correlation analysis (data not shown), with no evidence of a linear

relationship. Kent et al. (2010) investigated vector competence for WNV of *Culex quinquefasciatus* mosquitoes intrathoracically injected with CxFV (Izabal strain) 7 d prior to receiving a WNV infectious blood meal, and detected no significant differences in WNV titers between CxFV-positive and CxFV-negative mosquitoes at 14 d post WNV infection. The significant differences we detected in dissemination rates at 7 dpi had disappeared at 14 dpi, as seen with the CxFV (Izabal strain) study. The results from our vector competence study suggest a competitive interaction between CxFV and WNV early in WNV infection, as the arbovirus is amplified in the midgut and begins to disseminate. It should be noted that the *Cx. pipiens* colonies used for these experiments are from different geographic locations (Colorado and Iowa), so differences detected in vector competence for WNV could be due to factors other than co-infection with CxFV. Vector competence studies for dengue virus conducted in *A. aegypti* mosquitoes collected from different geographic regions in Mexico revealed infection rates ranging from 24% to 83%, perhaps resulting from genetic differences between mosquito populations (Bennett et al., 2002). It should also be taken into consideration that laboratory colonization can affect the genetic diversity and fitness of a population, which may result in altered vector competence profiles compared to freshly collected specimens (Lambrechts et al., 2010; Lorenz et al., 1984). If extrapolated to a mosquito-borne virus transmission cycle, the difference we observed between dissemination rates at 7 dpi could result in an increase in the extrinsic incubation period for WNV in vectors coinfecting with CxFV. There was, however, no difference in transmission rates between the two colonies at 7 dpi and the transmission rates at 14 dpi were not significantly different, so the overall impact on vector competence is unclear at this time.

The significant differences seen in our study could indicate that the presence of CxFV suppresses WNV replication in mosquitoes up to 7 dpi. Potential mechanisms of suppression could include an activated mosquito innate immune response or competition for essential cellular factors. The differences in dissemination rates seen in our study at 7 dpi seem to contrast with results from field collections in Chicago, Ill., which revealed a positive correlation between WNV and CxFV infection rates, with a four-fold increased likelihood of CxFV infection of WNV-positive mosquito pools compared to WNV-negative pools (Newman et al., 2011). Another field study conducted in the Southeastern United States revealed no association between WNV and CxFV infection rates (Crockett et al., 2012). Further studies are needed to determine if there are consistent trends for WNV vector competence in CxFV-infected *Culex* spp. populations. An important factor to consider is that for within-host competition among viruses to occur, they likely must infect the same cells in the host (Pepin et al., 2008). The specific tissue tropisms of CxFV in naturally infected mosquitoes are unclear at this time, although viral RNA has been detected by RT-PCR in salivary glands, ovaries, testes, head, fat bodies and midguts from experimentally infected *Cx. pipiens* mosquitoes (Saiyasombat et al., 2011). With numerous insect-specific flaviviruses apparently causing persistent infections in natural populations worldwide, it will be important to investigate and determine mechanisms of interactions between these viruses and other viruses, particularly arboviruses transmissible to vertebrates.

## Materials and methods

### *Mosquitoes and isolation of CxFV*

The Colorado *Cx. pipiens* laboratory colony (CPCO) was established in 2005 from egg rafts collected along lake margins at Dixon Reservoir and Riverbend Ponds Natural Area in Fort Collins, Colorado. Egg rafts were hatched in the laboratory in individual pans and larvae were reared to fourth instar stage for species identification. Approximately thirty-five egg rafts were used to begin the *Cx. pipiens* laboratory colony. After the adults emerged into the cage, 20 individuals were removed and pooled by sex. Total RNA was extracted and tested negative for

WNV infection by standard RT-PCR using WNV-specific primers (Gubler et al., 2000; Lanciotti et al., 2000). After it was determined from 2006 to 2007 surveillance collections that two insect-specific flaviviruses were circulating in local mosquitoes, the *Cx. pipiens* colony was tested using universal flavivirus primers and found to be positive. The RNA originally isolated at the time of colony establishment was tested using CxFV-specific primers (Hoshino et al., 2007), resulting in a positive PCR product. A mosquito sample was removed from the CPCO colony in 2008 and triturated as previously described, passed through a 0.45 µm filter and the filtrate placed on C6/36 cells to attempt virus isolation. Four blind passages were completed and cell culture medium was harvested after each passage. Total RNA was extracted from cell culture medium aliquots using a QIAamp Viral RNA Minikit (Qiagen, Inc., Valencia, CA) and tested by standard RT-PCR using CxFV-specific primers. Medium was positive for CxFV RNA after all four passages, confirming a virus isolate from the CO *Cx. pipiens* colony.

The Iowa *Cx. pipiens* colony (CPIA) was established from egg rafts sent from a laboratory colony at Iowa State University in 2002. Adult mosquitoes from the Iowa colony have consistently tested negative for the presence of CxFV RNA by standard RT-PCR. All *Culex* colonies are maintained at 27 °C, 75% relative humidity, with a photocycle of 16:8 (light:dark).

#### Preparation of mosquito RNA

Total RNA was extracted from *Culex pipiens* mosquitoes at different life stages (individual egg rafts, larvae, and adults) using Trizol Reagent (Invitrogen, Carlsbad, CA). Briefly, individual specimens were homogenized in 500 µl of Trizol Reagent using a motorized pestle and microcentrifuge tube. RNA was extracted following the manufacturer's protocol and eluted in 20 µl of nuclease-free water (Ambion, Inc., Austin, TX). Samples were stored at –80 °C until virus RNA titration by quantitative one-step RT-PCR assay.

#### Quantitative real-time reverse transcription (qRT)-PCR to assay CxFV RNA

Primers were designed to target a 168 nt region of the NS5 gene for CxFV detection (CxFV-forward 5'-CTACGCTCTCAACACCGTGA-3', CxFV-reverse 5'-GTTGCCACAACACATCATC-3'). Primers used to quantify WNV targeted a 70 nt portion of the envelope gene (WNENV-forward 5'-TCAGCGATCTCTCCACAAAG-3', WNENV-reverse 5'-GGGTCAGCAGTTTGTGATTG-3') (Lanciotti et al., 2000). DNA standards were prepared by cloning PCR products into the pCR 2.1 plasmid with the TA cloning kit (Invitrogen). Plasmids were purified by the QIAprep Spin Miniprep kit (Qiagen, Inc.) and concentrations were measured spectrophotometrically. Plasmids were diluted to 10<sup>10</sup> copies/µl and 10-fold serial dilutions were used to construct standard curves, ranging from 10 to 10<sup>9</sup> copies. RNA samples were quantified using the Quantitect SYBR Green RT-PCR kit (Qiagen, Inc.) on a Bio-Rad iCycler iQ5 real-time PCR detection system (BioRad, Hercules, CA). Duplicate reactions were set up for each sample, containing 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), 3.8 µl of nuclease-free water (Ambion, Inc.), and 100 ng of template RNA. No template and uninfected mosquito RNA controls were included for each run. The thermal profile consisted of reverse transcription at 50 °C for 30 min, RT inactivation at 95 °C for 15 min, and 40 cycles of polymerase chain reaction at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Dissociation analysis was conducted to detect non-specific amplicons and primer dimers. To avoid the incorporation of non-specific fluorescence into 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 estimate initial copy numbers of viral genomes in the RNA samples.

#### Transmission studies

##### Vertical transmission

Egg rafts, fourth instar larvae, and adults were removed from the CPCO colony and tested individually for the presence of CxFV. Male and female adult mosquitoes were tested at 5, 10, 20, and 30 days post emergence. Bloodfed females, varying in age, were removed from the colony 24 h post noninfectious bloodmeal for testing. *Culex* flavivirus titers per individual were estimated as RNA copies using qRT-PCR.

##### Larval horizontal transmission

Fifteen 1st instar larvae from the CPCO colony were combined with 15 1st instar larvae from the CPIA colony into one plastic larval pan (15×25×15 cm), containing approximately 500 ml of tap water. This test was conducted in triplicate. Larvae were fed *ad libitum* with a mixture of fish food and rabbit food. Mosquitoes were reared to fourth instar larvae, removed from the pan and placed in ethanol at –80 °C. Larvae were processed individually to detect CxFV infection by qRT-PCR. Additionally, water samples (1 ml) were collected from CPCO larval pans in which approximately 100 larvae had been reared. Total RNA was extracted using Trizol Reagent (Invitrogen) and tested for the presence of CxFV RNA by qRT-PCR.

##### Venereal transmission

Mosquitoes from the CPCO and CPIA colonies were sexed and separated as pupae. After the adults emerged and sex determinations were confirmed, CPCO females (n=39) were placed in a 30×30×30 cm cage with CPIA males (n=38). In a separate cage, CPCO males (n=40) were combined with CPIA females (n=42). Mosquitoes were maintained for 20 d with water and sugar *ad libitum*, removed from cages, and females were dissected to determine insemination rates. Females were considered inseminated if sperm were observed in the spermathecae. Mosquitoes were tested individually for CxFV RNA by qRT-PCR using CxFV-specific primers.

##### Contact transmission

Mosquitoes from the CPCO and CPIA colonies were sexed and separated as pupae. After the adults emerged and sex determinations were confirmed, CPCO females (n=41) were placed in a 30×30×30 cm cage with CPIA females (n=29). In a separate cage, CPCO males (n=44) were combined with CPIA males (n=40). Mosquitoes were held for 20 d with access to water and sugar, then removed and stored at –80 °C. Mosquitoes were tested individually for CxFV RNA by qRT-PCR using CxFV-specific primers.

##### Vector competence

##### Viral interference in cell culture

*A. albopictus* (C6/36) cells were maintained at 28 °C in L-15 medium supplemented with 7% FBS, L-glutamine, nonessential amino acids and penicillin/streptomycin. The WNV was isolated from a pool of *Cx. tarsalis* mosquitoes collected in Fort Collins, CO in 2004. It had been passaged 5 times in Vero cells and had a titer of 1.0×10<sup>7</sup> pfu/ml, as determined by plaque assay in Vero cells. The CxFV had been isolated from *Cx. pipiens* mosquitoes in the CPCO laboratory colony and passaged 4 times in C6/36 cells, with a titer of 8.79×10<sup>7</sup> RNA ge/ml, as determined by qRT-PCR. Twelve-well plates of confluent C6/36 cells were inoculated with CxFV at a dose of 0.1 RNA copy/well and placed on a rocker for 1 h at room temperature. Two milliliters of medium were added to each well and plates were incubated at 28 °C. At 48 h post infection, medium was removed and cells were challenged with WNV at doses of 0.1 or 0.01 pfu/well. After 1 h at room temperature, inocula were removed, cells were washed with PBS, and fresh medium was added to each well. Aliquots of medium were removed for titration every 12 h for 5 d. Titers for CxFV were determined as

RNA copies by qRT-PCR and titers for WNV were determined by plaque assay in Vero cells. Growth curves were analyzed using linear regression, controlling for time and additionally, titers at each time-point were compared using the Wilcoxon rank sum test (Stata, Stata Statistical Software: Release 10, College Station, TX).

#### Viral interference in mosquitoes

Mosquitoes from the CPO and CPIA laboratory colonies were transferred to a BSL-3 environmental chamber as pupae and maintained at 28 °C with 75% relative humidity. At approximately 3–5 d after emergence, mosquitoes were offered WNV-infected cell culture medium, with a titer of  $1 \times 10^7$  pfu/ml, mixed 1:1 with defibrinated sheep blood (Colorado Serum Co., Boulder, CO) with 1 mM ATP. Engorged females were returned to the environmental chamber with access to sugar and water. In the first experiment, 24 females from each colony were processed to determine infection rates at 14 d post blood meal. For the second experiment, approximately 30 females were removed from each colony at 7 d post infection for processing, and 24 females from each colony were processed at 14 d post infection. Each specimen was assayed to determine WNV infection (body: thorax and abdomen), dissemination (head, 6 legs, and 2 wings), and transmission (saliva). Females were immobilized by cold, wings and legs were removed, and the proboscis was inserted into a capillary tube containing 5  $\mu$ l of immersion oil (Type B) for saliva collection. After 30 min of salivation, females were removed from the capillary tubes and heads were separated and placed in a microcentrifuge tube containing legs and wings. Bodies were placed in separate tubes. Immersion oil was expelled from the capillary tubes into 200  $\mu$ l MEM containing 2% FBS and mixed by vortexing for 30 s. Saliva samples were then centrifuged at 12,000 rpm for 10 min at 4 °C to allow separation of the immersion oil from the mixture. The medium containing the saliva was removed by pipetting and stored at –80 °C. Total RNA was extracted from each sample using Trizol reagent. All samples were tested for WNV RNA by qRT-PCR and all CPO mosquito samples were additionally tested for CxV RNA by qRT-PCR.

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