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Vertical and Horizontal Transmission of Cell Fusing Agent Virus in *Aedes aegypti*

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ABSTRACT Cell fusing agent virus (CFAV) is an insect-specific flavivirus (ISF) found in *Aedes aegypti* mosquitoes. ISFs have demonstrated the ability to modulate the infection or transmission of arboviruses such as dengue, West Nile, and Zika viruses. It is thought that vertical transmission is the main route for ISF maintenance in nature. This has been observed with CFAV, but there is evidence of horizontal and venereal transmission in other ISFs. Understanding the route of transmission can inform strategies to spread ISFs to vector populations as a method of controlling pathogenic arboviruses. We crossed individually reared male and female mosquitoes from both a naturally occurring CFAV-positive *Ae. aegypti* colony and its negative counterpart to provide information on maternal, paternal, and horizontal transmission. RT-PCR was used to detect CFAV in individual female pupal exuviae and was 89% sensitive, but only 42% in male pupal exuviae. This is a possible way to screen individuals for infection without destroying the adults. Female-to-male horizontal transmission was not observed during this study. However, there was a 31% transmission rate from mating pairs of CFAV-positive males to negative female mosquitoes. Maternal vertical transmission was observed with a filial infection rate of 93%. The rate of paternal transmission was 85% when the female remained negative, 61% when the female acquired CFAV horizontally, and 76% overall. Maternal and paternal transmission of CFAV could allow the introduction of this virus into wild *Ae. aegypti* populations through male or female mosquito releases, and thus provides a potential strategy for ISF-derived arbovirus control.

IMPORTANCE Insect-specific flaviviruses (ISFs), are a group of nonpathogenic flaviviruses that only infect insects. ISFs can have a high prevalence in mosquito populations, but their transmission routes are not well understood. The results of this study confirm maternal transmission of cell fusing agent virus (CFAV) and demonstrate that paternal transmission is also highly efficient. Horizontal transmission of CFAV was also observed, aided by evaluation of the pupal infection status before mating with an infected individual. This technique of detecting infection in discarded pupae exuviae has not been evaluated previously and will be a useful tool for others in the field of studying viral transmission in mosquitoes. Identifying these routes of transmission provides information about how CFAV could be maintained in wild populations of mosquitoes and can aid future studies focusing on interactions of CFAV with their hosts and other viruses that infect mosquitoes.

KEYWORDS cell fusing agent virus, flavivirus, insect-specific flavivirus, insect-specific virus, mosquito, virus transmission

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The genus *Flavivirus* contains many arboviruses of medical and veterinary importance such as dengue, West Nile, and Zika viruses, as well as insect-specific flaviviruses (ISFs) that are only known to infect insect hosts. ISFs have been detected in a range of mosquito species and we are becoming increasingly aware of their interactions with other mosquito viruses because of recent reports evaluating their potential to modulate arbovirus infection or transmission in mosquitoes (1–9) and their use as chimeric vaccines (10, 11).

While the *Flavivirus* genus contains both arboviruses and ISFs, transmission is markedly different. Arboviruses are dual-host flaviviruses transmitted through the blood-feeding of arthropods on viremic vertebrate hosts. After the acquisition of the virus from the host, only a small fraction of subsequent transmission is vertical, where the female mosquito passes the virus to its offspring after feeding on an infected host (12–16). In contrast, vertical transmission is thought to be the main route of ISF transmission.

A small number of studies have observed vertical transmission of ISFs in naturally and experimentally infected mosquito colonies (9, 17–21), including for cell fusing agent virus (CFAV), an ISF identified in *Aedes aegypti* cell culture lines (22), field (23, 24) and laboratory colony mosquitoes (25), as well as in field-caught *Aedes albopictus* and *Culex* mosquitoes (26–28). The observed vertical transmission rate was higher in naturally infected colonies of *Ae. aegypti* with CFAV compared to experimentally infected colonies (19), and similar results were seen in *Culex pipiens* with Culex flavivirus (CxFV) (20). Filial infection rate – the percentage of offspring that were infected through vertical transmission from an infected parent – from experimentally infected females ranged from 0 to 50% for CFAV in *Ae. aegypti*, and 0 to 22% for CxFV in *Cx. pipiens*. For Kamiti River virus (KRV), an ISF isolated from *Aedes macintoshi*, the filial infection rate from *Ae. aegypti* females following an infectious blood meal was 4% (21). The disparate range in these experiments suggests that other forms of transmission may also occur because the individual modes of transmission do not account for the prevalence seen in naturally infected populations. ISFs can also infect male and female reproductive tissues, and salivary glands (1, 3, 20, 29), which can implicate additional vertical and horizontal transmission routes.

Horizontal transmission of ISFs has also been observed in laboratory mosquito colonies. KRV was able to infect a high proportion of *Ae. aegypti* larvae when exposed to infected cell culture (21), while Aedes flavivirus (AeFV) only infected a low proportion of *Ae. aegypti* larvae and adults when feeding on infected cell cultures and sugar meals, respectively (17). However, the virus was not detected in water used to rear CxFV-infected *Cx. pipiens* larvae and infection was not detected in coreared, negative larvae (9), suggesting infected individuals did not shed virus into their larval environment. Venereal transmission of CxFV and AeFV was demonstrated in both directions, from male-to-female as well as female-to-male, in experiments that crossed infected mosquito colonies with naive colonies (9, 17). These rates were generally low, except for male-to-female crosses in *Ae. albopictus*, which led to an 18% infection rate (17).

Further knowledge of the transmission and maintenance of ISFs in mosquito populations is of high relevance, particularly in the context of interactions with pathogenic arboviruses. CFAV infects important vector species and its relation to many human-pathogenic flaviviruses may allow it to be used to control arbovirus transmission through superinfection exclusion – blocking subsequent infection of a similar virus – (2) or as a vehicle for paratransgenesis – using a microbe to express transgenes in its host – as has been proposed for other insect-specific viruses (30–34). CFAV is maternally transmitted with experimentally infected female mosquitoes (19), but it is not known if CFAV is paternally or horizontally transmitted. Given the reduced rates of transmission seen in experimental infections, we hypothesized that multiple modes of transmission occur in naturally infected colonies. To assess this, we used a laboratory colony of CFAV-infected *Ae. aegypti* and a known uninfected colony to quantify maternal, paternal, and horizontal transmission of CFAV. Our results provide insights into the transmission routes of CFAV which could be used to inform strategies to spread pathogen-blocking ISFs into mosquito populations.

RESULTS

Detection of CFAV in pupal exuviae. Assessment of horizontal and vertical transmission of CFAV requires the confirmation of infected and noninfected mosquitoes before

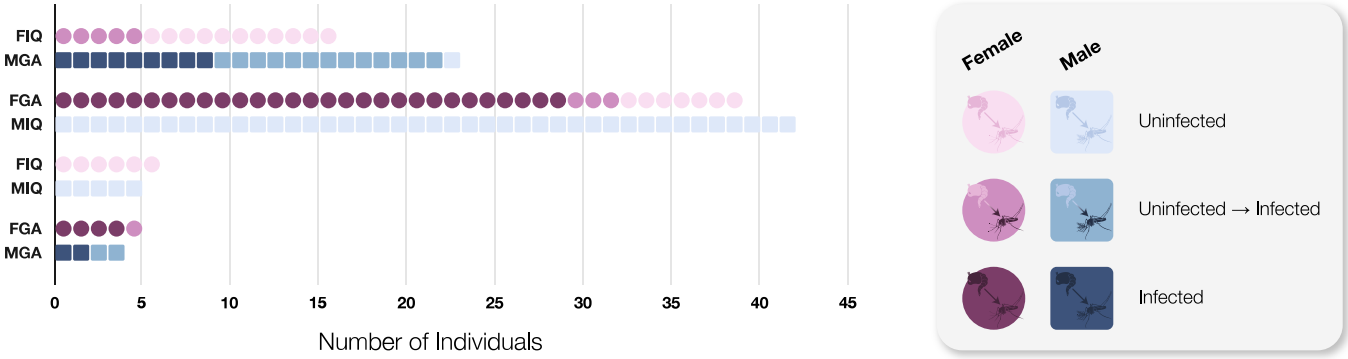

FIG 1 Comparison of CFAV infection status in pupal exuviae versus adult. Red circles indicate the female mosquito and blue squares represent the male in each grouped mating pair. Only mosquitoes that survived to the collection time point were tested, leading to an uneven number of males and females. The shading gradient indicates infection status, where the lightest shade indicates no infection detected in the pupae exuviae and adult, intermediate shade indicates a no infection detected at pupae and positive for infection at adult (pupae result does not agree with the adult result), and the darkest shade indicates positive for infection detected at both pupae and adult stages. The combination of each mating pair is on the y-axis. The 5 females with negative pupae and positive adults in the FIQ-MGA group were cases of horizontal transmission. FIQ, female Iquitos; MIQ, male Iquitos; FGA, female Galveston; MGA, male Galveston.

the transmission event. This is typically performed by surveying mosquitoes from the colonies to determine a baseline colony infection rate, rather than detection of virus in the individuals involved in the experiment. To assess the experimental individuals directly, pupae exuviae of the parental generation were tested for the presence of CFAV (Fig. 1). The pupal exuviae from all CFAV-negative adults were negative, which indicates that the PCR assay has a specificity of 100% in both female and male pupae exuviae. This included 17/17 females and 47/47 males from the Iquitos colony, and 7/7 females and 1/1 male from the Galveston colony. Only individuals from the Galveston colony were considered for comparison between pupae exuviae and CFAV-positive adults. The resulting sensitivity was 89% (33/37; CI, 74 to 97%) for females and 42% (11/26; CI, 26 to 61%) for males, with an overall sensitivity of 70% (44/63; CI, 58 to 80%) (Table 1). The positive predictive value for CFAV detection in pupae exuviae was 100% for both females (33/33) and males (11/11), and the negative predictive value was 86% (24/28) for females, 76% (48/63) for males, and 79% (72/91) overall. When only considering samples from the Galveston colony, the negative predictive value was 64% (7/11) for females, 6% (1/16) for males, and 30% (8/27) overall.

Horizontal transmission in paired mosquitoes. Adults from the parental generation were grouped into mating pairs and assessed for CFAV infection (Fig. 2). All paired adults from the Galveston colony positive control group were positive, and all paired adults from the Iquitos colony negative control group were negative for CFAV. No female-to-male transmission was observed in mating pairs consisting of a Galveston female and an Iquitos male when cohoused for either 3 days (0/13) or 14 days (0/15). All Galveston females were positive, and all Iquitos males were negative for CFAV in these pairs. Male-to-female transmission was observed at a rate of 31% (5/16; CI, 11 to 59%) in mating pairs with an Iquitos female and a Galveston male. All Galveston males in these mating pairs were positive for CFAV. The pupae exuviae corresponding to Iquitos female adults where CFAV was detected were all negative.

Vertical transmission from paired mosquitoes. Offspring from all four mating pair groups were assessed for CFAV to determine if vertical transmission was possible through both maternal and paternal routes (Fig. 3). Vertical transmission from the Galveston

TABLE 1 Analysis of detection of CFAV in pupae exuviae versus emerged adult mosquitoes[a]

Metric	Female	Male	All
Sensitivity (%, n)	89 (33/37)	42 (11/26)	70 (44/63)
Specificity (%, n)	100 (24/24)	100 (48/48)	100 (72/72)
PPV (%)	100 (33/33)	100 (11/11)	100 (44/44)
NPV, all samples (%)	86 (24/28)	76 (48/63)	79 (72/91)
NPV, Galveston only (%)	64 (7/11)	6 (1/16)	30 (8/27)

[a]PPV = positive predictive value, NPV = negative predictive value.

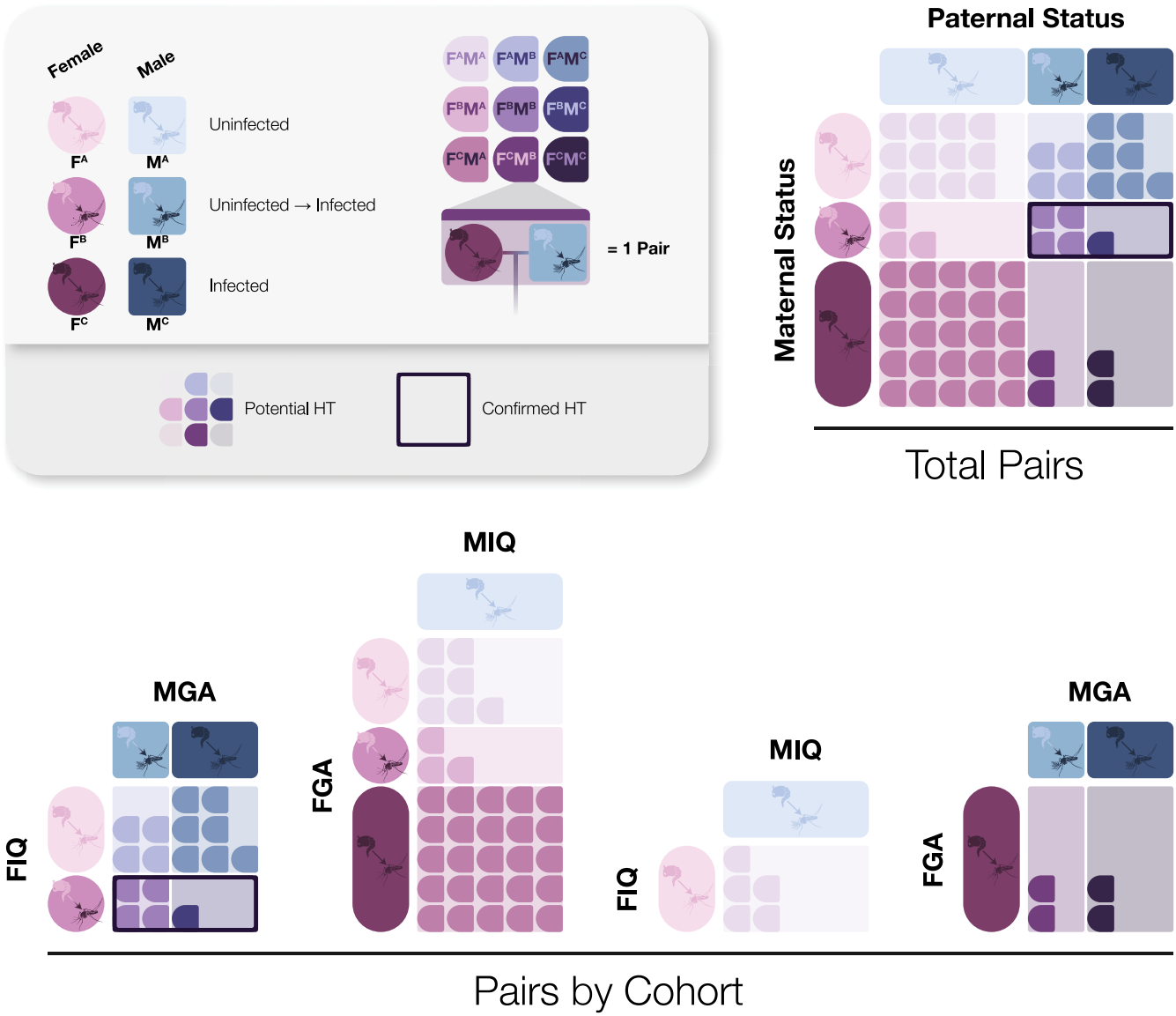

FIG 2 Evidence of horizontal transmission of CFAV between mating pairs. CFAV infection status of F0 mosquitoes in each mating pair. Shading of red circles and blue squares represent the infection status of each adult in the mating pair, with the lightest icons (F^A and M^A) indicating that both mosquitoes in the mating pair tested negative at both the pupa and adult stage and the darkest icons (F^C and M^C) indicating that both mosquitoes in the mating pair tested positive at both the pupa and adult stage. Mating pairs were assigned a color on the red-to-blue gradient based on the combined infection status of the female and male (9 potential outcomes). Mosquitoes that tested negative at the pupal stage and positive as an adult (F^B or M^B) were examples of potential horizontal transmission. Samples surrounded by the black border were confirmed cases of horizontal male-to-female transmission. Horizontal transmission can only be confirmed for mosquitoes from the Iquitos colony as they were from an uninfected colony and negative at the pupal stage, whereas mosquitoes from the Galveston colony could have had an undetected infection at the pupal stage. FIQ, female Iquitos; MIQ, male Iquitos; FGA, female Galveston; MGA, male Galveston.

colony control group was 100% (56/56; CI, 94 to 100%) for offspring from three different mating pairs (Fig. 4). Offspring from three Iquitos colony control mating pairs were all negative for CFAV (0/39; CI, 0 to 9%). Maternal transmission was observed from five mating pairs with a Galveston female and an Iquitos male. The filial infection rate from the five mating pairs ranged from 80 to 100%, with an overall filial infection rate of 93% (63/68; CI, 84 to 98%). Paternal transmission was also observed from eight mating pairs with an Iquitos female and a Galveston male, including three mating pairs in which the Iquitos female also became positive. The filial infection rate from mating pairs where the Iquitos female was negative varied from 33 to 100%, with an overall rate of 85% (56/66; CI, 74 to 92%). For the three mating pairs with positive Iquitos female adults, the filial infection rate varied from 25 to 80% with an overall rate of 61% (23/38; CI, 43 to 76%). The overall filial infection rate from all eight mating pairs with Iquitos female and Galveston male was 76% (79/104; CI, 67 to 84%).

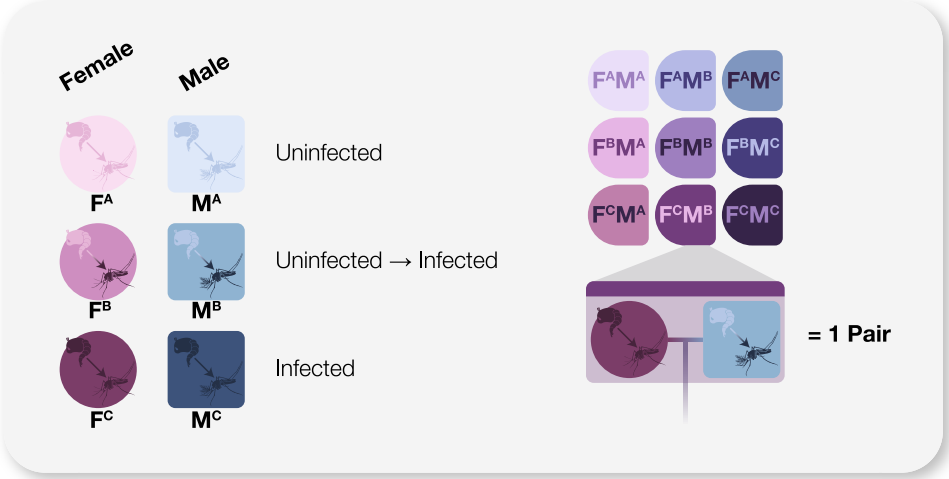

FIG 3 Filial infection of CFAV to assess maternal and paternal transmission. Red circles and blue squares indicate the female and male in each grouped mating pair, respectively. The shading gradient indicates infection status. Shading of red and blue on the bar above the icons represent the infection status of each pupa and adult in the mating pair. A histogram represents the infection status of the adult offspring, with light red or blue representing a negative female or male, respectively, and dark red or blue representing a positive female or male. Pupae exuviae were not examined for offspring. FIQ, female Iquitos; MIQ, male Iquitos; FGA, female Galveston; MGA, male Galveston.

DISCUSSION

There has been a rapid expansion of known members of ISFs and other insect-specific viruses, but little is known about their biology and maintenance in mosquito populations. This is true even for CFAV, an ISF first discovered in 1975 and with global distribution in a major vector species (23, 25, 27, 28, 35–38) and sustained seasonal infection (24). The

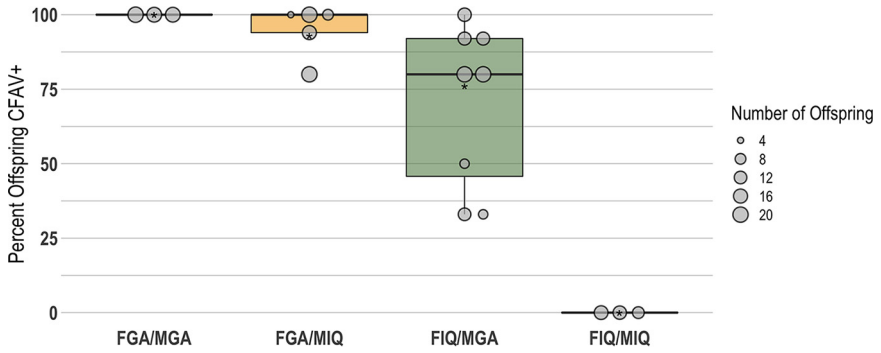

FIG 4 Vertical transmission of CFAV from different mating pair combinations. The size of the grey dots indicates the number of offspring from each mating pair in the group. Asterisks indicate the overall mean of vertical transmission seen from all offspring from the group. FIQ, female Iquitos; MIQ, male Iquitos; FGA, female Galveston; MGA, male Galveston.

detection of infected larvae or pupae and lack of other known hosts has led to speculation that ISFs are maintained primarily by vertical transmission. Although the results vary by virus and mosquito colony, experimental infections have demonstrated that maternal transmission occurs, as well as venereal transmission and the potential for other modes of horizontal transmission.

Crossing mosquitoes from CFAV-positive and CFAV-negative colonies confirmed maternal transmission and revealed paternal and horizontal transmission of CFAV. Maternal transmission of CFAV was first demonstrated by Contreras-Gutierrez et al. (19). Adult females from a CFAV-negative colony were injected with CFAV, which produced an overall F1 filial infection rate of 28%, and a range of 0 to 50% for individual females. Rearing offspring from the F1 generation increased the overall filial infection rate to 74% in the F2 generation (range of 60 to 93%), which was similar to the control rates of 78% to 100% from previous experiments with the Galveston colony (19) and the current rate of 100% in our Galveston colony. The increase from F1 to F2 infection rates in the experimentally infected colony may be due to the contributions of undetected paternal transmission and chronic infection of CFAV increasing the likelihood of infecting reproductive organs in F1 mosquitoes. Similarly, discrepancies between maternal transmission of 28% compared to 93% in the current experiments may be because the chronic infection of CFAV in Galveston females is more likely to infect reproductive organs compared to injection and 4-day incubation period employed in prior experiments, although ovaries from *Cx. pipiens* were infected with CxFV 4 days post-injection (20). Allowing sufficient time for systemic infection has also been suggested with Anopheles gambiae densovirus (AgDNV), where vertical transmission was observed when parent mosquitoes were infected at the larval stage (33), but not when females were infected through venereal transmission (39). The high levels of vertical transmission seen in the Galveston colony are also maintained by paternal transmission, which was responsible for an overall filial infection rate of 76%. While paternal transmission has not previously been evaluated in ISFs there are other well-documented examples of paternal transmission, such as for verdadero virus, a partitivirus in mosquitoes (40), and rice gall dwarf virus, an aphid-plant reovirus that binds to host sperm to infect offspring (41).

Horizontal transmission has also been demonstrated as a viable transmission route for ISFs. Transmission rates from male-to-female adults were 31% for CFAV. This rate is more similar to the 18% male-to-female venereal transmission for AeFV in *Ae. aegypti* (17) than the 2.4% rate observed with CxFV in *Cx. pipiens* (9). Neither the current CFAV experiments nor the AeFV experiments excluded other forms of contact transmission, such as sharing sugar meal sources. While transmission through food sharing did not occur with CxFV (9) and feeding on infected sugar meals rarely resulted in AeFV infection (17), KRV is known to have high oral infection rates (21). No female-to-male transmission was observed, although this occurred at a rate of 5.3% with CxFV (9), and 2% with AeFV (17). Increasing the sample size may reveal some female-to-male transmission, but the rate is likely low. Additional forms of horizontal transmission may have also occurred, such as infection through larval cannibalism or mating among emerged offspring, which would not be differentiated from vertical transmission based on our experiments.

Our horizontal transmission results were strengthened by testing pupae exuviae to demonstrate the lack of infection before being cohoused and mating with a positive male. Prior studies have not confirmed the infection status of individual mosquitoes before the potential transmission events. Although improvements for testing male pupae exuviae would be desirable, the sensitivity of 89% for female pupae exuviae provides the ability to assess prior infection in mosquitoes by testing pupae exuviae, which will be useful for future experiments. It is unknown why sensitivity differs between female and male pupae exuviae, but a previous study has shown that virus levels have a wider range and are lower titer, on average, in males (24).

CFAV may be a useful tool to limit secondary infections with arboviruses in *Ae. aegypti* mosquitoes. Superinfection exclusion has been demonstrated in cells and mosquitoes infected with CFAV, other ISFs, and insect-specific viruses. Previous studies showed that initial infection with a field-derived CFAV isolate resulted in reduced dengue virus and

Zika virus replication and dissemination (2). However, the lack of knowledge on the transmission of ISFs is a limitation in their potential use for pathogen control (31). Because both maternal and paternal transmission has been confirmed, our results offer the potential to establish CFAV infection in wild *Ae. aegypti* populations through the release of infected females or males. Releasing males would be most desirable because they do not contribute to the transmission of arboviruses and CFAV transmission by the male-to-female horizontal route may also improve overall infection levels in the field.

MATERIALS AND METHODS

Mosquitoes and viruses. Established laboratory colonies of *Ae. aegypti* Galveston and Iquitos colonies (kindly provided by Nikos Vasilakis from the University of Texas Medical Branch) were maintained in a 12-h light:12-h dark cycle with 1-h dawn and dusk, at 25°C and 75% relative humidity. A previous report identified a persistent infection of CFAV in the *Ae. aegypti* Galveston colony and no virus infection was detected in the *Ae. aegypti* Iquitos colony (25, 42). The presence and absence of CFAV in these colonies were confirmed by RT-PCR before performing the following experiments. The sequence for the CFAV isolate from the Galveston colony is available on GenBank (CFAV-Galveston strain accession no. KJ741267).

Mosquito rearing to assess vertical and horizontal CFAV transmission. Eggs collected from standard colony maintenance were floated out and larvae were fed ground fish food until reaching the pupal stage. Pupae from each colony were sexed and individually placed in 50 mL conical tubes with fresh water. Once emerged, water was removed from the tube and the pupae exuviae were stored at −80°C, the sex of the adults was confirmed, and individual males were removed from their tube and placed in a tube with an individual female. Mating pairs consisted of the following cohorts: (i) 1 Galveston female + 1 Galveston male for CFAV transmission positive control; (ii) 1 Iquitos female + 1 Iquitos male for CFAV transmission negative control; (iii) 1 Galveston female + 1 Iquitos male for female-to-male horizontal transmission and maternal transmission; or (iv) 1 Iquitos female + 1 Galveston male for male-to-female transmission and paternal transmission. Individual mating pairs were provided with 10% sucrose and allowed to mate for 3 days before the males were removed and stored at −80°C. Subsequent replicates to confirm the lack of female-to-male transmission involved cohousing mating pairs from the cohort (iii) 1 Galveston female + 1 Iquitos male for 14 days. Females were presented with a blood meal consisting of 1:1 human red blood cells and plasma via a Hemotek membrane feeding system to stimulate egg laying. After 2 days, individual blood-fed females were transferred to a 30 mL egg-laying tube containing water and filter paper. After another 2 days, females were collected and stored at −80°C. Egg papers were collected and dried for storage in the insectary until ready to rear offspring. The offspring were reared as normal, and adults were collected after all pupae in the cup had emerged and individually stored at −80°C. Adults in the F0 or F1 generation that were deceased before collection were not used for the detection of CFAV.

Detection of CFAV by reverse transcription PCR (RT-PCR). Pupal exuviae or adults were homogenized in a 2 mL Safe-lock microcentrifuge tube with a stainless-steel ball and RNA lysis buffer from the Zymo Quick-RNA Miniprep kit for 5 min at 26 Hz. RNA purification with the Zymo Quick-RNA Miniprep kit was performed per the manufacturer's protocol.

One-step RT-PCR assays without denaturation were prepared with the Jena Bioscience SCRIPT RT-PCR kit according to the manufacturer's instructions using CFAV forward and reverse primers as previously described (43). Thermocycler settings were as follows: 1 h at 50°C, 5 min at 95°C, 40 cycles of 10 s at 95°C, 20 s at 60°C, and 2 min at 72°C, with a final extension of 5 min at 72°C. An expected amplicon of 367 bp was visualized by gel electrophoresis.

Statistical analysis. All statistical analyses were conducted in Microsoft Excel and the R statistical software package (http://www.r-project.org; (44)). Binomial 95% confidence intervals (CI) were calculated for sensitivity and transmission efficiencies. Graphics were generated using the package ggplot2 (45).

Data availability. All data used for analysis is in the manuscript.

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