1 **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 field and laboratory populations of *Aedes aegypti*. ISFs have recently demonstrated the ability to block the transmission of arboviruses such as dengue, West Nile and Zika viruses. It is thought that vertical transmission is the main route for ISF infections. 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 wild 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 mosquito pupal exuviae and was 89% sensitive, but only 41% in male mosquito 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.

41 **Introduction**

42 The genus *Flavivirus* contains many arboviruses of medical and veterinary
43 importance such as dengue, West Nile and Zika viruses, as well as insect-specific flaviviruses
44 (ISFs) that are only known to infect insect hosts. ISFs are known to be present in a range of
45 mosquito species and we are becoming increasingly aware of their functions and uses
46 because of recent reports showing their ability to block arbovirus transmission in mosquitoes
47 and their use as chimeric vaccines (Harrison et al. 2021; Baidaliuk et al. 2019; Hobson-Peters
48 et al. 2019; Romo et al. 2018; Hall-Mendelin et al. 2016; Goenaga et al. 2015; Kenney et al.
49 2014; Hobson-Peters et al. 2013).

50 Whilst both arboviruses and ISFs belong to the *Flavivirus* genus, transmission is
51 markedly different. Arboviruses are dual-host flaviviruses transmitted through the blood-
52 feeding of arthropods on viremic vertebrate hosts. After acquisition of the virus from the
53 host, only a small fraction of subsequent transmission is vertical, with female mosquitoes
54 passing the virus to their offspring after feeding on an infected host (Phumee et al. 2019;
55 Tesh et al. 2016; Thangamani et al. 2016; Baqar et al. 1993; Rosen et al. 1983). Contrary to
56 this, vertical transmission is thought to be the main route of ISF transmission because the
57 viruses do not replicate in vertebrate hosts.

58 A small number of studies have observed vertical transmission of ISFs in naturally or
59 experimentally infected mosquito colonies (Peinado et al. 2022; McClean et al. 2021;
60 Contreras-Gutierrez et al. 2017; Bolling et al. 2012; Saiyasombat et al. 2011; Lutomiah et al.
61 2007), including for cell fusing agent virus (CFAV), an ISF identified in several *Aedes*
62 *aegypti* cell culture lines, and in field and laboratory colony mosquitoes (Baidaliuk et al.
63 2020; Martin et al. 2020; Bolling et al. 2015; Stollar and Thomas, 1975). The observed
64 vertical transmission rate was higher in naturally infected colonies of *Ae. aegypti* with CFAV
65 compared to experimentally infected colonies, and similar results were seen in *Culex pipiens*

66 with Culex flavivirus (CxFV) (Contreras-Gutierrez et al. 2017; Saiyasombat et al. 2011).
67 Filial transmission from experimentally infected females ranged from 0-50% for CFAV in
68 *Ae. aegypti*, and 0-22% for CxFV in *Cx. pipiens*. For Kamiti River virus (KRV), an ISF
69 isolated from *Aedes macintoshi*, the filial infection rate from *Ae. aegypti* females following
70 an infectious blood meal was 4% (Lutomiah et al. 2007). The disparate range in these
71 experiments suggest that other forms of transmission may also occur.
72 Horizontal transmission of ISFs has also been observed in mosquito colonies. KRV
73 was able to infect a high proportion of *Ae. aegypti* larvae when exposed to infected cell
74 culture, while Aedes flavivirus (AeFV) only infected a low proportion of *Ae. aegypti* larvae
75 and adults when feeding on infected cell cultures and sugar meals, respectively (Peinado et
76 al. 2022; Lutomiah et al. 2007). However, the virus was not detected in water used to rear
77 CxFV-infected *Cx. pipiens* larvae and infection was not detected in co-reared, negative larvae
78 (Bolling et al. 2012), suggesting infected individuals did not shed virus into their larval
79 environment. Venereal transmission of CxFV and AeFV was demonstrated in both directions,
80 from male-to-female as well as female-to-male, in experiments that crossed infected
81 mosquito colonies with naïve colonies (Peinado et al. 2022; Bolling et al. 2012). These rates
82 were generally low, except for male-to-female crosses in *Aedes albopictus* which lead to an
83 18% infection rate (Peinado et al. 2022).
84 Further knowledge of the transmission and maintenance of ISFs in mosquito
85 populations, especially for mosquito species that are competent vectors where ISFs are of
86 interest for pathogen control approaches, are of high relevance for this group of viruses.
87 CFAV infects an important vector species, *Ae. aegypti*, and its relation to many dangerous
88 flaviviruses may allow it to be used to control arbovirus transmission through superinfection
89 exclusion – blocking subsequent infection of a similar virus – (Baidaliuk et al. 2019) or as a
90 vehicle for paratransgenesis – using a microbe to express transgenes in its host – as has been

91 proposed for other insect-specific viruses (Patterson et al. 2021; Patterson et al. 2020; Gu et
92 al. 2010; Ren et al. 2008; Ward et al. 2001). CFAV has been shown to be maternally
93 transmitted with experimentally infected female mosquitoes (Contreras-Gutierrez et al.
94 2017), but it is not known if CFAV is paternally or horizontally transmitted. Given the
95 reduced rates of transmission seen in experimental infections, we hypothesized that multiple
96 modes of transmission occur in naturally infected colonies. To assess this, we used a
97 laboratory colony of CFAV-infected *Ae*. *aegypti* and a known uninfected colony to quantify
98 maternal, paternal, and horizontal transmission of CFAV. Our results provide insights into
99 the transmission routes of CFAV which could be used to inform strategies to spread
100 pathogen-blocking ISFs into mosquito populations.
101
102 **Materials and Methods**
103 *Mosquitoes and virus*
104 Established laboratory colonies of *Ae. aegypti* Galveston and Iquitos colonies were
105 maintained in a 12-hour light:12-hour dark cycle with a 1-hour dawn and dusk, at 25°C and
106 75% relative humidity. A previous report identified a persistent infection of CFAV in the *Ae.*
107 *aegypti* Galveston colony and no virus infection was detected in the *Ae. aegypti* Iquitos
108 colony (Ma et al. 2021; Bolling et al. 2015). The presence and absence of CFAV in these
109 colonies was confirmed prior to performing the following experiments. The sequence for the
110 CFAV isolate from the Galveston colony is available on GenBank (CFAV-Galveston strain
111 accession no. KJ741267).
112
113 *Mosquito rearing to assess vertical and horizontal CFAV transmission*
114 Eggs collected from standard colony maintenance were floated out and larvae were
115 fed ground fish food until reaching pupal stage. Pupae from each colony were sexed and

116 individually placed in 50 mL conical tubes with fresh water. Once emerged, water was
117 removed from the tube and the pupae exuviae were stored at -80 °C, the sex of the adults was
118 confirmed, and individual males were removed from their tube and placed in a tube with an
119 individual female. Mating pairs consisted of the following cohorts: i) 1 Galveston female + 1
120 Galveston male for CFAV transmission positive control; ii) 1 Iquitos female + 1 Iquitos male
121 for CFAV transmission negative control; iii) 1 Galveston female + 1 Iquitos male for female-
122 to-male horizontal transmission and maternal transmission; or iv) 1 Iquitos female + 1
123 Galveston male for male-to-female transmission and paternal transmission. Individual mating
124 pairs were provided with 10% sucrose and allowed to mate for three days before the males
125 were removed and stored at -80 °C. Subsequent replicates to confirm the lack of female-to-
126 male transmission involved cohousing mating pairs from cohort iii) 1 Galveston female + 1
127 Iquitos male for 14 days. Females were presented with a blood meal consisting of 1:1 human
128 red blood cells and plasma via a Hemotek membrane feeding system to stimulate egg laying.
129 After two days, individual blood fed females were transferred to a 30 mL egg laying tube
130 containing water and filter paper. After another two days, females were collected and stored
131 at -80 °C. Egg papers were collected and dried for storage in the insectary until ready to rear
132 offspring. The offspring were reared as normal and emerged adults were collected and
133 individually stored at -80 °C. Adults in the F0 or F1 generation that were deceased prior to
134 collection were not used for detection of CFAV.
135
136 *Detection of CFAV by reverse transcription polymerase chain reaction (RT-PCR)*
137 Pupae exuviae or adults were homogenized in a 2 mL Safe-lock microcentrifuge tube
138 with a stainless-steel ball and RNA lysis buffer from the Zymo Quick-RNA Miniprep kit for
139 5 min at 26 Hz. RNA purification with the Zymo Quick-RNA Miniprep kit was performed
140 per the manufacturer's protocol.

141 One-step RT-PCR assays without denaturation were prepared with the Jena
142 Bioscience SCRIPT RT-PCR kit according to the manufacturer's instructions using CFAV
143 forward and reverse primers as previously described (Weger-Lucarelli et al. 2018).
144 Thermocycler settings were as follows: 1 h at 50 °C, 5 min at 95 °C, 40 cycles of 10 s at 95
145 °C, 20 s at 60 °C and 2 min at 72 °C, with a final extension of 5 min at 72 °C. An expected
146 amplicon of 367 bp was visualized by gel electrophoresis.
147
148 *Statistical Analysis*
149 All statistical analyses were conducted in Microsoft Excel and the R statistical
150 software package (http://www.r-project.org; R Core Team 2020). Binomial 95% confidence
151 intervals (CI) were calculated for sensitivity and transmission efficiencies. Graphics were
152 generated using the package ggplot2 (Wickham 2016).
153
154 **Results**
155 *Detection of CFAV in pupal exuviae*
156 Assessment of horizontal and vertical transmission of CFAV requires the
157 confirmation of infected and non-infected mosquitoes prior to the transmission event. This is
158 typically performed by surveying mosquitoes from the colonies to determine a baseline
159 colony infection rate, rather than detection of virus in the individuals involved in the
160 experiment. To assess the experimental individuals directly, pupae exuviae of the parental
161 generation were tested for the presence of CFAV (Figure 1). The pupae exuviae from all
162 CFAV-negative adults were negative, which indicates that the PCR assay has a specificity of
163 100% in both female and male pupae exuviae. This includes 17/17 females and 47/47 males
164 from the Iquitos colony, and 7/7 females and 1/1 male from the Galveston colony. Only
165 individuals from the Galveston colony were considered for comparison between pupae

exuviae and CFAV-positive adults. The resulting sensitivity was 89% (33/37; CI: 74-97%) for females and 41% (11/27; CI: 22-61%) for males, with an overall sensitivity of 69% (44/64; CI: 58-80%) (Table 1). The positive predictive value for CFAV detection in pupae exuviae was 100% for both females and males, and the negative predictive value was 86% for females, 75% for males, and 78% overall. When only considering samples from the Galveston colony, the negative predictive value is 64% for females, 6% for males, and 29% overall.

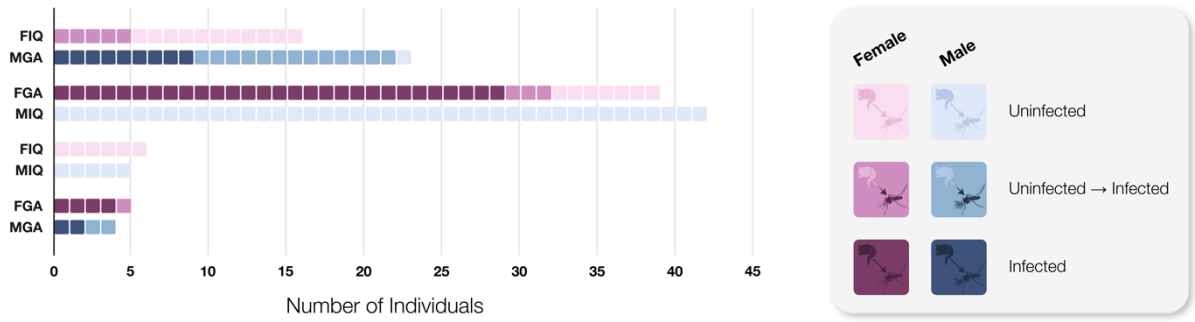

Figure 1. Comparison of CFAV infection status in pupal exuviae versus adult. Red icons indicate the female mosquito and blue icons represent the male in each grouped mating pair. Only mosquitoes that survived to the collection time point were tested, leading to an uneven number of males and females. 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 adult result), and the darkest shade indicates positive for infection detected at both pupae and adult stages. The combination of each individual mating pair is provided on the Y-axis. The 5 females with negative pupae and positive adult in the FIQ-MGA group are cases of horizontal transmission. FIQ = female Iquitos, MIQ = male Iquitos, FGA = female Galveston, MGA = male Galveston.

187 Table 1. Analysis of detection of CFAV in pupae exuviae versus emerged adult mosquitoes.

188 PPV = positive predictive value, NPV = negative predictive value.

Metric	**Female**	**Male**	**All**
Sensitivity (%, n)	89 (33/37)	41 (11/27)	69 (44/64)
Specificity (%, n)	100 (17/17)	100 (47/47)	100 (64/64)
PPV (%)	100	100	100
NPV – all samples (%)	85	62	69
NPV – Galveston only (%)	64	6	29

189

190 *Horizontal transmission in paired mosquitoes*

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

201

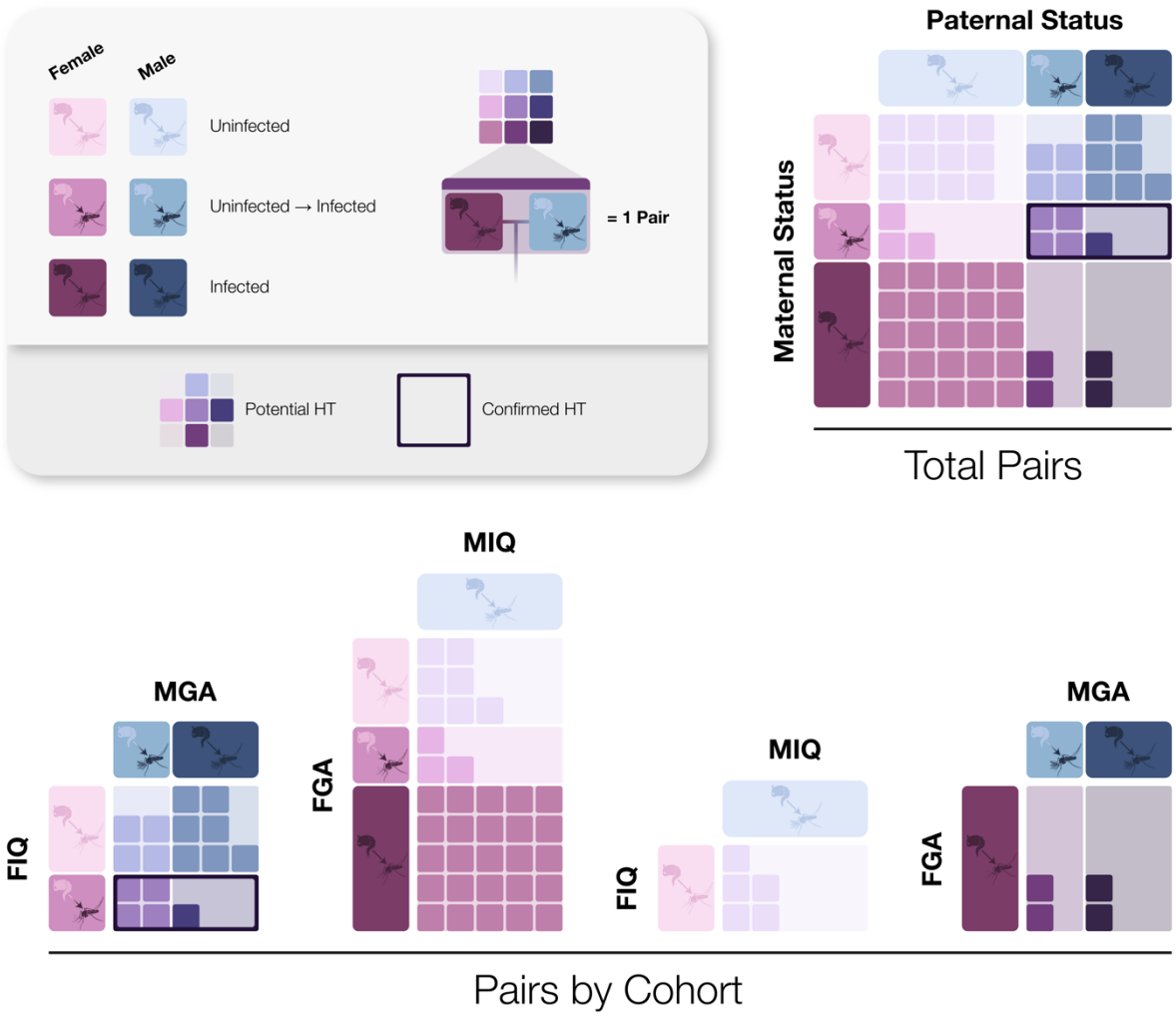

Figure 2. Evidence of horizontal transmission of CFAV between mating pairs. CFAV infection status of F0 mosquitoes in each mating pair. Shading of red and blue on the icons represent the infection status of each adult in the mating pair, with the lightest icons indicating that both mosquitoes in the mating pair tested negative at both the pupa and adult stage, and the darkest icons indicating that both mosquitoes in the mating pair tested positive at both the pupa and adult stage. Mating pairs are assigned a colour 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 pupa stage and positive as an adult are examples of potential horizontal transmission. Samples surrounded by the black border are confirmed cases of horizontal male-to-female transmission. FIQ = female Iquitos, MIQ = male Iquitos, FGA = female Galveston, MGA = male Galveston.

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 (Figure 3). Vertical transmission from the Galveston colony control group was 100% (56/56; CI: 94-100%) for offspring from three different mating pairs (Figure 4). Offspring from three Iquitos colony control mating pairs were all negative for CFAV (0/39; CI: 0-9%). 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 80-100%, with an overall filial infection rate of 93% (63/68; CI: 84-98%). 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 33-100%, with an overall rate of 85% (56/66; CI: 74-92%). For the three mating pairs with positive Iquitos female adults, the filial infection rate varied from 25-80% with an overall rate of 61% (23/38; CI: 43-76%). The overall filial infection rate from all eight mating pairs with Iquitos female and Galveston male was 76% (79/104; CI: 67-84%).

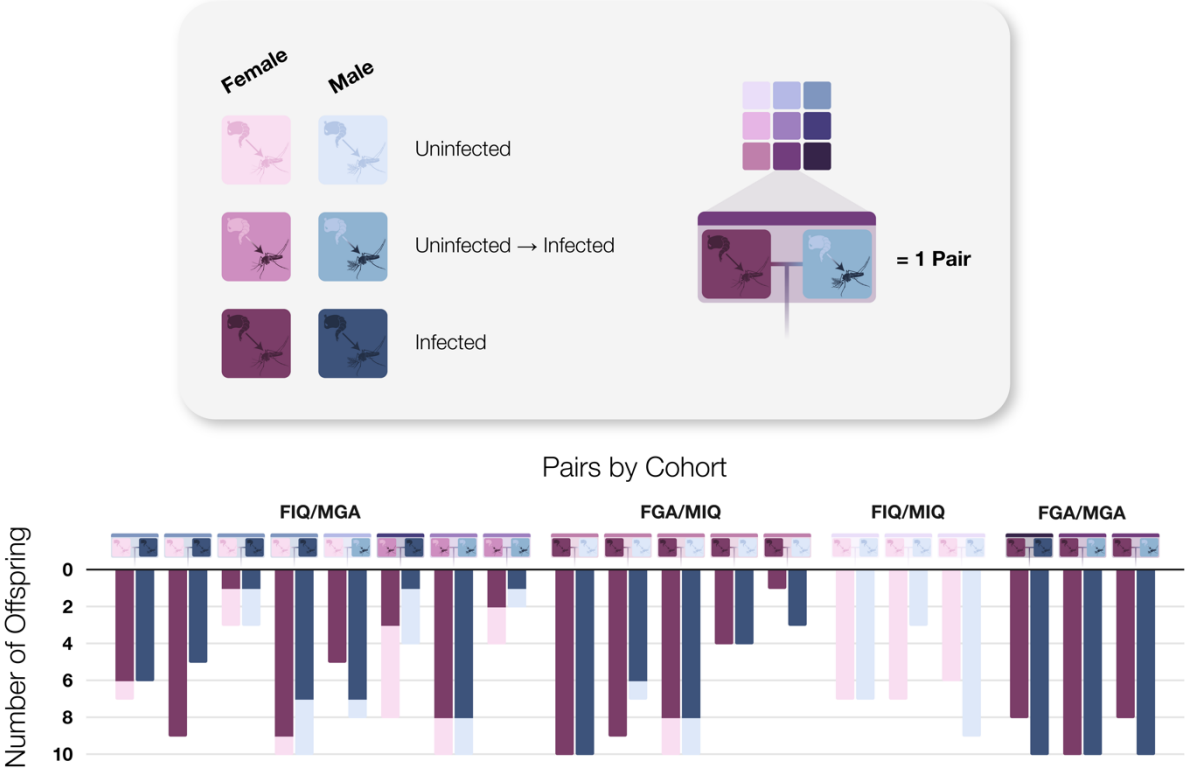

Figure 3. Filial infection of CFAV to assess maternal and paternal transmission. Red and blue icons indicate the female and male in each grouped mating pair, respectively. The shading gradient indicates infection status. Shading of red and blue on the bar above the icons represent the infection status of each pupa and adult in the mating pair. 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.

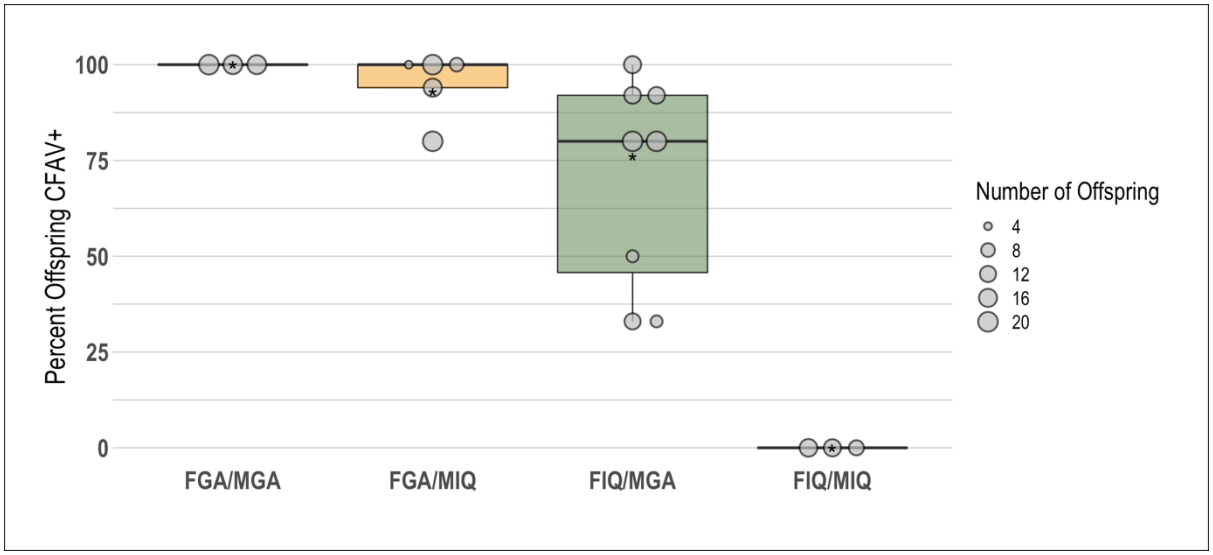

Figure 4. Vertical transmission of CFAV from different mating pair combinations. The size of the grey dots indicates the number of offspring from each mating pair in the group. Asterisk indicates the overall mean of vertical transmission seen from all offspring from the group. FIQ = female Iquitos, MIQ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

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 and sustained seasonal infection (Baidaliuk et al. 2020; Jeffries et al. 2020; Martin et al. 2020; Ajamma et al. 2018; Fernandes et al. 2018; Bolling et al. 2015; Yamanaka et al. 2013; Espinoza-Gomez et al. 2011; Cook et al. 2006). The detection of infected larvae or pupae and lack of other known hosts has led to speculation that ISFs are vertically transmitted. 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.

260 Crossing mosquitoes from CFAV-positive and -negative colonies confirmed maternal
261 transmission and revealed paternal and horizontal transmission of CFAV. Maternal
262 transmission of CFAV was first demonstrated by Contreras-Gutierrez et al. (2017). Adult
263 females from a CFAV-negative colony were injected with CFAV, which produced an overall
264 F1 filial infection rate of 28%, and range of 0-50% for individual females. Rearing offspring
265 from the F1 generation increased the overall filial infection rate to 74% in the F2 generation
266 (range of 60-93%), similar to the control rates of 78% to 100% from previous experiments
267 with the Galveston colony (Contreras-Gutierrez et al. 2017) and the current rate of 100% in
268 our Galveston colony. The increase from F1 to F2 infection rates in the experimentally
269 infected colony may be due to the contributions of undetected paternal transmission and
270 chronic infection of CFAV increasing the likelihood of infecting reproductive organs in F1
271 mosquitoes. Similarly, discrepancies between maternal transmission of 28% compared to
272 93% in the current experiments may be because the chronic infection of CFAV in Galveston
273 females are more likely to infect reproductive organs compared to injection and 4-day
274 incubation period employed in prior experiments, although ovaries from *Cx. pipiens* were
275 infected with CxFV 4 days post-injection (Saiyasombat et al. 2011). Allowing sufficient time
276 for systemic infection has also been suggested with Anopheles gambiae densovirus
277 (AgDNV), where vertical transmission was observed when parent mosquitoes were infected
278 at the larval stage, but not when females were infected through venereal transmission
279 (Werling et al. 2022; Ren et al. 2008). The high levels of vertical transmission seen in the
280 Galveston colony are also maintained by paternal transmission, which was responsible for an
281 overall filial infection rate of 76% and is the first observation of paternal transmission in
282 ISFs. While paternal transmission has not previously been evaluated in ISFs there are other
283 well-documented examples of paternal transmission, such as for verdadero virus, a

284 partitivirus in mosquitoes (Cross et al. 2020) and rice gall dwarf virus, an aphid-plant
285 reovirus that binds to host sperm to infect offspring (Mao et al. 2019).
286 Horizontal transmission has also been demonstrated as a viable transmission route for
287 ISFs. Transmission rates from male-to-female adults were 31% for CFAV. This rate is more
288 similar to the 18% male-to-female venereal transmission for AeFV in *Ae. aegypti* than the
289 2.4% rate observed with CxFV in *Cx. pipiens* (Peinado et al. 2022; Bolling et al. 2012).
290 Neither the current CFAV experiments nor the AeFV experiments excluded other forms of
291 contact transmission, such as sharing sugar meal sources. While transmission through food
292 sharing did not occur with CxFV and feeding on infected sugar meals rarely resulted in AeFV
293 infection (Peinado et al. 2022; Bolling et al. 2012), KRV is known to have high oral infection
294 rates (Lutomiah et al. 2007). No female-to-male transmission was observed, although this
295 occurred at a rate of 5.3% with CxFV, and 2% with AeFV (Peinado et al. 2022; Bolling et al.
296 2012). Increasing the sample size may reveal some female-to-male transmission, but the rate
297 is likely low.
298 Our horizontal transmission results were strengthened by testing pupae exuviae to
299 demonstrate the lack of infection before being cohoused and mating with a positive male.
300 Prior studies have not confirmed the infection status of individual mosquitoes before the
301 potential transmission events. Although improvements for testing male pupae exuviae would
302 be desirable, the sensitivity of 89% for female pupae exuviae provides the ability to assess
303 prior infection in mosquitoes by testing pupae exuviae, which will be useful for future
304 experiments. It is unknown why sensitivity differs between female and male pupae exuviae,
305 but a previous study has shown that virus levels have a wider range and are lower titer, on
306 average, in males (Martin et al. 2020).
307 CFAV may be a useful tool to limit secondary infections with arboviruses in *Ae.*
308 *aegypti* mosquitoes. Superinfection exclusion has been demonstrated in cells and mosquitoes

309 infected with CFAV, other ISFs and insect-specific viruses. Previous studies showed that
310 initial infection with a field-derived CFAV isolate resulted in reduced dengue virus and Zika
311 virus replication and dissemination (Baidaliuk et al. 2019). However, the lack of knowledge
312 on the transmission of ISFs is a limitation in their potential use for pathogen control
313 (Patterson et al. 2020). As both maternal and paternal transmission have been confirmed, our
314 results offer the potential to establish CFAV infection in wild *Ae. aegypti* populations
315 through the release of infected females or males. Releasing males would be most desirable
316 because they do not contribute to the transmission of arboviruses and CFAV transmission by
317 the male-to-female horizontal route may also improve overall infection levels in the field.
318

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