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*

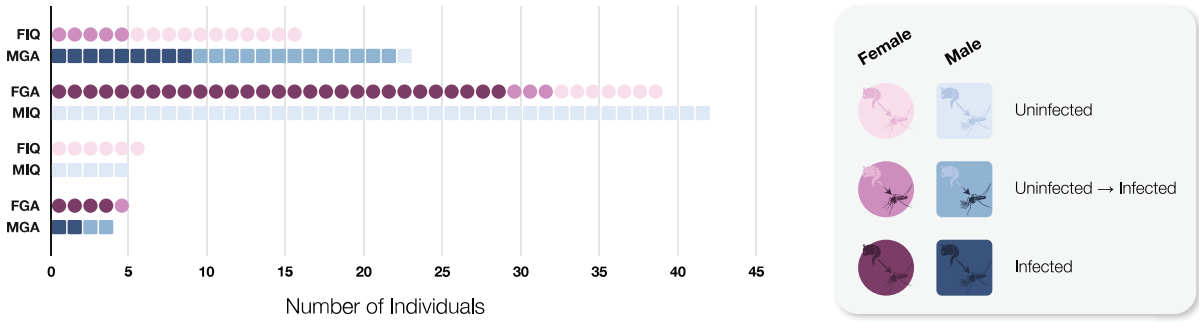
104 Established laboratory colonies of *Ae. aegypti* Galveston and Iquitos colonies (kindly
105 provided by Prof. Nikos Vasilakis from the University of Texas Medical Branch) were
106 maintained in a 12-hour light:12-hour dark cycle with a 1-hour dawn and dusk, at 25°C and
107 75% relative humidity. A previous report identified a persistent infection of CFAV in the *Ae.*
108 *aegypti* Galveston colony and no virus infection was detected in the *Ae. aegypti* Iquitos
109 colony (Ma et al. 2021; Bolling et al. 2015). The presence and absence of CFAV in these
110 colonies was confirmed prior to performing the following experiments. The sequence for the
111 CFAV isolate from the Galveston colony is available on GenBank (CFAV-Galveston strain
112 accession no. KJ741267).
113

114 *Mosquito rearing to assess vertical and horizontal CFAV transmission*

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

140 5 min at 26 Hz. RNA purification with the Zymo Quick-RNA Miniprep kit was performed
141 per the manufacturer's protocol.
142 One-step RT-PCR assays without denaturation were prepared with the Jena
143 Bioscience SCRIPT RT-PCR kit according to the manufacturer's instructions using CFAV
144 forward and reverse primers as previously described (Weger-Lucarelli et al. 2018).
145 Thermocycler settings were as follows: 1 h at 50 °C, 5 min at 95 °C, 40 cycles of 10 s at 95
146 °C, 20 s at 60 °C and 2 min at 72 °C, with a final extension of 5 min at 72 °C. An expected
147 amplicon of 367 bp was visualized by gel electrophoresis.
148
149 *Statistical Analysis*
150 All statistical analyses were conducted in Microsoft Excel and the R statistical
151 software package (http://www.r-project.org; R Core Team 2020). Binomial 95% confidence
152 intervals (CI) were calculated for sensitivity and transmission efficiencies. Graphics were
153 generated using the package ggplot2 (Wickham 2016).
154
155 **Results**
156 *Detection of CFAV in pupal exuviae*
157 Assessment of horizontal and vertical transmission of CFAV requires the
158 confirmation of infected and non-infected mosquitoes prior to the transmission event. This is
159 typically performed by surveying mosquitoes from the colonies to determine a baseline
160 colony infection rate, rather than detection of virus in the individuals involved in the
161 experiment. To assess the experimental individuals directly, pupae exuviae of the parental
162 generation were tested for the presence of CFAV (Figure 1). The pupae exuviae from all
163 CFAV-negative adults were negative, which indicates that the PCR assay has a specificity of
164 100% in both female and male pupae exuviae. This includes 17/17 females and 47/47 males

165 from the Iquitos colony, and 7/7 females and 1/1 male from the Galveston colony. Only
166 individuals from the Galveston colony were considered for comparison between pupae
167 exuviae and CFAV-positive adults. The resulting sensitivity was 89% (33/37; CI: 74-97%)
168 for females and 41% (11/27; CI: 22-61%) for males, with an overall sensitivity of 69%
169 (44/64; CI: 58-80%) (Table 1). The positive predictive value for CFAV detection in pupae
170 exuviae was 100% for both females and males, and the negative predictive value was 86%
171 for females, 75% for males, and 78% overall. When only considering samples from the
172 Galveston colony, the negative predictive value is 64% for females, 6% for males, and 29%
173 overall.
174



175
176 Figure 1. Comparison of CFAV infection status in pupal exuviae versus adult. Red circles
177 indicate the female mosquito and blue squares represent the male in each grouped mating
178 pair. Only mosquitoes that survived to the collection time point were tested, leading to an
179 uneven number of males and females. The shading gradient indicates infection status, where
180 the lightest shade indicates no infection detected in the pupae exuviae and adult, intermediate
181 shade indicates a no infection detected at pupae and positive for infection at adult (pupae
182 result does not agree with adult result), and the darkest shade indicates positive for infection
183 detected at both pupae and adult stages. The combination of each individual mating pair is
184 provided on the Y-axis. The 5 females with negative pupae and positive adult in the FIQ-

185 MGA group are cases of horizontal transmission. FIQ = female Iquitos, MIQ = male Iquitos,
186 FGA = female Galveston, MGA = male Galveston.
187
188 Table 1. Analysis of detection of CFAV in pupae exuviae versus emerged adult mosquitoes.
189 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

190
191 *Horizontal transmission in paired mosquitoes*
192 Adults from the parental generation were grouped into mating pairs and assessed for
193 CFAV infection (Figure 2). All paired adults from the Galveston colony positive control
194 group were positive, and all paired adults from the Iquitos colony negative control group
195 were negative for CFAV. No female-to-male transmission was observed in mating pairs
196 consisting of a Galveston female and an Iquitos male when cohoused for either 3 days (0/14)
197 or 14 days (0/15). All Galveston females were positive, and all Iquitos males were negative
198 for CFAV. Male-to-female transmission was observed at a rate of 31% (5/16; CI: 11-59%) in
199 mating pairs with an Iquitos female and a Galveston male. All Galveston males in these
200 mating pairs were positive for CFAV. The pupae exuviae corresponding to Iquitos female
201 adults where CFAV was detected were all negative.
202

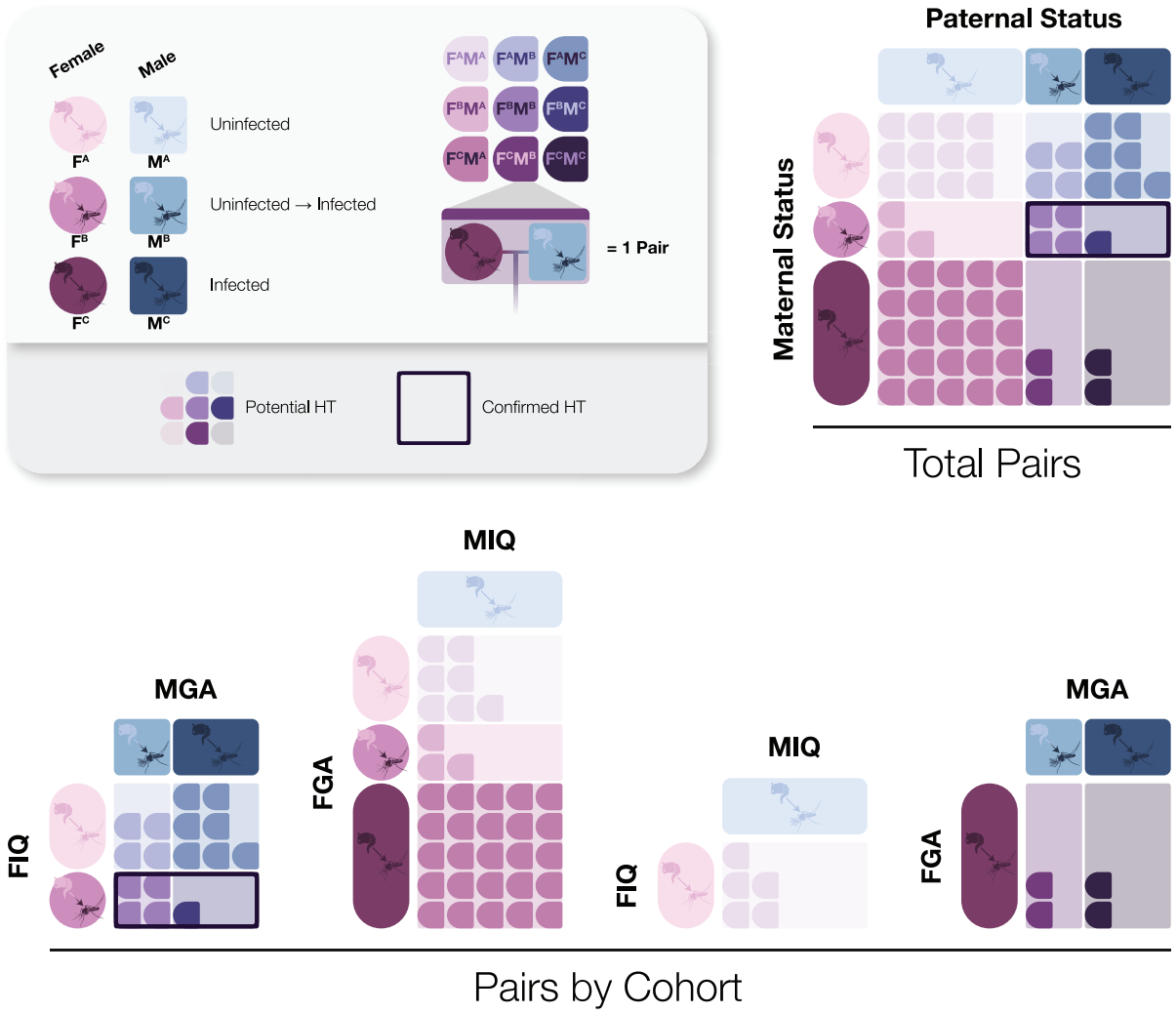


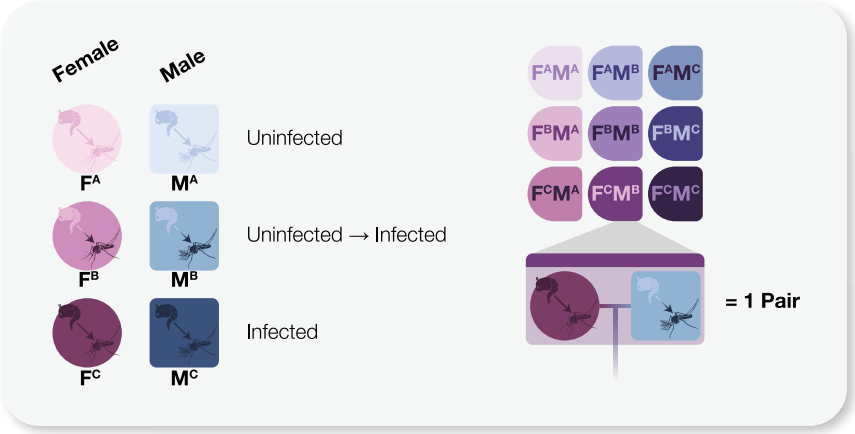
Figure 2. Evidence of horizontal transmission of CFAV between mating pairs. CFAV infection status of F0 mosquitoes in each mating pair. Shading of red circles and blue squares represent the infection status of each adult in the mating pair, with the lightest icons (F^A and M^A) indicating that both mosquitoes 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 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 (F^B or M^B) 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.

215

216 *Vertical transmission from paired mosquitoes*

217 Offspring from all four mating pair groups were assessed for CFAV to determine if
218 vertical transmission was possible through both maternal and paternal routes (Figure 3).
219 Vertical transmission from the Galveston colony control group was 100% (56/56; CI: 94-
220 100%) for offspring from three different mating pairs (Figure 4). Offspring from three Iquitos
221 colony control mating pairs were all negative for CFAV (0/39; CI: 0-9%). Maternal
222 transmission was observed from five mating pairs with a Galveston female and an Iquitos
223 male. The filial infection rate from the five mating pairs ranged from 80-100%, with an
224 overall filial infection rate of 93% (63/68; CI: 84-98%). Paternal transmission was also
225 observed from eight mating pairs with an Iquitos female and a Galveston male, including
226 three mating pairs in which the Iquitos female also became positive. The filial infection rate
227 from mating pairs where the Iquitos female was negative varied from 33-100%, with an
228 overall rate of 85% (56/66; CI: 74-92%). For the three mating pairs with positive Iquitos
229 female adults, the filial infection rate varied from 25-80% with an overall rate of 61% (23/38;
230 CI: 43-76%). The overall filial infection rate from all eight mating pairs with Iquitos female
231 and Galveston male was 76% (79/104; CI: 67-84%).

232



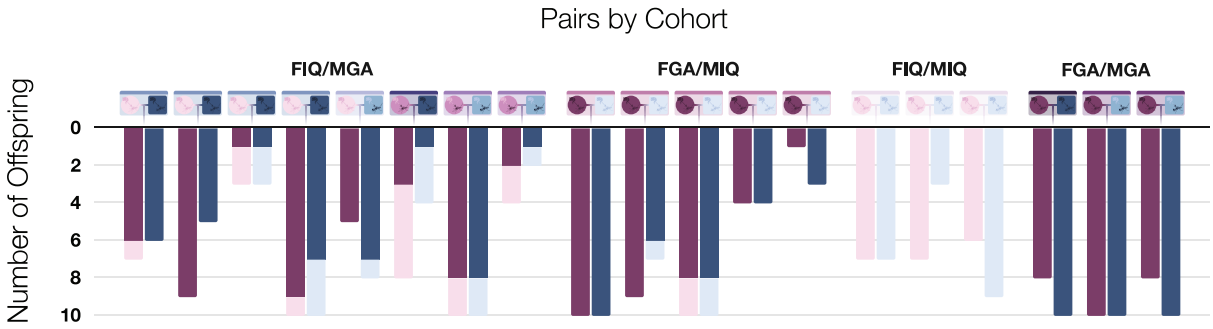


Figure 3. Filial infection of CFAV to assess maternal and paternal transmission. Red circles and blue squares indicate the female and male in each grouped mating pair, respectively. The shading gradient indicates infection status. Shading of red and blue on the bar above the icons represent the infection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

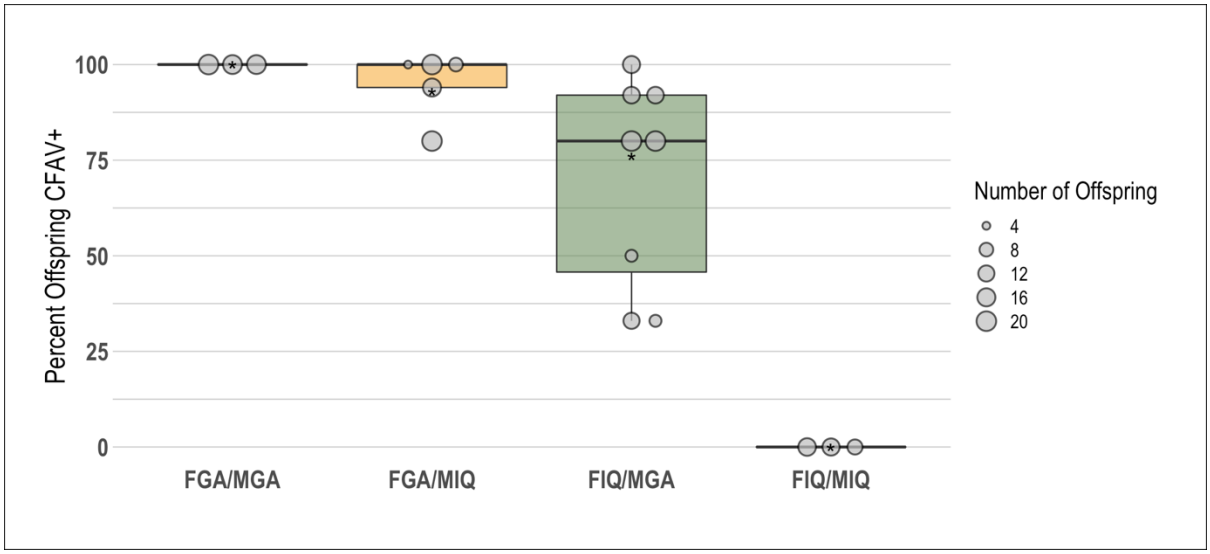


Figure 4. Vertical transmission of CFAV from different mating pair combinations. The size of the grey dots indicates the number of offspring from each mating pair in the group. Asterisk indicates the overall mean of vertical transmission seen from all offspring from the group. FIQ = female Iquitos, MIQ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

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

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

310 infected with CFAV, other ISFs and insect-specific viruses. Previous studies showed that
311 initial infection with a field-derived CFAV isolate resulted in reduced dengue virus and Zika
312 virus replication and dissemination (Baidaliuk et al. 2019). However, the lack of knowledge
313 on the transmission of ISFs is a limitation in their potential use for pathogen control
314 (Patterson et al. 2020). As both maternal and paternal transmission have been confirmed, our
315 results offer the potential to establish CFAV infection in wild *Ae. aegypti* populations
316 through the release of infected females or males. Releasing males would be most desirable
317 because they do not contribute to the transmission of arboviruses and CFAV transmission by
318 the male-to-female horizontal route may also improve overall infection levels in the field.
319

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330 **References**

331 1. Harrison, J. J., Hobson-Peters, J., Bielefeldt-Ohmann, H., & Hall, R. A. (2021). Chimeric
332 Vaccines Based on Novel Insect-Specific Flaviviruses. *Vaccines*, *9*(11), 1230.
333
334 2. Baidaliuk, A., Miot, E. F., Lequime, S., Moltini-Conclois, I., Delaigue, F., Dabo, S., ... &
335 Lambrechts, L. (2019). Cell-fusing agent virus reduces arbovirus dissemination in Aedes
336 aegypti mosquitoes in vivo. *Journal of virology*, *93*(18), e00705-19.
337
338 3. Hobson-Peters, J., Harrison, J. J., Watterson, D., Hazlewood, J. E., Vet, L. J., Newton, N.
339 D., ... & Hall, R. A. (2019). A recombinant platform for flavivirus vaccines and diagnostics
340 using chimeras of a new insect-specific virus. *Science translational medicine*, *11*(522).
341
342 4. Romo, H., Kenney, J. L., Blitvich, B. J., & Brault, A. C. (2018). Restriction of Zika virus
343 infection and transmission in Aedes aegypti mediated by an insect-specific
344 flavivirus. *Emerging microbes & infections*, *7*(1), 1-13.
345
346 5. Hall-Mendelin, S., McLean, B. J., Bielefeldt-Ohmann, H., Hobson-Peters, J., Hall, R. A.,
347 & van den Hurk, A. F. (2016). The insect-specific Palm Creek virus modulates West Nile
348 virus infection in and transmission by Australian mosquitoes. *Parasites & Vectors*, *9*(1), 1-
349 10.
350
351 6. Goenaga, S., Kenney, J. L., Duggal, N. K., Delorey, M., Ebel, G. D., Zhang, B., ... &
352 Brault, A. C. (2015). Potential for co-infection of a mosquito-specific flavivirus, Nhumirim
353 virus, to block West Nile virus transmission in mosquitoes. *Viruses*, *7*(11), 5801-5812.
354

7. Kenney, J. L., Solberg, O. D., Langevin, S. A., & Brault, A. C. (2014). Characterization of a novel insect-specific flavivirus from Brazil: potential for inhibition of infection of arthropod cells with medically important flaviviruses. *The Journal of general virology*, *95*(0 12), 2796.

8. Hobson-Peters, J., Yam, A. W. Y., Lu, J. W. F., Setoh, Y. X., May, F. J., Kurucz, N., ... & Hall, R. A. (2013). A new insect-specific flavivirus from northern Australia suppresses replication of West Nile virus and Murray Valley encephalitis virus in co-infected mosquito cells. *PloS one*, *8*(2), e56534.

9. Phumee, A., Chompoosri, J., Intayot, P., Boonserm, R., Boonyasuppayakorn, S., Buathong, R., ... & Siriyasatien, P. (2019). Vertical transmission of Zika virus in Culex quinquefasciatus Say and Aedes aegypti (L.) mosquitoes. *Scientific reports*, *9*(1), 1-9.

10. Tesh, R. B., Bolling, B. G., & Beaty, B. J. (2016). Role of vertical transmission in mosquito-borne arbovirus maintenance and evolution. *Arbovirus: Molecular Biology, Evolution and Control; Vasilakis, N., Gubler, DJ, Eds*, 191-217.

11. Thangamani, S., Huang, J., Hart, C. E., Guzman, H., & Tesh, R. B. (2016). Vertical transmission of Zika virus in Aedes aegypti mosquitoes. *The American journal of tropical medicine and hygiene*, *95*(5), 1169.

12. Baqar, S., Hayes, C. G., Murphy, J. R., & Watts, D. M. (1993). *Vertical transmission of West Nile virus by Culex and Aedes species mosquitoes*. NAVAL MEDICAL RESEARCH UNIT NO 3 CAIRO (EGYPT) DEPT OF MEDICAL ZOOLOGY.

380

381 13. Rosen, L., Shroyer, D. A., Tesh, R. B., Freier, J. E., & Lien, J. C. (1983). Transovarial
382 transmission of dengue viruses by mosquitoes: Aedes albopictus and Aedes aegypti. *The*
383 *American journal of tropical medicine and hygiene*, *32*(5), 1108-1119.
384

385 14. Peinado, S. A., Aliota, M. T., Blitvich, B. J., & Bartholomay, L. C. (2022). Biology and
386 Transmission Dynamics of Aedes flavivirus. *Journal of Medical Entomology*.
387

388 15. McLean, B. J., Hall-Mendelin, S., Webb, C. E., Bielefeldt-Ohmann, H., Ritchie, S. A.,
389 Hobson-Peters, J., ... & van den Hurk, A. F. (2021). The insect-specific Parramatta River
390 virus is vertically transmitted by Aedes vigilax mosquitoes and suppresses replication of
391 pathogenic flaviviruses in vitro. *Vector-Borne and Zoonotic Diseases*, *21*(3), 208-215.
392

393 16. Contreras-Gutierrez, M. A., Guzman, H., Thangamani, S., Vasilakis, N., & Tesh, R. B.
394 (2017). Experimental infection with and maintenance of cell fusing agent virus (Flavivirus) in
395 Aedes aegypti. *The American journal of tropical medicine and hygiene*, *97*(1), 299.
396

397 17. Bolling, B. G., Olea-Popelka, F. J., Eisen, L., Moore, C. G., & Blair, C. D. (2012).
398 Transmission dynamics of an insect-specific flavivirus in a naturally infected Culex pipiens
399 laboratory colony and effects of co-infection on vector competence for West Nile
400 virus. *Virology*, *427*(2), 90-97.
401

402 18. Saiyasombat, R., Bolling, B. G., Brault, A. C., Bartholomay, L. C., & Blitvich, B. J.
403 (2011). Evidence of efficient transovarial transmission of Culex flavivirus by Culex pipiens
404 (Diptera: Culicidae). *Journal of medical entomology*, *48*(5), 1031-1038.

405

406 19. Lutomiah, J. J., Mwandawiro, C., Magambo, J., & Sang, R. C. (2007). Infection and
407 vertical transmission of Kamiti river virus in laboratory bred Aedes aegypti
408 mosquitoes. *Journal of Insect Science*, *7*(1).
409

410 20. Baidaliuk, A., Lequime, S., Moltini-Conclois, I., Dabo, S., Dickson, L. B., Prot, M., ... &
411 Lambrechts, L. (2020). Novel genome sequences of cell-fusing agent virus allow comparison
412 of virus phylogeny with the genetic structure of Aedes aegypti populations. *Virus*
413 *evolution*, *6*(1), veaa018.
414

415 21. Martin, E., Tang, W., Briggs, C., Hopson, H., Juarez, J. G., Garcia-Luna, S. M., ... &
416 Hamer, G. L. (2020). Cell fusing agent virus (Flavivirus) infection in Aedes aegypti in Texas:
417 seasonality, comparison by trap type, and individual viral loads. *Archives of Virology*, *165*(8),
418 1769-1776.
419

420 22. Bolling, B. G., Vasilakis, N., Guzman, H., Widen, S. G., Wood, T. G., Popov, V. L., ... &
421 Tesh, R. B. (2015). Insect-specific viruses detected in laboratory mosquito colonies and their
422 potential implications for experiments evaluating arbovirus vector competence. *The*
423 *American journal of tropical medicine and hygiene*, *92*(2), 422.
424

425 23. Stollar, V., & Thomas, V. L. (1975). An agent in the Aedes aegypti cell line (Peleg)
426 which causes fusion of Aedes albopictus cells. *Virology*, *64*(2), 367-377.
427

428 24. Patterson, E. I., Kautz, T. F., Contreras-Gutierrez, M. A., Guzman, H., Tesh, R. B.,
429 Hughes, G. L., & Forrester, N. L. (2021). Negeviruses reduce replication of alphaviruses
430 during coinfection. *Journal of Virology*, *95*(14), e00433-21.
431
432 25. Patterson, E. I., Villinger, J., Muthoni, J. N., Dobel-Ober, L., & Hughes, G. L. (2020).
433 Exploiting insect-specific viruses as a novel strategy to control vector-borne disease. *Current*
434 *opinion in insect science*, *39*, 50-56.
435
436 26. Gu, J. B., Dong, Y. Q., Peng, H. J., & Chen, X. G. (2010). A recombinant AeDNA
437 containing the insect-specific toxin, BmK IT1, displayed an increasing pathogenicity on
438 Aedes albopictus. *The American journal of tropical medicine and hygiene*, *83*(3), 614.
439
440 27. Ren, X., Hoiczyk, E., & Rasgon, J. L. (2008). Viral paratransgenesis in the malaria vector
441 Anopheles gambiae. *PLoS pathogens*, *4*(8), e1000135.
442
443 28. Ward, T. W., Jenkins, M. S., Afanasiev, B. N., Edwards, M., Duda, B. A., Suchman, E.,
444 ... & Carlson, J. O. (2001). Aedes aegypti transducing densovirus pathogenesis and
445 expression in Aedes aegypti and Anopheles gambiae larvae. *Insect molecular biology*, *10*(5),
446 397-405.
447
448 29. Ma, Q., Srivastav, S. P., Gamez, S., Dayama, G., Feitosa-Suntheimer, F., Patterson, E. I.,
449 ... & Lau, N. C. (2021). A mosquito small RNA genomics resource reveals dynamic
450 evolution and host responses to viruses and transposons. *Genome research*, *31*(3), 512-528.
451

452 30. Weger-Lucarelli, J., Rückert, C., Grubaugh, N. D., Misencik, M. J., Armstrong, P. M.,
453 Stenglein, M. D., ... & Brackney, D. E. (2018). Adventitious viruses persistently infect three
454 commonly used mosquito cell lines. *Virology*, *521*, 175-180.
455
456 31. R Core Team R: a language and environment for statistical computing. R foundation for
457 statistical computing, 2020. Available: https://www.r-project.org/
458
459 32. Wickham H ggplot2: elegant graphics for data analysis. New York: Springer-Verlag,
460 2016. https://ggplot2.tidyverse.org
461
462 33. Jeffries, C. L., White, M., Wilson, L., Yakob, L., & Walker, T. (2020). Detection of Cell-
463 Fusing Agent virus across ecologically diverse populations of Aedes aegypti on the
464 Caribbean island of Saint Lucia. *Wellcome Open Research*, *5*.
465
466 34. Ajamma, Y. U., Onchuru, T. O., Ouso, D. O., Omondi, D., Masiga, D. K., & Villinger, J.
467 (2018). Vertical transmission of naturally occurring Bunyamwera and insect-specific
468 flavivirus infections in mosquitoes from islands and mainland shores of Lakes Victoria and
469 Baringo in Kenya. *PLoS Neglected Tropical Diseases*, *12*(11), e0006949.
470
471 35. Natal Fernandes, L., de Moura Coletti, T., Julio Costa Monteiro, F., Octavio da Silva
472 Rego, M., Soares D'Athaide Ribeiro, E., de Oliveira Ribeiro, G., ... & Charlys da Costa, A.
473 (2018). A novel highly divergent strain of cell fusing agent virus (CFAV) in mosquitoes from
474 the Brazilian Amazon region. *Viruses*, *10*(12), 666.
475

476 36. Yamanaka, A., Thongrungkiat, S., Ramasoota, P., & Konishi, E. (2013). Genetic and
477 evolutionary analysis of cell-fusing agent virus based on Thai strains isolated in 2008 and
478 2012. *Infection, Genetics and Evolution*, *19*, 188-194.
479
480 37. Espinoza-Gómez, F., López-Lemus, A. U., Rodriguez-Sanchez, I. P., Martinez-Fierro, M.
481 L., Newton-Sánchez, O. A., Chávez-Flores, E., & Delgado-Enciso, I. (2011). Detection of
482 sequences from a potentially novel strain of cell fusing agent virus in Mexican Stegomyia
483 (Aedes) aegypti mosquitoes. *Archives of virology*, *156*(7), 1263-1267.
484
485 38. Cook, S., Bennett, S. N., Holmes, E. C., De Chesse, R., Moureau, G., & De Lamballerie,
486 X. (2006). Isolation of a new strain of the flavivirus cell fusing agent virus in a natural
487 mosquito population from Puerto Rico. *Journal of General Virology*, *87*(4), 735-748.
488
489 39. Werling, K. L., Johnson, R. M., Metz, H. C., & Rasgon, J. L. (2022). Sexual transmission
490 of Anopheles gambiae densovirus (AgDNV) leads to disseminated infection in mated
491 females. bioRxiv.
492
493 40. Cross, S. T., Maertens, B. L., Dunham, T. J., Rodgers, C. P., Brehm, A. L., Miller, M. R.,
494 ... & Stenglein, M. D. (2020). Partitiviruses infecting Drosophila melanogaster and Aedes
495 aegypti exhibit efficient biparental vertical transmission. *Journal of Virology*, *94*(20),
496 e01070-20.
497
498 41. Mao, Q., Wu, W., Liao, Z., Li, J., Jia, D., Zhang, X., ... & Wei, T. (2019). Viral
499 pathogens hitchhike with insect sperm for paternal transmission. *Nature*
500 *communications*, *10*(1), 1-10.