1	Vertical and horizontal transmission of cell fusing agent virus in Aedes aegypti
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19 Abstract

20 Cell fusing agent virus (CFAV) is an insect-specific flavivirus (ISF) found in Aedes aegypti 21 mosquitoes. ISFs have demonstrated the ability to modulate the infection or transmission of 22 arboviruses such as dengue, West Nile and Zika viruses. It is thought that vertical 23 transmission is the main route for ISF maintenance in nature. This has been observed with 24 CFAV, but there is evidence of horizontal and venereal transmission in other ISFs. 25 Understanding the route of transmission can inform strategies to spread ISFs to vector populations as a method of controlling pathogenic arboviruses. We crossed individually 26 27 reared male and female mosquitoes from both a naturally occurring CFAV-positive Ae. 28 aegypti colony and its negative counterpart to provide information on maternal, paternal, and 29 horizontal transmission. RT-PCR was used to detect CFAV in individual female pupal 30 exuviae and was 89% sensitive, but only 41% in male pupal exuviae. This is a possible way 31 to screen individuals for infection without destroying the adults. Female-to-male horizontal 32 transmission was not observed during this study, however there was a 31% transmission rate 33 from mating pairs of CFAV-positive males to negative female mosquitoes. Maternal vertical 34 transmission was observed with a filial infection rate of 93%. The rate of paternal 35 transmission was 85% when the female remained negative, 61% when the female acquired 36 CFAV horizontally, and 76% overall. Maternal and paternal transmission of CFAV could 37 allow the introduction of this virus into wild Ae. aegypti populations through male or female 38 mosquito releases, and thus provides a potential strategy for ISF-derived arbovirus control.

40 **Importance**

Insect-specific flaviviruses (ISFs), are a group of non-pathogenic flaviviruses that only infect 41 42 insects. ISFs can have high prevalence in mosquito populations, but their transmission routes 43 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. 44 45 Horizontal transmission of CFAV was also observed, aided by evaluation of the pupal infection status prior to mating with an infected individual. This technique of detecting 46 47 infection in discarded pupae exuviae has not been evaluated previously and will be a useful 48 tool for others in the field studying viral transmission in mosquitoes. Identifying these routes of transmission provides information about how CFAV could be maintained in wild 49 50 populations of mosquitoes, and can aid future studies focusing on interactions of CFAV with 51 their hosts and other viruses that infect mosquitoes.

53 Introduction

54 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 55 56 (ISFs) that are only known to infect insect hosts. ISFs have been detected in a range of 57 mosquito species and we are becoming increasingly aware of their interactions with other 58 mosquito viruses because of recent reports evaluating their potential to modulate arbovirus 59 infection or transmission in mosquitoes (Koh et al. 2021; Baidaliuk et al. 2019; Romo et al. 2018; Talavera et al. 2018; Hall-Mendelin et al. 2016; Goenaga et al. 2015; Kenney et al. 60 61 2014; Hobson-Peters et al. 2013; Bolling et al. 2012) and their use as chimeric vaccines 62 (Harrison et al. 2021; Hobson-Peters et al. 2019). Whilst the Flavivirus genus contains both arboviruses and ISFs, transmission is 63 64 markedly different. Arboviruses are dual-host flaviviruses transmitted through the blood-65 feeding of arthropods on viremic vertebrate hosts. After acquisition of the virus from the host, only a small fraction of subsequent transmission is vertical, where the female mosquito 66 67 passes the virus to their offspring after feeding on an infected host (Phumee et al. 2019; Tesh 68 et al. 2016; Thangamani et al. 2016; Baqar et al. 1993; Rosen et al. 1983). In contrast, vertical 69 transmission is thought to be the main route of ISF transmission.

70 A small number of studies have observed vertical transmission of ISFs in naturally 71 and experimentally infected mosquito colonies (Peinado et al. 2022; McLean et al. 2021; 72 Contreras-Gutierrez et al. 2017; Bolling et al. 2012; Saiyasombat et al. 2011; Lutomiah et al. 73 2007), including for cell fusing agent virus (CFAV), an ISF identified in several Aedes 74 aegypti cell culture lines (Stollar and Thomas, 1975), field (Baidaliuk et al. 2020; Martin et 75 al. 2020) and laboratory colony mosquitoes (Bolling et al. 2015), as well as in field caught Aedes albopictus and Culex mosquitoes (Martin et al. 2019; Fernandes et al. 2018; Cooke et 76 77 al. 2006). The observed vertical transmission rate was higher in naturally infected colonies of 78 Ae. aegypti with CFAV compared to experimentally infected colonies (Contreras-Gutierrez et 79 al. 2017), and similar results were seen in *Culex pipiens* with Culex flavivirus (CxFV) 80 (Saiyasombat et al. 2011). Filial infection rate – the percentage of offspring that were 81 infected through vertical transmission from an infected parent - from experimentally infected 82 females ranged from 0-50% for CFAV in Ae. aegypti, and 0-22% for CxFV in Cx. pipiens. For Kamiti River virus (KRV), an ISF isolated from Aedes macintoshi, the filial infection rate 83 84 from Ae. aegypti females following an infectious blood meal was 4% (Lutomiah et al. 2007). The disparate range in these experiments suggest that other forms of transmission may also 85 86 occur because the individual modes of transmission do not account for the prevalence seen in 87 naturally infected populations. ISFs can also infect male and female reproductive tissues, and 88 salivary glands (Koh et al. 2021; Frangeul et al. 2020; Romo et al. 2018; Saiyasombat et al. 89 2011), which can implicate additional vertical and horizontal transmission routes. 90 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 91 92 infected cell culture (Lutomiah et al. 2007), while Aedes flavivirus (AeFV) only infected a 93 low proportion of Ae. aegypti larvae and adults when feeding on infected cell cultures and 94 sugar meals, respectively (Peinado et al. 2022). However, the virus was not detected in water 95 used to rear CxFV-infected Cx. pipiens larvae and infection was not detected in co-reared, 96 negative larvae (Bolling et al. 2012), suggesting infected individuals did not shed virus into 97 their larval environment. Venereal transmission of CxFV and AeFV was demonstrated in 98 both directions, from male-to-female as well as female-to-male, in experiments that crossed 99 infected mosquito colonies with naïve colonies (Peinado et al. 2022; Bolling et al. 2012). 100 These rates were generally low, except for male-to-female crosses in Ae. albopictus which 101 led to an 18% infection rate (Peinado et al. 2022).

102 Further knowledge of the transmission and maintenance of ISFs in mosquito 103 populations is of high relevance, particularly in the context of interactions with pathogenic 104 arboviruses. CFAV infects important vector species and its relation to many human-105 pathogenic flaviviruses may allow it to be used to control arbovirus transmission through 106 superinfection exclusion – blocking subsequent infection of a similar virus – (Baidaliuk et al. 107 2019) or as a vehicle for paratransgenesis – using a microbe to express transgenes in its host 108 - as has been proposed for other insect-specific viruses (Patterson et al. 2021; Patterson et al. 109 2020; Gu et al. 2010; Ren et al. 2008; Ward et al. 2001). CFAV has been shown to be 110 maternally transmitted with experimentally infected female mosquitoes (Contreras-Gutierrez 111 et al. 2017), but it is not known if CFAV is paternally or horizontally transmitted. Given the 112 reduced rates of transmission seen in experimental infections, we hypothesized that multiple 113 modes of transmission occur in naturally infected colonies. To assess this, we used a 114 laboratory colony of CFAV-infected Ae. aegypti and a known uninfected colony to quantify 115 maternal, paternal, and horizontal transmission of CFAV. Our results provide insights into 116 the transmission routes of CFAV which could be used to inform strategies to spread 117 pathogen-blocking ISFs into mosquito populations.

118

119 Materials and Methods

120 Mosquitoes and virus

Established laboratory colonies of *Ae. aegypti* Galveston and Iquitos colonies (kindly provided by Prof. Nikos Vasilakis from the University of Texas Medical Branch) were maintained in a 12-hour light:12-hour dark cycle with a 1-hour 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 (Ma et al. 2021; Bolling et al. 2015). The presence and absence of CFAV in these 127 colonies was confirmed by RT-PCR prior to performing the following experiments. The
128 sequence for the CFAV isolate from the Galveston colony is available on GenBank (CFAV129 Galveston strain accession no. KJ741267).

130

131 Mosquito rearing to assess vertical and horizontal CFAV transmission

132 Eggs collected from standard colony maintenance were floated out and larvae were 133 fed ground fish food until reaching pupal stage. Pupae from each colony were sexed and 134 individually placed in 50 mL conical tubes with fresh water. Once emerged, water was 135 removed from the tube and the pupae exuviae were stored at -80 °C, the sex of the adults was 136 confirmed, and individual males were removed from their tube and placed in a tube with an 137 individual female. Mating pairs consisted of the following cohorts: i) 1 Galveston female + 1 138 Galveston male for CFAV transmission positive control; ii) 1 Iquitos female + 1 Iquitos male 139 for CFAV transmission negative control; iii) 1 Galveston female + 1 Iquitos male for female-140 to-male horizontal transmission and maternal transmission; or iv) 1 Iquitos female + 1141 Galveston male for male-to-female transmission and paternal transmission. Individual mating 142 pairs were provided with 10% sucrose and allowed to mate for three days before the males 143 were removed and stored at -80 °C. Subsequent replicates to confirm the lack of female-to-144 male transmission involved cohousing mating pairs from cohort iii) 1 Galveston female + 1 Iquitos male for 14 days. Females were presented with a blood meal consisting of 1:1 human 145 146 red blood cells and plasma via a Hemotek membrane feeding system to stimulate egg laying. 147 After two days, individual blood fed females were transferred to a 30 mL egg laying tube 148 containing water and filter paper. After another two days, females were collected and stored 149 at -80 °C. Egg papers were collected and dried for storage in the insectary until ready to rear 150 offspring. The offspring were reared as normal and adults were collected after all pupae in

151 the cup had emerged and individually stored at -80 °C. Adults in the F0 or F1 generation that 152 were deceased prior to collection were not used for detection of CFAV.

153

154 Detection of CFAV by reverse transcription polymerase chain reaction (RT-PCR)

Pupal exuviae or adults were homogenized in a 2 mL Safe-lock microcentrifuge tube 155

156 with a stainless-steel ball and RNA lysis buffer from the Zymo Quick-RNA Miniprep kit for

157 5 min at 26 Hz. RNA purification with the Zymo Quick-RNA Miniprep kit was performed

158 per the manufacturer's protocol.

One-step RT-PCR assays without denaturation were prepared with the Jena 159

160 Bioscience SCRIPT RT-PCR kit according to the manufacturer's instructions using CFAV

161 forward and reverse primers as previously described (Weger-Lucarelli et al. 2018).

162 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 163

164 amplicon of 367 bp was visualized by gel electrophoresis.

165

166 Statistical Analysis

All statistical analyses were conducted in Microsoft Excel and the R statistical 167 168 software package (http://www.r-project.org; R Core Team 2020). Binomial 95% confidence 169 intervals (CI) were calculated for sensitivity and transmission efficiencies. Graphics were 170

171

172 Data Availability

All data used for analysis is available in the manuscript. 173

generated using the package ggplot2 (Wickham 2016).

174

175 **Results**

176 Detection of CFAV in pupal exuviae

177 Assessment of horizontal and vertical transmission of CFAV requires the confirmation of infected and non-infected mosquitoes prior to the transmission event. This is 178 179 typically performed by surveying mosquitoes from the colonies to determine a baseline 180 colony infection rate, rather than detection of virus in the individuals involved in the 181 experiment. To assess the experimental individuals directly, pupae exuviae of the parental 182 generation were tested for the presence of CFAV (Figure 1). The pupal exuviae from all 183 CFAV-negative adults were negative, which indicates that the PCR assay has a specificity of 184 100% in both female and male pupae exuviae. This includes 17/17 females and 47/47 males 185 from the Iquitos colony, and 7/7 females and 1/1 male from the Galveston colony. Only 186 individuals from the Galveston colony were considered for comparison between pupae 187 exuviae and CFAV-positive adults. The resulting sensitivity was 89% (33/37; CI: 74-97%) 188 for females and 41% (11/27; CI: 22-61%) for males, with an overall sensitivity of 69% 189 (44/64; CI: 58-80%) (Table 1). The positive predictive value for CFAV detection in pupae 190 exuviae was 100% for both females (33/33) and males (11/11), and the negative predictive 191 value was 86% (24/28) for females, 76% (48/63) for males, and 79% (72/91) overall. When 192 only considering samples from the Galveston colony, the negative predictive value is 64% (7/11) for females, 6% (1/16) for males, and 30% (8/27) overall. 193

194

195 Horizontal transmission in paired mosquitoes

Adults from the parental generation were grouped into mating pairs and assessed for CFAV infection (Figure 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 (0/14) or 14 days (0/15). All Galveston females were positive, and all Iquitos males were negative
for CFAV. Male-to-female transmission was observed at a rate of 31% (5/16; CI: 11-59%) 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.

206

207 Vertical transmission from paired mosquitoes

208 Offspring from all four mating pair groups were assessed for CFAV to determine if 209 vertical transmission was possible through both maternal and paternal routes (Figure 3). 210 Vertical transmission from the Galveston colony control group was 100% (56/56; CI: 94-211 100%) for offspring from three different mating pairs (Figure 4). Offspring from three Iquitos 212 colony control mating pairs were all negative for CFAV (0/39; CI: 0-9%). Maternal 213 transmission was observed from five mating pairs with a Galveston female and an Iquitos 214 male. The filial infection rate from the five mating pairs ranged from 80-100%, with an 215 overall filial infection rate of 93% (63/68; CI: 84-98%). Paternal transmission was also 216 observed from eight mating pairs with an Iquitos female and a Galveston male, including 217 three mating pairs in which the Iquitos female also became positive. The filial infection rate 218 from mating pairs where the Iquitos female was negative varied from 33-100%, with an 219 overall rate of 85% (56/66; CI: 74-92%). For the three mating pairs with positive Iquitos 220 female adults, the filial infection rate varied from 25-80% with an overall rate of 61% (23/38; 221 CI: 43-76%). The overall filial infection rate from all eight mating pairs with Iquitos female 222 and Galveston male was 76% (79/104; CI: 67-84%).

223

224 Discussion

225 There has been a rapid expansion of known members of ISFs and other insect-specific 226 viruses, but little is known about their biology and maintenance in mosquito populations. 227 This is true even for CFAV, an ISF first discovered in 1975 and with global distribution in a 228 major vector species (Baidaliuk et al. 2020; Jeffries et al. 2020; Ajamma et al. 2018; 229 Fernandes et al. 2018; Bolling et al. 2015; Yamanaka et al. 2013; Espinoza-Gomez et al. 230 2011; Cook et al. 2006) and sustained seasonal infection (Martin et al. 2020). The detection 231 of infected larvae or pupae and lack of other known hosts has led to speculation that ISFs are 232 maintained primarily by vertical transmission. Although the results vary by virus and 233 mosquito colony, experimental infections have demonstrated that maternal transmission 234 occurs, as well as venereal transmission and the potential for other modes of horizontal 235 transmission.

236 Crossing mosquitoes from CFAV-positive and -negative colonies confirmed maternal 237 transmission and revealed paternal and horizontal transmission of CFAV. Maternal 238 transmission of CFAV was first demonstrated by Contreras-Gutierrez et al. (2017). Adult 239 females from a CFAV-negative colony were injected with CFAV, which produced an overall F1 filial infection rate of 28%, and range of 0-50% for individual females. Rearing offspring 240 241 from the F1 generation increased the overall filial infection rate to 74% in the F2 generation (range of 60-93%), similar to the control rates of 78% to 100% from previous experiments 242 243 with the Galveston colony (Contreras-Gutierrez et al. 2017) and the current rate of 100% in 244 our Galveston colony. The increase from F1 to F2 infection rates in the experimentally 245 infected colony may be due to the contributions of undetected paternal transmission and 246 chronic infection of CFAV increasing the likelihood of infecting reproductive organs in F1 247 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 248 249 females are more likely to infect reproductive organs compared to injection and 4-day

250 incubation period employed in prior experiments, although ovaries from Cx. pipiens were 251 infected with CxFV 4 days post-injection (Saiyasombat et al. 2011). Allowing sufficient time for systemic infection has also been suggested with Anopheles gambiae densovirus 252 253 (AgDNV), where vertical transmission was observed when parent mosquitoes were infected 254 at the larval stage (Ren et al. 2008), but not when females were infected through venereal 255 transmission (Werling et al. 2022). The high levels of vertical transmission seen in the 256 Galveston colony are also maintained by paternal transmission, which was responsible for an 257 overall filial infection rate of 76%. While paternal transmission has not previously been 258 evaluated in ISFs there are other well-documented examples of paternal transmission, such as 259 for verdadero virus, a partitivirus in mosquitoes (Cross et al. 2020) and rice gall dwarf virus, 260 an aphid-plant reovirus that binds to host sperm to infect offspring (Mao et al. 2019).

261 Horizontal transmission has also been demonstrated as a viable transmission route for 262 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 (Peinado et 263 264 al. 2022) than the 2.4% rate observed with CxFV in Cx. pipiens (Bolling et al. 2012). Neither 265 the current CFAV experiments nor the AeFV experiments excluded other forms of contact 266 transmission, such as sharing sugar meal sources. While transmission through food sharing did not occur with CxFV (Bolling et al. 2012) and feeding on infected sugar meals rarely 267 268 resulted in AeFV infection (Peinado et al. 2022), KRV is known to have high oral infection 269 rates (Lutomiah et al. 2007). No female-to-male transmission was observed, although this 270 occurred at a rate of 5.3% with CxFV (Bolling et al. 2012), and 2% with AeFV (Peinado et 271 al. 2022). Increasing the sample size may reveal some female-to-male transmission, but the 272 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 273 274 be differentiated from vertical transmission based on our experiments.

275 Our horizontal transmission results were strengthened by testing pupae exuviae to 276 demonstrate the lack of infection before being cohoused and mating with a positive male. 277 Prior studies have not confirmed the infection status of individual mosquitoes before the 278 potential transmission events. Although improvements for testing male pupae exuviae would 279 be desirable, the sensitivity of 89% for female pupae exuviae provides the ability to assess 280 prior infection in mosquitoes by testing pupae exuviae, which will be useful for future 281 experiments. It is unknown why sensitivity differs between female and male pupae exuviae, 282 but a previous study has shown that virus levels have a wider range and are lower titer, on 283 average, in males (Martin et al. 2020).

284 CFAV may be a useful tool to limit secondary infections with arboviruses in Ae. 285 aegypti mosquitoes. Superinfection exclusion has been demonstrated in cells and mosquitoes 286 infected with CFAV, other ISFs and insect-specific viruses. Previous studies showed that 287 initial infection with a field-derived CFAV isolate resulted in reduced dengue virus and Zika 288 virus replication and dissemination (Baidaliuk et al. 2019). However, the lack of knowledge 289 on the transmission of ISFs is a limitation in their potential use for pathogen control 290 (Patterson et al. 2020). As both maternal and paternal transmission have been confirmed, our 291 results offer the potential to establish CFAV infection in wild Ae. aegypti populations 292 through the release of infected females or males. Releasing males would be most desirable 293 because they do not contribute to the transmission of arboviruses and CFAV transmission by 294 the male-to-female horizontal route may also improve overall infection levels in the field.

295

296 Acknowledgments

297 EIP was supported by the Liverpool School of Tropical Medicine Director's Catalyst Fund

award, Brock University Research Initiative Award, and NSERC (RGPIN-2022-05099).

299 JNM was supported by the Wellcome Trust International Masters fellowship

- 300 (219672/Z/19/Z). GLH was supported by the BBSRC (BB/T001240/1 and BB/W018446/1),
- 301 the UKRI (20197), the EPSRC (V043811/1), a Royal Society Wolfson Fellowship
- 302 (RSWF\R1\180013), and the NIHR (NIHR2000907). SH was supported by a Liverpool
- 303 School of Tropical Medicine Director's Catalyst Fund award. EH, LEB and GLH were
- 304 supported by the BBSRC (V011278/1). We would like to thank Prof. Nikos Vasilakis and the
- 305 UTMB Insectary Services Core for providing the mosquito colonies used in these
- 306 experiments

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496 Tables

- 497 Table 1. Analysis of detection of CFAV in pupae exuviae versus emerged adult mosquitoes.
- 498 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 (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 (8/28)

499

501 Figures



503 Figure 1. Comparison of CFAV infection status in pupal exuviae versus adult. Red circles 504 indicate the female mosquito and blue squares represent the male in each grouped mating 505 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 506 507 the lightest shade indicates no infection detected in the pupae exuviae and adult, intermediate 508 shade indicates a no infection detected at pupae and positive for infection at adult (pupae 509 result does not agree with adult result), and the darkest shade indicates positive for infection 510 detected at both pupae and adult stages. The combination of each individual mating pair is 511 provided on the Y-axis. The 5 females with negative pupae and positive adult in the FIQ-512 MGA group are cases of horizontal transmission. FIQ = female Iquitos, MIQ = male Iquitos, FGA = female Galveston, MGA = male Galveston. 513



515

Pairs by Cohort

Figure 2. Evidence of horizontal transmission of CFAV between mating pairs. CFAV 516 infection status of F0 mosquitoes in each mating pair. Shading of red circles and blue squares 517 represent the infection status of each adult in the mating pair, with the lightest icons (F^A and 518 519 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 520 521 pair tested positive at both the pupa and adult stage. Mating pairs are assigned a colour on the 522 red-to-blue gradient based on the combined infection status of the female and male (9 523 potential outcomes). Mosquitoes that tested negative at the pupal stage and positive as an adult (F^B or M^B) are examples of potential horizontal transmission. Samples surrounded by 524 525 the black border are confirmed cases of horizontal male-to-female transmission. Horizontal transmission can only be confirmed for mosquitoes from the Iquitos colony as they were 526

from an uninfected colony and negative at the pupal stage, whereas mosquitoes from the
Galveston colony could have had an undetected infection at pupal stage. FIQ = female
Iquitos, MIQ = male Iquitos, FGA = female Galveston, MGA = male Galveston.



Pairs by Cohort



531 Figure 3. Filial infection of CFAV to assess maternal and paternal transmission. Red circles 532 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 533 534 represent the infection status of each pupa and adult in the mating pair. Histogram represents 535 the infection status of the adult offspring, with light red or blue representing a negative 536 female or male, respectively, and dark red or blue representing a positive female or male. 537 Pupae exuviae were not examined for offspring. FIQ = female Iquitos, MIQ = male Iquitos, 538 FGA = female Galveston, MGA = male Galveston.



541 Figure 4. Vertical transmission of CFAV from different mating pair combinations. The size

542 of the grey dots indicates the number of offspring from each mating pair in the group.

543 Asterisk indicates the overall mean of vertical transmission seen from all offspring from the

544 group. FIQ = female Iquitos, MIQ = male Iquitos, FGA = female Galveston, MGA = male

545 Galveston.