

1 **Running Title:** *Anopheles* mosquitoes are competent MAYV vectors

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3 **Keywords:** *Aedes*, *Anopheles*, *Culex*, Mayaro, vector competence, transmission.

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5 ***Anopheles* Mosquitoes May Drive Invasion and Transmission of Mayaro Virus across**

6 **Geographically Diverse Regions**

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36 **Abstract**

37
38 The Togavirus (Alphavirus) Mayaro virus (MAYV) was initially described in 1954 from Mayaro
39 County (Trinidad) and has been responsible for outbreaks in South America and the Caribbean.
40 Imported MAYV cases are on the rise, leading to invasion concerns similar to Chikungunya and
41 Zika viruses. Little is known about the range of mosquito species that are competent MAYV
42 vectors. We tested vector competence of 2 MAYV genotypes for six mosquito species (*Aedes*
43 *aegypti*, *Anopheles gambiae*, *An. stephensi*, *An. quadrimaculatus*, *An. freeborni*, *Culex*
44 *quinquefasciatus*). *Ae. aegypti* and *Cx. quinquefasciatus* were poor MAYV vectors, and either were
45 poorly infected or poorly transmitted. In contrast, all *Anopheles* species were able to transmit
46 MAYV, and 3 of the 4 species transmitted both genotypes. The *Anopheles* species tested are
47 divergent and native to widely separated geographic regions, suggesting that *Anopheles* may be
48 important in the invasion and spread of MAYV across diverse regions of the world.

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63 **Introduction**

64 Mayaro virus (MAYV) is a member of the genus *Alphavirus* (family Togaviridae) which
65 was first isolated from the blood of five febrile workers in Mayaro County, Trinidad, in 1954 (1).
66 MAYV is a single-stranded positive-sense RNA virus of approximately 11.7 kb and is classified in
67 three genotypes: D, L, and N (2,3). Genotype D (dispersed) includes strains isolated in several
68 South American countries, whereas genotype L (limited) includes strains isolated only in Brazil. In
69 2010, a minor genotype called N (new), was isolated in Peru, but it is limited to one known
70 sequence. Since its first isolation, MAYV has caused sporadic outbreaks and small epidemics in
71 several countries of South and Central America (reviewed in 4). In 2015, the case of an 8-year-old
72 child from Haiti co-infected with MAYV and Dengue virus (DENV) suggested that MAYV may
73 also be actively circulating in the Caribbean (5). Several imported cases recently reported in the
74 Netherlands (6), Germany (7), France (8), and Switzerland (9) highlight the need to survey naive
75 regions, such as the United States, for possible introductions of this neglected arthropod-borne virus
76 (arbovirus).

77 The symptoms of Mayaro fever (MAYF) include rash, fever, myalgia, retro-orbital pain,
78 headache, diarrhea, and arthralgia, which may persist for months or even years (10), and are similar
79 to those caused by other arboviruses, such as DENV or Chikungunya virus (CHIKV). Due to the absence of
80 routine differential diagnostics, reported cases of MAYV likely underestimate the real prevalence,
81 and the circulation of the virus can pass undetected in areas with ongoing DENV or CHIKV
82 outbreaks (4,11).

83 MAYV is thought to be principally transmitted by the bite of diurnal canopy-dwelling
84 mosquitoes of the genus *Haemagogus* (4). These mosquitoes are responsible for maintaining the
85 sylvatic cycle involving nonhuman primates and birds as primary and secondary hosts, respectively.
86 Human infections are sporadic, likely because *Haemagogus* spp. rarely display anthropophilic
87 behaviors, and they possess a preference for rural areas with proximity to forests (12). Vector
88 competence (VC) studies demonstrated that anthropophilic and urban-adapted species, such as

89 *Aedes aegypti* and *Ae. albopictus*, are competent vectors for MAYV in laboratory conditions
90 (*13,14*). *Culex quinquefasciatus* mosquitoes positive for MAYV have also been identified from
91 field collections during a DENV outbreak in Mato Grosso County, Brazil (*15*); however, their
92 capacity to transmit MAYV has not been demonstrated.

93 Overall, little data is available about the VC of mosquitoes for MAYV (*16–18*) and,
94 furthermore, there have been no studies about the VC of autochthonous vector species of the United
95 States. To address this knowledge gap, we evaluated the ability of *Anopheles stephensi* (Liston,
96 1901), *An. gambiae* (Giles, 1902), *An. freeborni* (Aitken, 1939), *An. quadrimaculatus* (Say, 1824),
97 *Cx. quinquefasciatus* (Say, 1823), and *Ae. aegypti* (Linnaeus, 1762) to become infected with and
98 transmit MAYV after feeding on a viremic blood meal. Our results demonstrate that while *Ae.*
99 *aegypti* and *Cx. quinquefasciatus* are poor vectors for MAYV, all tested *Anopheles* species were
100 competent laboratory vectors for MAYV, including species that they have the potential to support
101 the transmission cycle if the virus is introduced into the United States. Additionally, the results of
102 our study provide useful information to improve entomologic surveillance programs and prevent
103 future outbreaks of this emerging neglected pathogen.

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105 **Material and Methods**

106 Six mosquito species were used in this experimental study. The *An. gambiae* (NIH strain)
107 were originally obtained from The National Institutes of Health (Bethesda, MD, USA). *An.*
108 *stephensi* (Liston strain) were provided by Johns Hopkins University (Baltimore, MD, USA). *Cx.*
109 *quinquefasciatus* (Benzon strain) were provided by the Wadsworth Center (Slingerlands, NY, USA)
110 and was initially derived from a colony maintained by Benzon Research (Carlisle, PA, USA). *An.*
111 *quadrimaculatus* (Orlando strain, MRA-139) and *An. freeborni* (F1 strain, MRA-130) were
112 provided by BEI Resources (Manassas, VA, USA). *Ae. aegypti* (Rockefeller strain) were provided
113 by Johns Hopkins University.

114 Mosquito colonies were reared and maintained at the Millennium Sciences Complex
115 insectary (The Pennsylvania State University, University Park, PA, USA) at $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$, 12:12 h
116 light:dark diurnal cycle at 80% relative humidity in 30×30×30-cm cages. Ground fish flakes
117 (TetraMin, Melle, Germany) were used to feed *Anopheles* spp. and *Aedes* sp. larvae. A 1:1:1
118 mixture of bovine liver powder (MP Biomedicals, Solon, OH, USA), koi pellets (TetraPond Koi
119 Vibrance; TetraPond, Prestons, Australia), and rabbit pellets (Kaytee, Chilton, WI, USA) was used
120 for *Culex* sp. larvae. Adult mosquitoes were provided with 10% sucrose solution *ad libitum* for
121 maintenance. For reproduction and virus infection purposes, adults were fed with expired
122 anonymous human blood (Biological Specialty Corporation, Colmar, PA, USA).

123 Two strains of MAYV were used for the experimental infections: BeAr 505411 (BEI
124 Resources, Manassas, VA, USA), a genotype L strain isolated from *Haemagogus janthinomys*
125 mosquitoes in Para, Brazil, in March 1991, and BeAn 343102 (BEI Resources, Manassas, VA,
126 USA), a genotype D strain originally isolated from a monkey in Para, Brazil, in May 1978. Both
127 viruses were passed once in African green monkey kidney (Vero) cells. Virus-infected supernatant
128 was aliquoted and stored at -70°C until used for mosquito infections. Viral stock titers were
129 obtained by the focus forming unit (FFU) technique, as described below.

130 Five- to seven-day-old females that had not previously blood-fed were used in this
131 experiment. The mosquitoes were allowed to feed on infected human blood via a glass feeder
132 jacketed with 37°C distilled water for 1 h. Aliquots of the infectious bloodmeals were collected and
133 titers of MAYV were determined by FFU (Table 1). After blood feeding, mosquitoes were
134 anesthetized and fully engorged females were selected and placed in cardboard cages. Infection rate
135 (IR), dissemination rate (DIR), transmission rate (TR), and transmission efficiency (TE) were
136 assessed at 7 and 14 days post-infection (dpi). IR was measured as the rate of mosquitoes with
137 infected bodies among the total number of analyzed mosquitoes. DIR was measured as the rate of
138 mosquitoes with infected legs among the mosquitoes with positive bodies. TR was measured as the
139 rate of mosquitoes with infectious saliva among the mosquitoes with positive legs, and TE

140 measured as the rate of mosquitoes with infectious saliva among the total number of analyzed
141 mosquitoes (19).

142 At 7 and 14 dpi, mosquitoes were anesthetized with triethylamine (Sigma, St. Louis, MO,
143 USA). Legs were detached from each body and placed in 2-mL tubes filled with 1 mL of mosquito
144 diluent (20% heat-inactivated fetal bovine serum [FBS] in Dulbecco's phosphate-buffered saline
145 [PBS], 50 µg/mL penicillin/streptomycin, 50 µg/mL gentamicin, and 2.5 µg/mL fungizone) and a
146 single zinc-plated, steel, 4.5-mm bead (Daisy, Rogers, AR, USA), and tubes immediately placed on
147 ice. Saliva was collected using a capillary technique as previously described (20), expelled into in a
148 2-mL tube filled with 100 µL of mosquito diluent, and immediately placed on ice. Body and leg
149 samples were homogenized at 30□Hz for 2□mi using a TissueLyser II (QIAGEN GmbH, Hilden,
150 Germany) and centrifuged for 30 sec at 11,000 rpm. All samples were stored at –70°C until tested.

151 The presence of infectious MAYV particles in the body, legs, and saliva samples was tested
152 by FFU assay in Vero cells. Vero cells were grown to a confluent monolayer in 96-well plates at
153 37°C with 5% CO₂ in complete media (1× Dulbecco's modified-essential media [DMEM], 100
154 units/mL penicillin/streptomycin, and 10% FBS). The next day, wells were washed with DMEM
155 without FBS and incubated with a 30-µL aliquot of each homogenized tissue sample for 2 h at
156 37°C. After the incubation step, the 30-µL aliquot was removed from the cell monolayer and any
157 unattached viral particles were removed with a DMEM wash. A total of 100 µL of overlay medium
158 (1% methyl cellulose in complete growth medium) was dispensed into each well, and plates were
159 incubated at 37°C inside the CO₂ incubator. Cells were fixed at 24 h (bodies and legs samples) or
160 48 h (saliva samples) post-infection with 4% paraformaldehyde (Sigma, St. Louis, MO, USA).
161 Fixed cells were blocked and permeabilized for 30 min with blocking solution containing detergent
162 (3% bovine serum albumin and 0.05% Tween 20 in PBS) and washed with cold PBS. Viral antigens
163 in infected cells were labeled using the monoclonal anti-Chikungunya virus E2 envelope
164 glycoprotein clone CHK-48 (which reacts with Alphaviruses) (BEI Resources, Manassas, VA,
165 USA) diluted 1:500 in blocking solution. Subsequently, cells were washed 4 times with cold PBS to

166 remove unbound primary antibodies. The primary antibody was labeled with the Alexa-488 goat
167 anti-mouse IgG secondary antibody (Invitrogen, Life Science, Eugene OR, USA) at a dilution of
168 1:500, and green fluorescence was observed and evaluated with an Olympus BX41 inverted
169 microscope equipped with an UPlanFI 4× objective and a FITC filter (Figure 1). Fluorescent foci
170 were counted for each well, and virus titers were calculated and expressed as FFU/mL.

171 Data were analyzed using GraphPad Prism version 7.04. Differences in the IR, DIR, TR,
172 and TE of mosquitoes challenged with BeAr 505411 and BeAn 343102 were analyzed by Fisher's
173 exact test. The Mann-Whitney U test was used to compare the body, legs, and saliva viral titers of
174 mosquitoes exposed to BeAr 505411 or BeAn 343102.

175

176 **Results**

177 A total of 115 *Ae. aegypti*, 132 *An. stephensi*, 31 *An. gambiae*, 29 *An. quadrimaculatus*, 19
178 *An. freeborni*, and 60 *Cx. quinquefasciatus* were analyzed in this study. Details of analyzed
179 mosquitoes and the IR, DIR, TR, and TE are in Table 1.

180 All six mosquito species were susceptible to infection with MAYV to some degree, although
181 there were MAYV strain-specific differences. IRs for *Ae. aegypti* exposed to strain BeAr 505411
182 were significantly higher compared to strain BeAn 343102 ($p<0.0001$) at 7 dpi, and IRs for strain
183 BeAr 505411 at 7 dpi were significantly higher than 14 dpi ($p<0.0001$). Moreover, no *Ae. aegypti*
184 exposed to strain BeAn 343102 became infected at 14 dpi despite the presence of positive
185 mosquitoes at 7 dpi. IRs for *An. stephensi* and *An. gambiae* were similar across MAYV strains, and
186 IRs increased over time in *An. gambiae*. *An. quadrimaculatus* and *An. freeborni* were susceptible to
187 infection with both strains of MAYV, and *Cx. quinquefasciatus* was susceptible only to a low-
188 frequency infection with strain BeAr 505411.

189 Once infected, all tested mosquito species developed a disseminated infection. Disseminated
190 infection was generally detected as early as 7 dpi, with the exception of *An. freeborni* exposed to
191 the BeAr 505411 strain. DIRs were similar for both virus strains in *An. stephensi* and *An. gambiae*

192 at both timepoints and for *Ae. aegypti* at 7 dpi. There was a trend toward higher DIRs for strain
193 BeAn 343102 compared to strain BeAr 505411 in *An. quadrimaculatus* and *An. freeborni* at day 7.
194 There was also a trend toward a higher DIR at 14 dpi than at 7 dpi for strain BeAr 505411 in *Ae.*
195 *aegypti*, both strains in *An. gambiae*, and strain BeAr 505411 in *An. freeborni*.

196 Transmission was detected in all *Anopheles* species and *Ae. aegypti* (albeit very poorly), but
197 not in *Cx. quinquefasciatus*. *An. stephensi*, *An. gambiae*, and *An. quadrimaculatus* were able to
198 transmit both MAYV strains tested. For *Ae. aegypti* only a single transmission event was detected
199 for virus strain BeAr 505411. Only virus strain BeAn 343102 was transmitted by *An. freeborni*.
200 Both virus strains were able to be transmitted by *An. gambiae*, *stephensi*, and *quadrimaculatus*.

201 MAYV titers for all samples were calculated and expressed as FFU/mL (Figure 2). *Ae.*
202 *aegypti* exposed to strain BeAr 505411 had significantly greater titers in the bodies (7 and 14 dpi)
203 and legs (7 and 14 dpi) compared to strain BeAn 343102 ($p<0.0001$) (Figure). Conversely, *An.*
204 *stephensi* exposed to strain BeAn 343102 had significantly greater titers in the bodies (7 dpi,
205 $p<0.05$; 14 dpi, $p<0.001$) and legs (7 dpi, $p<0.001$) compared to strain BeAr 505411 (Figure 2).
206 There were no significant differences in body, legs, or saliva titers between the MAYV strains in
207 *An. gambiae*, *An. quadrimaculatus*, *An. freeborni*, and *Cx. quinquefasciatus*.

208

209 Discussion

210 These results demonstrate that *An. stephensi*, *An. gambiae*, *An. quadrimaculatus*, and *An.*
211 *freeborni* are competent laboratory vectors for MAYV. The two viral strains tested present
212 significant differences in their ability to infect and disseminate in *Ae. aegypti* and *An. stephensi*. In
213 *An. stephensi*, the strain BeAn 343102 had a statistically higher titer in body and legs samples than
214 BeAr 505411. Conversely, strain BeAn 343102 has a statistically lower body titer in *Ae. aegypti*
215 and was not detected in legs, likely indicating the presence of a midgut escape barrier. Finally,
216 strain BeAn 343102 failed to infect *Cx. quinquefasciatus*, likely due to the presence of a midgut
217 infection barrier. The VC differences between the strains may be explained by the theory of the host

218 genotype and pathogen genotype ($G \times G$) interaction (21). $G \times G$ interactions have been found in
219 many systems, including DENV (22). For example, Lambrechts et al. (22) showed that DENV
220 vector competence varied greatly depending on the specific *Ae. aegypti* population and DENV
221 genotype combination. This provides evidence that $G \times G$ interactions may be responsible for the
222 adaptation of a lineage/strain to a specific population.

223 *Ae. aegypti*, *An. stephensi*, *An. quadrimaculatus*, and *An. freeborni* were able to transmit the
224 virus at 7 dpi but we did not detect transmission at 14 dpi. The short extrinsic incubation period
225 (EIP) of MAYV for these species might represent a notable increase in their vectorial capacity (23)
226 and must be considered when establishing a future surveillance plan. In *An. stephensi*, the absence
227 of transmission at 14 dpi corresponds with a decrease of the viral titer in the legs between 7 dpi and
228 14 dpi. These data suggest that in *An. stephensi*, MAYV infection may not persist, and may be
229 progressively eliminated or limited by the vector. Similar results were recently published for *Ae.*
230 *aegypti* infected with DENV (24). In that study, a progressive decrease of transmission began at 14
231 dpi and continued until 25 dpi, at which point no viral transmission was recorded. To test this
232 hypothesis and to better understand the kinetics of MAYV infection, a study with a longer EIP and
233 more intermediate timepoints should be performed. *An. gambiae* also is a competent laboratory
234 vector for MAYV but the longer EIP (14 dpi) required for the transmission of the virus might limit
235 the role of this species in the transmission cycle.

236 With the *Ae. aegypti* strain tested here, we obtained similar IR and DIR results compared to
237 those previously described with a different strain (16). However, the MAYV TR in this study is
238 considerably lower than that described by Long et al. (14) (6.7% vs. 88%). This discrepancy could
239 be due to the genetic differences in the mosquito population (salivary gland infection barrier in the
240 strain tested) or in the viral strain used for the experiment.

241 The global expansion of CHIKV due to a single point mutation (30) has previously
242 demonstrated that the adaptation of an arbovirus to a new vector species can be devastating. The
243 adaptation of MAYV to the *Aedes* vector has been analyzed (31), and the emergence of hybrid

244 genotypes D and L suggests that *Aedes* mosquitoes can play an important role in the urban diffusion
245 of MAYV. *Ae. aegypti* and *Ae. albopictus* are well adapted to urban and peri-urban habitats, and,
246 contrary to *Haemagogus* mosquitoes, consistently have anthropophilic feeding behavior. Our results
247 confirm that *Ae. aegypti* is a possible (if potentially inefficient) vector for MAYV, but more studies
248 are needed to understand the differences in the VC for the genotype D and genotype L strains.

249 We found that *Cx. quinquefasciatus* can be infected with MAYV strain BeAr 505411, but is
250 not able to transmit the virus. Conversely, another study found MAYV-positive *Cx.*
251 *quinquefasciatus* during an outbreak of DENV in Mato Grosso, Brazil, and suggested that this
252 species could sustain the transmission cycle (15). These results highlight the important point that
253 merely detecting virus in a mosquito does not necessarily implicate it as a vector.

254 Previously, only two alphaviruses were known to be transmitted by *Anopheles* mosquitoes:
255 O'nyong-nyong virus (25) and a single record for CHIKV (26). However, in the original paper
256 describing the isolation of MAYV, the authors present an anecdote (no data) stating that when
257 inoculated into *An. quadrimaculatus* from Trinidad, MAYV was able to replicate (although neither
258 oral infection nor transmission was investigated) (1). The capacity of *An. quadrimaculatus* and *An.*
259 *freeborni* to transmit MAYV is particularly relevant to the United States, because the estimated
260 geographic distribution of these species covers the entirety of the country (27,28). If MAYV was
261 introduced into the United States, these two mosquito species may have the capacity to sustain the
262 transmission cycle and spread the virus throughout the country. An interesting and important aspect
263 of *Anopheles* vector biology is their tendency to have multiple feeding events during a single
264 gonotrophic cycle (29). The bite frequency of *Anopheles* mosquitos increases their vectorial
265 capacity and make them a very effective vector (23). For these reasons, we highlight the need for
266 more studies on the possible role of *Anopheles* mosquitoes in spreading arboviruses in the United
267 States.

268 We tested 4 *Anopheles* species (2 from North America, one from Africa, and one from
269 Southeast Asia) for MAYV VC, and all were able to transmit the virus. Our results illustrate the
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270 knowledge gaps that remain about this important emerging virus. *Anopheles* mosquitoes in general
271 are currently neglected as potential vectors of arboviral pathogens. Our data suggest that *Anopheles*
272 sp. may be important vectors driving the emergence and invasion of MAYV (and potentially other
273 arboviruses) across geographically diverse regions of the globe, and their epidemiological role in
274 virus invasions should be further studied.

275

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Table . Infection, dissemination, and transmission rates for mosquitoes orally exposed to Mayaro virus.

Mosquito species	Strain	Viral dose (log ₁₀ FFU/mL)	7 dpi					14 dpi				
			N [*]	IR% [†]	DIR% [‡]	TR% [§]	TE% [¶]	N [*]	IR% [†]	DIR% [‡]	TR% [§]	TE% [¶]
<i>Ae. aegypti</i>	BeAr 505411	7	29	86.2	60	6.7	3.4	29	51.7	80	0	0
	BeAn 343102	7.1	28	7.1	50	0	0	29	0	0	0	0
<i>An. stephensi</i>	BeAr 505411	7	35	71.4	96	12.5	7.5	41	78.8	100	0	0
	BeAn 343102	7.2	28	89.3	96	12.5	8.57	28	85.7	95.8	0	0
<i>An. gambiae</i>	BeAr 505411	6.5	9	55.5	22.2	0	0	6	100	100	50	50
	BeAn 343102	7.1	12	75	16.7	0	0	4	100	100	100	100
<i>An. quadrimaculatus</i>	BeAr 505411	7	14	78.6	18.2	50	7.1	10	100	30	0	0
	BeAn 343102	6.8	5	20	100	100	20	NA	NA	NA	NA	NA
<i>An. freeborni</i>	BeAr 505411	7	6	16.7	0	0	0	4	75	66.7	0	0
	BeAn 343102	6.8	8	37.5	100	66.7	12.5	1	0	0	0	0
<i>Cx. quinquefasciatus</i>	BeAr 505411	7	19	5.3	100	0	0	20	10	100	0	0
	BeAn 343102	7	10	0	0	0	0	11	0	0	0	0

^{*}number of analyzed mosquitoes

[†]infection rate, percentage of mosquitoes with positive body/analyzed mosquitoes

[‡]dissemination rate, percentage of mosquitoes with positive legs/mosquitoes with positive body

[§]transmission rate, percentage of mosquitoes with positive saliva/mosquitoes with positive legs

[¶]transmission efficiency, percentage of mosquitoes with positive saliva/analyzed mosquitoes

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438 **Figure 1. Fluorescent foci formation in Vero cells following infection with Mayaro virus.**

439 Vero cells were incubated with samples homogenate (body, legs or saliva). After 24 (body and legs)
440 or 48 hours post infection (saliva) the monolayer was fixed, permeabilized, stained with antibody
441 for alphavirus-E2 and visualized by immunofluorescence microscopy.

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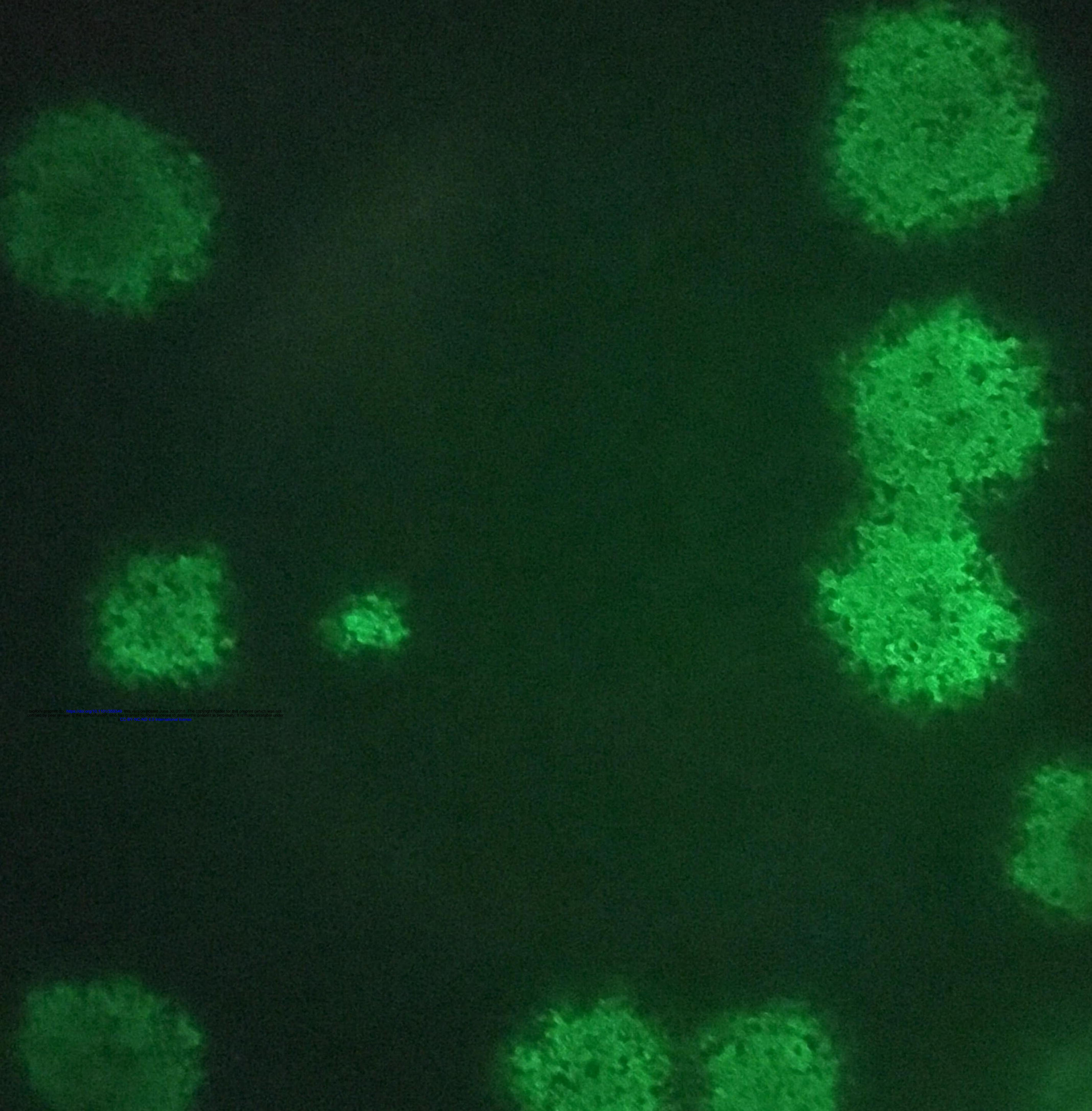
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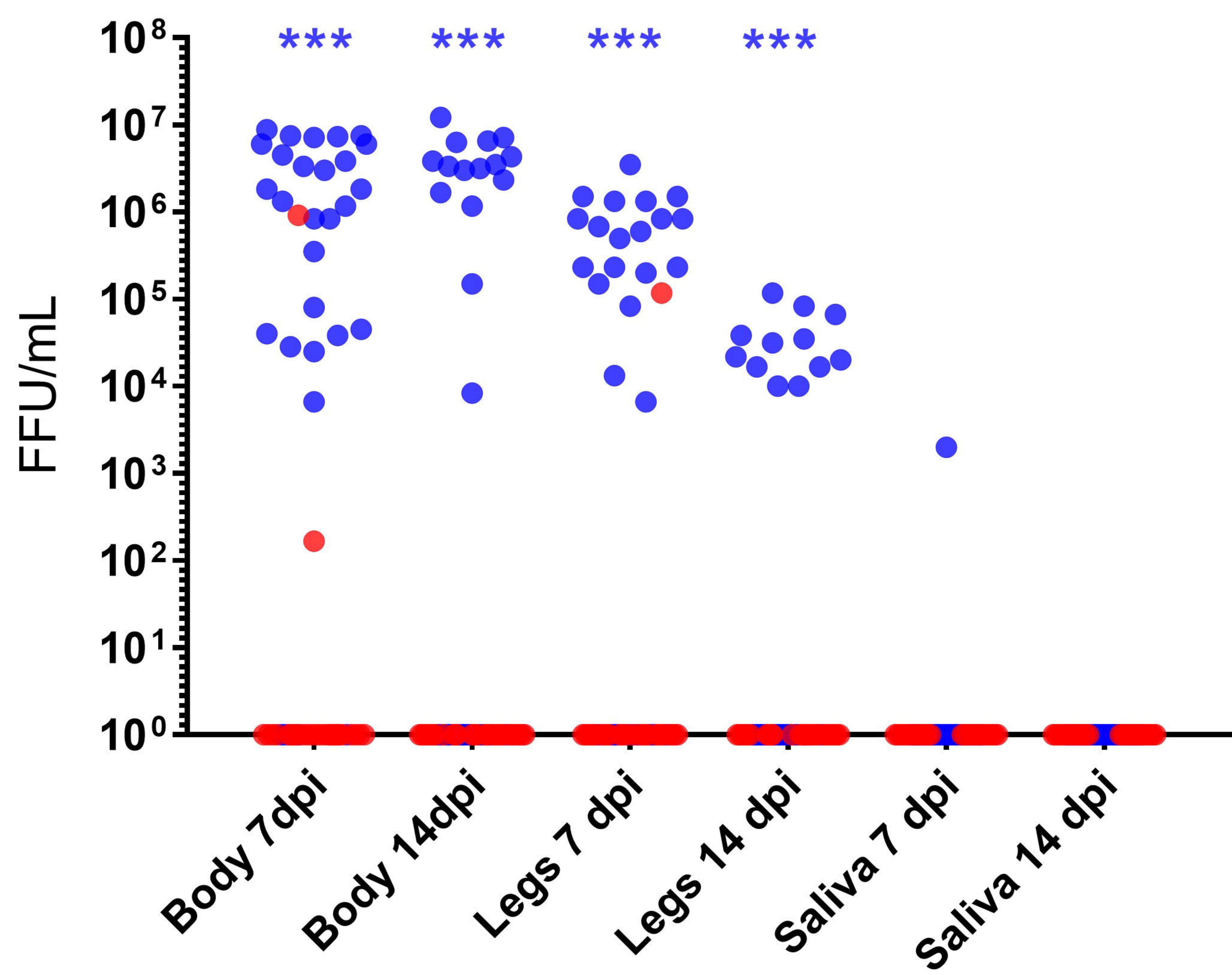
446 **Figure 2. Viral titer in body, legs, and saliva of six mosquito species mosquitoes exposed to**

447 **Mayaro virus.** Each dot corresponds to a single mosquito sample. Viral titers were statistically
448 compared between strains by Mann-Whitney U test.

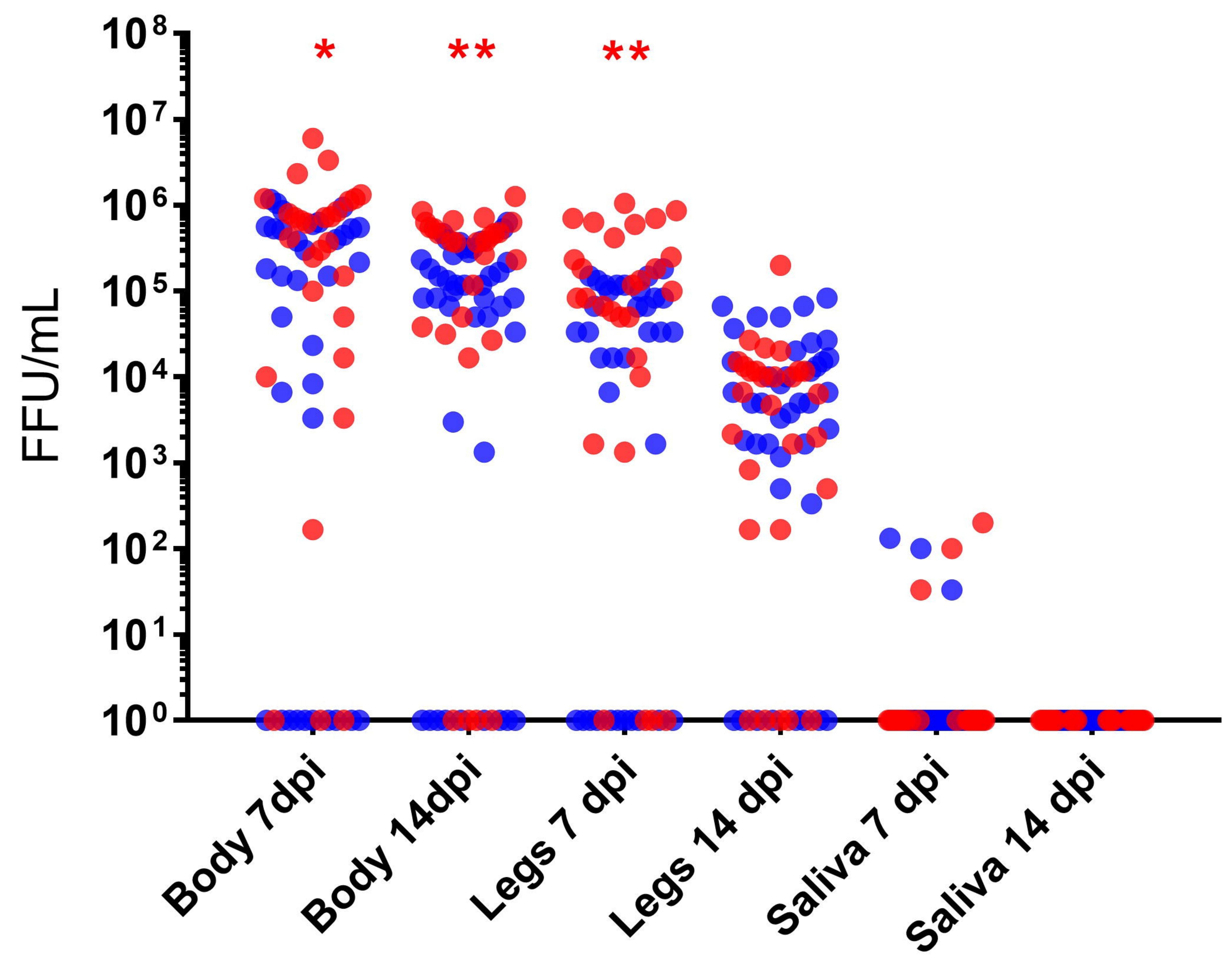
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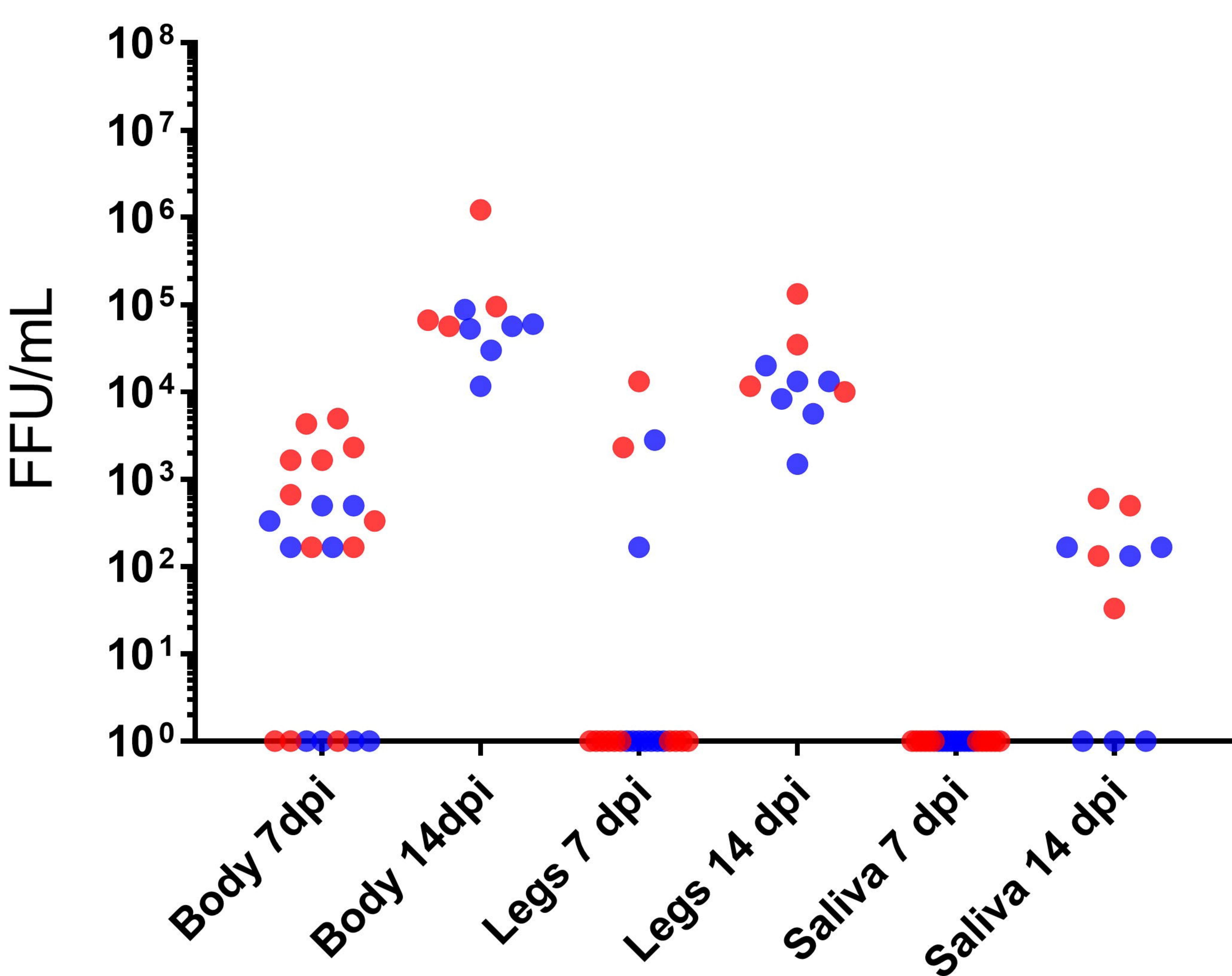
Ae. aegypti



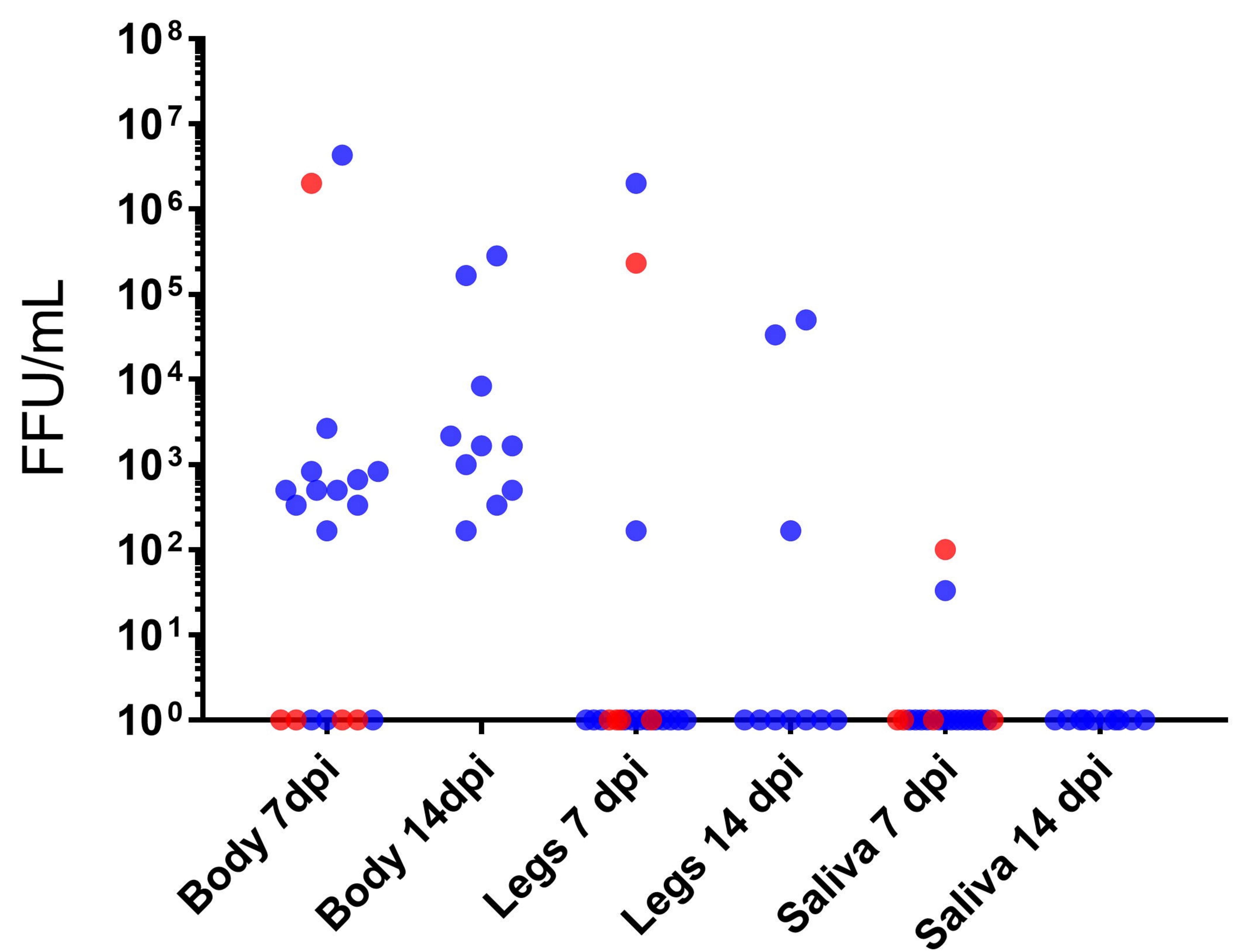
An. stephensi



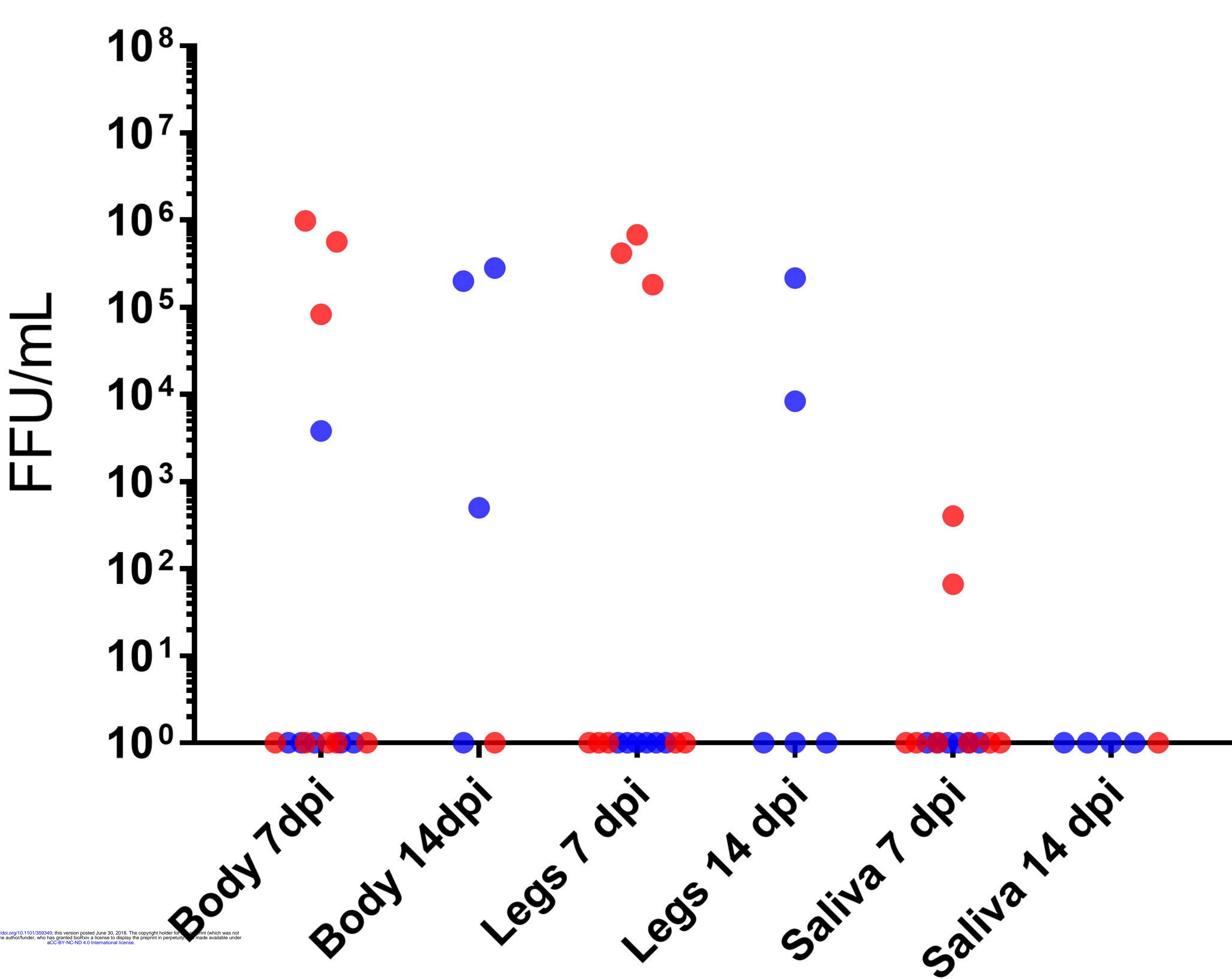
An. gambiae



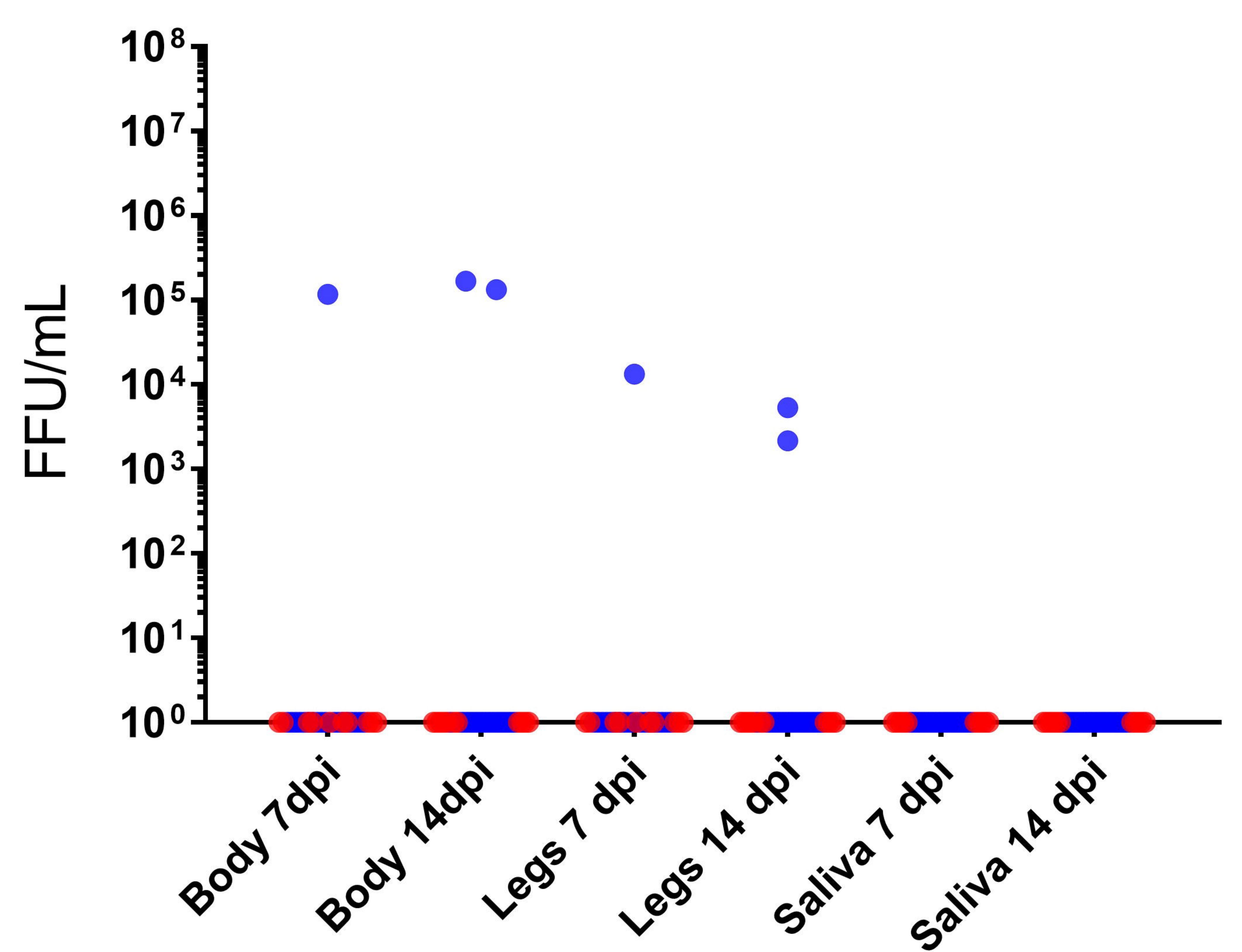
An. quadrimaculatus



An. freeborni



Cx. quinquefasciatus



● MAYV BeAr 505411

● MAYV BeAn 343102

*** = $P < 0.0001$; ** = $P < 0.001$; * = $P < 0.05$