

Three-way interactions between mosquito population, viral strain and temperature underlying chikungunya virus transmission potential

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1	Three-way interactions between mosquito population, viral strain and temperature
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21	Running title: Complex interactions influence chikungunya virus transmission
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26 Abstract

27

Interactions between pathogens and their insect vectors in nature are under the control of both 28 29 genetic and non-genetic factors, yet most studies on mosquito vector competence for human pathogens are conducted in laboratory systems that ignore genetic and environmental 30 variability. Evaluating the risk of emergence of arthropod-borne viruses (arboviruses) of 31 32 public health importance such as chikungunya virus (CHIKV) requires a more realistic 33 appraisal of genetic and environmental contributions to vector competence. In particular, sources of variation do not necessarily act additively and may combine in the form of 34 35 interactions. Here, we measured CHIKV transmission by the mosquito Aedes albopictus in all combinations of six worldwide vector populations, two virus strains and two ambient 36 temperatures (20°C and 28°C). Overall, CHIKV transmission by Ae. albopictus strongly 37 38 depended on the three-way combination of mosquito population, virus strain and temperature. 39 Such genotype-by-genotype-by-environment (G x G x E) interactions question the relevance 40 of vector competence studies conducted with a simpler set of conditions. Our results highlight 41 the need to account for the complex interplay between vectors, pathogens and environmental factors to accurately assess the potential of vector-borne diseases to emerge. 42 43 Keywords: mosquito, arbovirus, chikungunya, temperature, transmission, interaction 44

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51 **1. Introduction**

52 Our mechanistic understanding of interactions between pathogens and their insect vectors has made considerable progress in the last two decades. In particular, tremendous 53 54 knowledge has been generated on the immune responses of mosquitoes against pathogens of public health importance such as arthropod-borne viruses (arboviruses) and malaria parasites 55 reviewed in [1,2]. To date, however, the vast majority of studies on vector-pathogen 56 interactions have been conducted in laboratory models that ignore the genetic and 57 58 environmental variability of natural situations [3]. Only very recently, studies begun to consider that natural vector-pathogen interactions occur in a variable world. Vector 59 60 competence, defined as the ability of an insect to become infected and subsequently, transmit a pathogen, is a quantitative trait that displays substantial variation in natural populations. As 61 in many other host-parasite associations, vector-pathogen interactions are governed by 62 63 genotype-by-genotype (G x G) interactions, whereby the outcome of infection depends on the specific pairing of vector and pathogen genotypes [4,5]. The existence of such G x G 64 65 interactions implies that the effect of vector genes controlling competence depends on the pathogen genotype [6-8]. Several recent ecological studies have also emphasized the role of 66 environmental factors, such as ambient temperature, in shaping mosquito vector competence 67 for pathogens [9,10]. For example, the immune response and resistance of Anopheles 68 69 mosquitoes to bacterial challenge strongly depended on environmental drivers such as mean 70 temperature, diurnal temperature variation, and time of infection [11]. Likewise, both the mean and daily amplitude of temperature variation influenced Aedes aegypti vector 71 72 competence for dengue virus [12,13]. In the natural environment, vector-pathogen 73 interactions are likely governed by complex genotype-by-genotype-by environment (G x G x 74 E) interactions [9,14], but this has yet to be documented.

75 Accounting for such ecological complexity is especially important when assessing the 76 vector competence of natural insect populations to evaluate the risk of vector-borne disease emergence. For instance, the presence of competent insect vectors is the key factor in 77 78 assessing the risk of arboviral emergence [15], but vector competence for arboviruses is a dynamic process. This was illustrated during the recent emergence of chikungunya virus 79 80 (CHIKV), a mosquito-borne alphavirus usually transmitted among non-human primates by 81 forest-dwelling mosquitoes. In 2004, a chikungunya outbreak emerged on the coast of Kenya. 82 The mosquito incriminated was the typical vector Ae. aegypti ensuring inter-human CHIKV transmission. The virus subsequently spread to several islands of the Indian Ocean where 83 84 transmission was predominantly achieved by an alternative vector, Ae. albopictus [16,17]. The switch from Ae. aegypti to Ae. albopictus was associated with the selection of an amino-85 acid change from an alanine to a valine at position 226 of CHIKV E1 glycoprotein (E1-86 87 A226V) causing increased replication, midgut infection, dissemination and transmission in 88 Ae. albopictus but not in Ae. aegypti [18,19]. This adaptive mutation conferring enhanced 89 transmission by Ae. albopictus is thought to have occurred in at least three independent 90 occasions in the Indian Ocean region, the Indian subcontinent and Central Africa, supporting the hypothesis of evolutionary convergence [16,20]. Since the 2004 outbreak, CHIKV has 91 emerged worldwide including temperate regions, such as Italy where several hundred 92 93 autochthonous cases were reported in 2007 [21].

In this study, we investigated the combined influence of genetic and environmental variations on the risk of CHIKV transmission by a collection of *Ae. albopictus* populations from both tropical and temperate regions. We determined the respective contributions of virus genotype, mosquito genotype, ambient temperature and their interactions to variation in CHIKV transmission.

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101 **2. Material and methods**

102 (a) Ethics Statement

Laboratory mice were used to blood feed mosquitoes for egg production (see below). The Institut Pasteur animal facility has received accreditation from the French Ministry of Agriculture to perform experiments on live mice in appliance of the French and European regulations on care and protection of the Laboratory Animals (EC Directive 2010/63, French Law 2013-118, February 6th, 2013). Protocols were approved by the veterinary staff of the Institut Pasteur animal facility and were performed in compliance with the NIH Animal Welfare Insurance #A5476-01 issued on 31/07/2012.

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111 (b) Mosquito populations and viral strains

112 Mosquito populations were sampled in the field as eggs collected with ovitraps (more than 113 500 eggs per population) that were brought back to the laboratory and reared in an insectary 114 for less than 10 generations before experimental infections. Sampling locations included three 115 temperate regions in Europe: France (Bar-sur-Loup, F₃-F₄ generations), Italy (Castiglione-di-Cervia, F_3 generation), Montenegro (Ulcinj, F_1 - F_2 generation); and three tropical overseas 116 regions: Brazil (Manaus, F₃ generation), Vietnam (Bình Phước, F₈-F₉ generation) and La 117 118 Réunion island (Providence, F8-F9 generation). After hatching, larvae were split into pans of 119 150 individuals and supplied every two days with a yeast tablet dissolved in 1 liter of 120 dechlorinated tap water. Emerging adults were maintained at 28°C±1°C with a light: dark 121 cycle of 16h: 8h, 80% relative humidity and supplied with a 10% sucrose solution. Females 122 were blood fed three times a week on anaesthetized mice (OF1 mice, Charles River 123 laboratories, France).

Experimental infections of mosquitoes used two CHIKV strains, named 06-021 and 124 125 2010-1909 here after, that were kindly provided by the French National Reference Center for 126 Arboviruses at Institut Pasteur. The 06-021 strain was isolated on C6/36 cells (Ae. albopictus 127 cell line) from a patient on La Réunion island in 2005 [22]. The 2010-1909 strain was isolated 128 on Vero cells (African green monkey kidney cell line) from an autochthonous human case in 129 Southeast France in 2010 [23]. Following isolation, both strains were passaged twice on C6/36 cells and the viral stocks produced were stored at -80°C prior to their use in mosquito 130 131 oral infections [19,22]. The viral titer estimated by plaque assay on Vero cells [19], was 10^9 plaque-forming units per mL (PFU/mL) and 10⁸ PFU/mL for 06-021 and 2010-1909 CHIKV 132 strains, respectively. Phylogenetic analysis of the complete viral genome sequences (11,237 133 nucleotides) showed that the two strains belonged to the East-Central-South Africa 134 phylogroup (ECSA) (Figure 1; Tables S1 and S2). 135

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137 (c) Mosquito oral infections and temperature regimes

138 Each of the six Ae. albopictus populations (three temperate and three tropical populations) 139 was simultaneously challenged with the two CHIKV strains (06-021 or 2010-1909) and then 140 split into two subsets that were incubated at 20°C or 28°C. The entire experiment was repeated twice. For each experiment, 7- to 10-day-old females were fed on an infectious 141 blood-meal provided at a final titer of 10^{7.5} PFU/mL. The blood-meal mixture contained two 142 thirds of washed rabbit erythrocytes, one third of viral suspension and ATP as a 143 144 phagostimulant at a final concentration of 10 mM. Mosquito feeding was limited to 50 145 minutes and non-engorged females were discarded. Fully engorged females were transferred 146 to cardboard cups and maintained on 10% sucrose in climatic chambers (KB 53, Binder, 147 Tuttlingen, Germany) set at constant temperatures of 20°C±0.1°C or 28°C±0.1°C, with a light: dark cycle of 16h: 8h and 70% relative humidity. The temperature 20°C was chosen as 148

representative of the low-temperature threshold recorded when local transmission occurred during the Italian epidemic between June and September 2007 [21,24] and in Southeast France in September 2010 (http://www.meteociel.fr) [23], whereas 28°C was chosen as a typical average temperature in tropical regions and has been commonly used in vector competence assays to CHIKV [19, 25-27].

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155 (d) Vector competence phenotypes

Due to the relatively high blood-meal titer of 10^{7.5} PFU/mL, 100% of engorged mosquitoes 156 were considered to have established a midgut infection [25]. Vector competence was assessed 157 158 based on two conventional phenotypes: viral dissemination from the midgut and transmission 159 potential [26]. For each experimental condition, viral dissemination and transmission was 160 tested in 12-20 mosquitoes 6 days post-infection (pi). CHIKV dissemination and transmission 161 were previously found to reach a maximum at 6 days pi in Ae. albopictus maintained at 28°C 162 [25,27]. Transmission potential was measured by forced salivation as previously described 163 [26]. Briefly, legs and wings of each mosquito were removed and the mosquito's proboscis 164 was inserted into a micropipette tip containing 5 μ L of foetal bovine serum (FBS). After 45 165 minutes, the saliva-containing FBS was expelled into 45 μ L of Leibovitz L15 medium (Invitrogen Life Technologies, Carlsbad, CA). After salivation, the head of each mosquito 166 167 was removed and homogenized individually in 135 μ L of Leibovitz L15 medium. 168 Homogenates were then supplemented with 10% FBS and stored at -80°C before processing. 169 Transmission efficiency (TE) was calculated as the overall proportion of females that had 170 infectious saliva (*i.e.*, among all tested females with or without a disseminated infection). TE 171 was then broken down in two intermediate indices. Dissemination efficiency (DE) was 172 calculated as the proportion of females with infected head tissues (i.e., in which the virus successfully disseminated from the midgut). Transmission rate (TR) was defined as the 173

proportion of females with infectious saliva among those that developed a disseminatedinfection. Therefore, TE equals the product of DE and TR [27,28].

176

177 (e) Virus titration

Viral dissemination and transmission were determined by the presence of infectious virus in 178 179 heads and saliva extracts, respectively, by focus-forming assay (FFA) in C6/36 cells as previously described [27]. Briefly, 96-well plates were seeded with cells and each well was 180 181 inoculated with 50 μ L of saliva extract or head homogenate and incubated for 1 h at 28°C. Then, cells were overlaid with a 1:1 mix of carboxymethyl cellulose and Leibovitz L15 182 183 medium supplemented with 10% FBS and 1.5X of an antibiotic-antifungal solution (Dutscher, 184 Brumath, France). After three days of incubation, cells were fixed for 20 min at room temperature with formaldehyde 3.7%, washed 3 times in PBS 1X, and incubated 15 min with 185 186 0.5% Triton X-100 in PBS 1X. Cells were then incubated for 1 h with a hyper-immune 187 ascetic fluid specific to CHIKV as the primary antibody, washed three times with PBS 1X, 188 and incubated for 1 h at room temperature with a goat anti-mouse conjugate as the second 189 antibody (BioRad, Hercules, CA). The number of focus-forming units (FFU) was determined 190 under a fluorescence microscope. The data was analyzed qualitatively, that is, presence of 191 absence of infectious virus in the sample.

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193 (f) Phylogenetic analysis

Sequence analysis, contig assembly and CHIKV sequence alignments were performed using the program BioNumerics version 6.5 (Applied-Maths, Saint-Martens-Latem, Belgium). For phylogenetic analysis, a maximum-likelihood tree was constructed using MEGA version 5 (www.megasoftware.net), based on the Tamura-Nei model. Reliability of nodes was assessed by bootstrap resampling with 1,000 replicates. 199

200 (g) Statistical analyses

201 The study was run in two separate experiments that involved the same set of virus strains, 202 mosquito populations and temperature conditions, therefore experiment was included as a 203 covariate. Viral dissemination and transmission were analyzed as a binary response (0=absence and 1=presence of virus in heads or saliva) with a full-factorial generalized linear 204 205 model that included the factors experiment, mosquito population, viral strain, temperature and 206 all their interactions. The model was fitted with a binomial error structure and a logit link 207 function. Statistical significance of the effects was assessed by an analysis of deviance [29]. 208 Effects were considered statistically significant when P < 0.05. All analyses were performed in 209 the statistical environment R (http://www.r-project.org/).

210

211 **3. Results**

212 CHIKV dissemination and transmission were examined in a total of 940 Ae. 213 albopictus females from six mosquito populations (France, Italy, Montenegro, Brazil, La 214 Reunion, Vietnam) infected with two strains of CHIKV (2010-1909 and 06-021) following incubation for six days post-infection under two temperatures regimes (20°C or 28°C). The 215 study was run in two separate experiments that consisted of 464 and 476 individual females, 216 217 respectively. All twenty-four combinations of mosquito population, viral strain and 218 temperature were represented in both experiments. For each combination, 12-20 individual 219 females were tested by experiment.

Epidemiologically, the most important phenotype is the ability of mosquitoes to deliver infectious virus in their saliva following virus exposure during a blood meal (*i.e.*, vector competence). This is adequately measured by the transmission efficiency (TE), calculated as the proportion of all tested mosquitoes that had infectious virus in their saliva

extracts. Overall, TE depended strongly on the three-way interaction between mosquito 224 population, viral strain, and temperature (P = 0.00024; Table 1). The effect of the four-way 225 226 interaction between experiment, mosquito population, viral strain, and temperature was not statistically significant (P = 0.13281; Table 1), indicating that the three-way interaction was 227 228 consistent across experiments. The three-way interaction can be represented graphically as 229 differing patterns of two-way interactions between temperature and viral strain among mosquito populations (Figure 2). The strongest two-way interaction between temperature and 230 231 viral strain was observed for Ae. albopictus from Brazil: TE were 2.25 times lower at 20°C than at 28°C for CHIKV 06-021 (37.5% vs. 85%) and 2.15 times higher at 20°C than at 28°C 232 233 for CHIKV 2010-1909 (75% vs. 35%)(Figure 2). In other mosquito populations, TE values 234 ranged from 12.5% (Ae. albopictus from Vietnam infected by CHIKV 06-021 at 20°C) to 87.5% (Ae. albopictus from France infected by CHIKV 2010-1909 at 20°C)(figure 2). 235

236 TE is a composite phenotype that encapsulates the ability of the virus to disseminate 237 from the midgut, invade the salivary glands, and be released in the saliva. Failure to transmit 238 the virus can therefore result from lack of dissemination from the midgut, lack of salivary gland infection and/or lack of virus release in the saliva. To determine whether the strong 239 three-way interaction underlying TE could be specifically attributed to one of these 240 intermediate steps of transmission, we analyzed dissemination efficiency (DE) and 241 242 transmission rate (TR) separately. While TR was strongly influenced by the three-way 243 interaction between mosquito population, viral strain, and temperature (P = 0.0016; table 3), 244 DE was only slightly influenced (P = 0.0486; Table 2). The effect of the four-way interaction 245 between experiment, mosquito population, viral strain, and temperature did not significantly 246 affect TR (P = 0.2317; Table 3), indicating that the three-way interaction was consistent across 247 experiments. The four-way interaction could not be included in the analysis of DE because of 248 the disproportionately high frequency of dissemination that resulted in a strongly unbalanced response variable. Indeed, 90.2% of all females tested had virus-infected head tissues, which
prevented analysis of the full-factorial model. Together, the secondary analyses indicated that
the effect of the three-way interaction on TE resulted primarily from differences in TR.

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253 4. Discussion

254 In this study, we provide evidence that CHIKV transmission depends on a complex interaction between the mosquito vector population, the viral strain and the ambient 255 256 temperature. Prior and during our experiments, Ae. albopictus mosquitoes were maintained in controlled insectary conditions and we interpret phenotypic differences between populations 257 258 as primarily genetic variation. Likewise, we consider the viral strain effect to reflect the 259 underlying genetic make-up (Figure 1; Tables S1 and S2) because both virus isolates were prepared in an identical method and used to infect mosquitoes at the same infectious dose in 260 261 the blood meal. Therefore, we conclude that the three-way interaction between mosquito 262 population, viral strain and temperature that we observed reflects a G x G x E interaction. Our 263 analyses of intermediate phenotypes suggest that the G x G x E interaction influencing 264 CHIKV transmission that we have uncovered results primarily from differences in the ability of mosquitoes with a disseminated infection to deliver infectious virus in their saliva, rather 265 than differences in dissemination efficiency. Although the molecular mechanism(s) 266 267 underlying this complex interaction remains to be elucidated, our data suggest that it occurs 268 during viral invasion of the salivary glands and/or release in saliva.

The existence of G x G x E interactions has been documented in other cases of biological interactions [30-33]. To the best of our knowledge, however, this is the first time G x G x E interactions are documented in the case of a mosquito-borne pathogen of public heath relevance. It bears particular importance in the context of pathogen emergence because it suggests that the environment can profoundly modify adaptive properties of genotypes. For 274 example, the adaptive E1-A226V amino-acid change conferring enhanced transmission by Ae. 275 albopictus [18,19] might not be favored to the same extent in different environments. The two 276 CHIKV strains of this study differed mainly by three substitutions in E1 and E2 glycoproteins 277 at positions E1-211, E1-226 and E2-264 (see Table S1). CHIKV 2010-1909 has an alanine 278 whereas CHIKV 06-021 has a valine at position E1-226. Consistently with the Indian Ocean 279 emergence scenario [16,22,34], CHIKV 06-021 was always better or equally transmitted at 280 28°C than CHIKV 2010-1909 in our experiments (red lines in Figure 2). However, it was the 281 opposite pattern at 20°C, with CHIKV 2010-1909 being better or equally transmitted than 282 CHIKV 06-021 in all mosquito populations but one (Italy) (blue lines in Figure 2). Therefore, 283 the probability of emergence of a viral strain in a particular mosquito species or population 284 may vary according to the environmental temperature. It is worth noting that both CHIKV strains used in this study were relatively efficiently transmitted at 20°C by most Ae. 285 286 albopictus populations. Accordingly, we have previously demonstrated that TR and TE of Ae. 287 aegypti from temperate Argentina experimentally infected with dengue virus were higher 288 when mosquitoes were incubated at 20°C than at 28°C [28]. This is in contrast with the 289 notion that cooler temperature is less permissive to arboviral transmission [35]. However, 290 exposure to cooler temperatures was recently shown to increase mosquito susceptibility to CHIKV infection through destabilization of the antiviral immune response [36]. Thus, 291 292 temperature has multiple, and sometimes opposite effects on the efficiency of virus 293 transmission by mosquitoes.

Our study adds a new layer of complexity to the understanding of mosquito-borne pathogen transmission. It shows that temperature may alter G x G interactions that have previously observed between vectors and pathogens [4,5]. Temperature has long been considered an important environmental driver of insect-pathogen interactions [14]. There are, however, a wide variety of additional environmental factors that may also influence vector 299 competence [9]. These additional factors can be abiotic or biotic. For example, mosquito-300 pathogen interactions can be modulated by bacterial communities. Bacteria diversity, which is 301 mosquito population-specific [37], is an essential determinant of vector competence [38]. 302 Pathogens co-exist and/or directly interact with bacteria colonizing the midgut or with 303 intracellular symbionts such as Wolbachia. So, bacterial communities may alter vector 304 competence by acting directly on virus replication or by modulating the host immune system [40]. A change in the composition or density of bacteria in mosquitoes [39-41] triggered by 305 306 external factors such as temperature may alter mosquito susceptibility to pathogens [42].

307 In conclusion, we detected strong G x G x E interactions underlying CHIKV 308 transmission by Ae. albopictus. This finding questions the relevance of vector competence 309 studies conducted in laboratory systems that typically use one mosquito population and a 310 single virus strain under constant and single environmental conditions. It also underlines the 311 public health significance of questions that are usually addressed in an ecological or 312 evolutionary context. In future studies, it will be important to account for the complex 313 interplay between genetic and environmental variability to accurately assess the potential of 314 vector-borne diseases to emerge.

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Figure and Table Legends 483 484 Figure 1. Phylogenetic relationship among CHIKV strains inferred from complete 485 486 genome sequences (11,237 nucleotides) Bootstrap support values (1,000 replicates) are indicated at major nodes. Scale bar indicates 487 the number of base substitutions per site. French strains used in this study are shown in bold. 488 489 Figure 2. Effect of mosquito population, viral strain and temperature on transmission 490 491 efficiency 492 Transmission efficiency (TE) is the overall proportion of females that had infectious virus in their saliva six days after exposure to the virus during an infectious blood meal. Different 493 panels represent different mosquito populations (left column: temperate regions; right 494 495 column: tropical regions). In each panel, the average TE across two experiments is shown for 496 both viral strains (indicated on the x-axis) under two temperature regimes (blue line: 20°C; red line: 28°C). Vertical bars are the TE confidence intervals. 497 498 499 Table 1. Test statistics of virus transmission efficiency 500 d.f.: degrees of freedom; res. dev.: residual deviance. Transmission efficiency is calculated as 501 the overall proportion of females that had infectious saliva (i.e., among all tested females with 502 or without a disseminated infection). 503 504 Table 2. Test statistics of virus dissemination efficiency d.f.: degrees of freedom; res. dev.: residual deviance. Dissemination efficiency is calculated 505 as the proportion of tested females that had infected head tissues (i.e., in which the virus

507 successfully disseminated from the midgut). In this analysis, the four-way interaction between

508	experiment, population, viral strain and temperature could not be supported by the model, due
509	to the strongly unbalanced distribution of the response variable (>90% of mosquitoes had a
510	disseminated infection).
511	
512	Table 3. Test statistics of virus transmission rate
513	d.f.: degrees of freedom; res. dev.: residual deviance. Transmission rate is calculated as the
514	proportion of females with infectious saliva among females with a disseminated infection.
515	
516	Table S1. Amino acids differences in the structural protein (nsp) between CHIKV 2010-
517	1909, CHIKV 06-021 and different chikungunya strains*
518	*Molecular signatures were based on the analysis of complete amino-acid sequence of nsp1, nsp2 and
519	nsp3 (2474 amino acids). The numbering of amino-acid positions refers to the African isolate S27.
520	CHIKV 2010-1909 and CHIKV 06-021 strains are highlighted in red. Colors correspond to amino-
521	acid changes among ECSA phylogroup (green) or Asian phylogroup (pink). CHIKV, chikungunya
522	virus. ECSA, East Central South Africa.
523	
524	Table S2 Amino acids differences in the non structural protein between CHIKV 2010-
525	1909, CHIKV 06-021 and different chikungunya strains*
526	*Molecular signatures were based on the analysis of complete amino acid sequence of C, E3,
527	E2, 6K and E1 glycoprotein (1244 amino acids). The numbering of amino-acid positions
528	refers to the African isolate S27.
529	CHIKV 2010-1909 and CHIKV 06-021 strains are highlighted in red. Colors correspond to amino-
530	acid change among ECSA phylogroup (yellow) or Asian phylogroup (purple). CHIKV,
531	chikungunya virus. C, capsid. E, enveloppe (glycoprotein). ECSA, East Central South Africa.
532	[†] The amino acid substitution E2-V264A was unique to France 2010-1909 CHIKV isolate.

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537 Table 1. Test statistics of virus transmission efficiency

538

Factor	d.f.	res. dev.	<i>P</i> -value
Experiment	1	0.1	0.70797
Population	5	63.0	2.9e-12
Viral strain	1	0.2	0.6465
Temperature	1	2.6	0.10696
Experiment * Population	5	21.5	0.00066
Experiment * Viral strain	1	0.2	0.65539
Population* Viral strain	5	29.8	1.6e-5
Experiment * Temperature	1	0.2	0.64037
Population * Temperature	5	15.4	0.00893
Viral strain * Temperature	1	22.3	2.3e-6
Experiment * Population * Viral strain	5	7.3	0.19681
Experiment * Population * Temperature	5	4.1	0.53824
Experiment * Viral strain * Temperature	1	3.2	0.07471
Population * Viral strain * Temperature	5	23.8	0.00024
Experiment * Population * Viral strain * Temperature	5	8.5	0.13281

539 d.f.: degrees of freedom; res. dev.: residual deviance. Transmission efficiency is calculated as the overall 540 proportion of females that had infectious saliva (*i.e.*, among all tested females with or without a 541 disseminated infection).

543 Table 2. Test statistics of virus dissemination efficiency

544

Factor	d.f.	res. dev.	<i>P</i> -value
Experiment	1	4.9	0.0272
Population	5	60.4	1e-11
Viral strain	1	33	9.3e-9
Temperature	1	25.4	4.6e-7
Experiment * Population	5	21	0.0008
Experiment * Viral strain	1	10	0.0016
Experiment * Viral strain	5	13.3	0.0208
Experiment * Temperature	1	0.1	0.7547
Population * Temperature	5	2.7	0.7397
Viral strain * Temperature	1	0.6	0.4380
Experiment * Population * Viral strain	5	1.4	0.925
Experiment * Population * Temperature	5	10.2	0.0699
Experiment * Viral strain * Temperature	1	1.8	0.1782
Experiment * Viral strain * Temperature	5	11.1	0.0486

d.f.: degrees of freedom; res. dev.: residual deviance. Dissemination efficiency is calculated as the proportion of tested females that had infected head tissues (*i.e.*, in which the virus successfully disseminated from the midgut). In this analysis, the four-way interaction between experiment, population, viral strain and temperature could not be supported by the model, due to the strongly unbalanced distribution of the response variable (>90% of mosquitoes had a disseminated infection).

Factor	d.f.	res. dev.	<i>P</i> -value
Experiment	1	1.5	0.2235
Population	5	42.6	4.6e-8
Viral strain	1	1.8	0.1798
Temperature	1	0	0.9285
Experiment * Population	5	20.5	0.001
Experiment * Viral strain	1	0.1	0.7526
Population* Viral strain	5	17.4	0.0037
Experiment * Temperature	1	1.2	0.2718
Population * Temperature	5	11.3	0.0457
Viral strain * Temperature	1	17	3.8e-5
Experiment * Population * Viral strain	5	2.7	0.7396
Experiment * Population * Temperature	5	7.3	0.2020
Experiment * Viral strain * Temperature	1	2.3	0.1321
Population * Viral strain * Temperature	5	19.4	0.0016
Experiment * Population * Viral strain * Temperature	5	6.9	0.2317

551 **Table 3. Test statistics of virus transmission rate**

d.f.: degrees of freedom; res. dev.: residual deviance. Transmission rate is calculated as the
 proportion of females with infectious saliva among females with a disseminated



Bootstrap support values (1,000 replicates) are indicated at major nodes. Scale bar indicates the number of base substitutions per site. French strains used in this study are shown in bold. 172x128mm (300 x 300 DPI)



Transmission efficiency (TE) is the overall proportion of females that had infectious virus in their saliva six days after exposure to the virus during an infectious blood meal. Different panels represent different mosquito populations (left column: temperate regions; right column: tropical regions). In each panel, the average TE across two experiments is shown for both viral strains (indicated on the x-axis) under two temperature regimes (blue line: 20°C; red line: 28°C). Vertical bars are the TE confidence intervals. 188x239mm (300 x 300 DPI)