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Author for correspondence:
Karima Zouache
e-mail: karima.zouache@pasteur.fr

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

Karima Zouache[1], Albin Fontaine[2,3], Anubis Vega-Rua[1,4], Laurence Mousson[1], Jean-Michel Thiberge[5], Ricardo Lourenco-De-Oliveira[1,6], Valérie Caro[5], Louis Lambrechts[2] and Anna-Bella Failloux[1]

[1]Department of Virology, Institut Pasteur, Arboviruses and Insect Vectors Laboratory, Paris, France
[2]Department of Genomes and Genetics, Institut Pasteur–Centre National de la Recherche Scientifique URA 3012, Insect–Virus Interactions Group, Paris, France
[3]Institut de Recherche Biomédicale des Armées (IRBA), Unité d'Entomologie, Brétigny-sur-Orge, France
[4]Université Pierre et Marie Curie, Cellule Pasteur UPMC, Paris, France
[5]Institut Pasteur, Genotyping of Pathogens and Public Health, Paris, France
[6]Laboratório de Transmissores de Hematozoários, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil

Interactions between pathogens and their insect vectors in nature are under the control of both genetic and non-genetic factors, yet most studies on mosquito vector competence for human pathogens are conducted in laboratory systems that do not consider genetic and/or environmental variability. Evaluating the risk of emergence of arthropod-borne viruses (arboviruses) of public health importance such as chikungunya virus (CHIKV) requires a more realistic appraisal of genetic and environmental contributions to vector competence. In particular, sources of variation do not necessarily act independently and may combine in the form of interactions. Here, we measured CHIKV transmission potential by the mosquito *Aedes albopictus* in all combinations of six worldwide vector populations, two virus strains and two ambient temperatures (20°C and 28°C). Overall, CHIKV transmission potential by *Ae. albopictus* strongly depended on the three-way combination of mosquito population, virus strain and temperature. Such genotype-by-genotype-by-environment (G × G × E) interactions question the relevance of vector competence studies conducted with a simpler set of conditions. Our results highlight 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.

1. Introduction

Our mechanistic understanding of interactions between pathogens and their insect vectors has made considerable progress in the last two decades. In particular, tremendous 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 (reviewed in [1,2]). To date, however, the vast majority of studies on vector–pathogen interactions have been conducted in laboratory models that ignore the genetic and environmental variability of natural situations [3]. Only very recently have studies begun to consider that natural vector–pathogen interactions occur in a variable world. Vector 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 in many other host–parasite associations, vector–pathogen interactions are governed by genotype-by-genotype (G × G) interactions, whereby the outcome of infection depends on the specific pairing of vector and pathogen genotypes [4,5]. The existence of such G × G 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 environmental factors, such as ambient temperature, in shaping mosquito vector competence for pathogens [9,10]. For example, the immune response and resistance of *Anopheles* mosquitoes to bacterial challenge strongly depended on environmental drivers such as mean temperature, diurnal temperature variation and time of infection [11]. Likewise, both the mean and daily amplitude of temperature variation influenced *Aedes aegypti* vector competence for dengue virus [12,13]. In the natural environment, vector–pathogen interactions are likely to be governed by complex genotype-by-genotype-by-environment ($G \times G \times E$) interactions [9,14], but this has yet to be documented.

Accounting for such ecological complexity is especially important when assessing the 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 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 (CHIKV), a mosquito-borne alphavirus usually transmitted among non-human primates by forest-dwelling mosquitoes. In 2004, a chikungunya outbreak emerged on the coast of Kenya. The mosquito implicated was the vector *Ae. aegypti* that is typically responsible for inter-human CHIKV transmission. The virus subsequently spread to several islands of the Indian Ocean where 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 acid change from alanine to valine at position 226 of CHIKV E1 glycoprotein (E1-A226 V) causing increased replication, midgut infection, dissemination and transmission in *Ae. albopictus* but not in *Ae. aegypti* [18,19]. This adaptive mutation conferring enhanced transmission by *Ae. albopictus* is thought to have occurred on at least three independent 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 emerged worldwide, including temperate regions such as Italy, where several hundred 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 potential.

2. Material and methods

(a) Mosquito populations and viral strains

Mosquito populations were sampled in the field as eggs collected with ovitraps (more than 500 eggs per population) that were brought back to the laboratory and reared in an insectary for less than 10 generations before experimental infections were performed. Sampling locations included three temperate regions in Europe: France (Bar-sur-Loup, F_3–F_4 generations), Italy (Castiglione-di-Cervia, F_3 generation), Montenegro (Ulcinj, F_1–F_2 generations); and three tropical overseas regions: Brazil (Manaus, F_3 generation), Vietnam (Bình Phước, F_8–F_9 generations) and La Reunion island (Providence, F_8–F_9 generations). After hatching, larvae were split into pans of 150 individuals and supplied every 2 days with a yeast tablet dissolved in 1 l of dechlorinated tap water. All immature stages were reared at 26°C ± 1°C. Emerging adults were maintained at 28°C ± 1°C with a 16 L : 8 D cycle, 80% relative humidity and supplied with a 10% sucrose solution. Females were blood-fed three times a week on anaesthetized mice (OF1 mice, Charles River laboratories, France).

Experimental infections of mosquitoes used two CHIKV strains, named 06-021 and 2010-1909 hereafter, that were kindly provided by the French National Reference Center for Arboviruses at Institut Pasteur. The 06-021 strain was isolated on C6/36 cells (*Ae. albopictus* cell line) from a patient on La Reunion island in 2005 [22]. The 2010-1909 strain was isolated on Vero cells (African green monkey kidney cell line) from an autochthonous human case in 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 oral infections [19,22]. The viral titre estimated by plaque assay on Vero cells [19] was 10^9 plaque-forming units per millilitre (PFU ml^{-1}) and 10^8 PFU ml^{-1} for 06-021 and 2010-1909 CHIKV strains, respectively.

(b) Mosquito oral infections and temperature regimes

Each of the six *Ae. albopictus* populations (three temperate and three tropical populations) was simultaneously challenged with the two CHIKV strains (06-021 or 2010-1909) and then split into two subsets that were incubated at 20°C or 28°C for 6 days. The entire experiment was repeated twice. For each experiment, 7- to 10-day-old females were fed on an infectious blood-meal provided at a final titre of $10^{7.5}$ PFU ml^{-1}, in agreement with viraemia levels recorded in patients [24]. The blood-meal mixture contained two-thirds of washed rabbit erythrocytes, one-third of viral suspension and ATP as a phagostimulant at a final concentration of 10 mM. Mosquito feeding was limited to 50 min and non-engorged females were discarded. Fully engorged females were transferred to cardboard cups and maintained on 10% sucrose in climatic chambers (KB 53, Binder, Tuttlingen, Germany) set at constant temperatures of 20°C ± 0.1°C or 28°C ± 0.1°C, with a 16 L : 8 D cycle and 70% relative humidity. A temperature of 20°C was chosen as representative of the low-temperature threshold recorded when local CHIKV transmission occurred during the Italian epidemic between June and September 2007 [21,25] 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 CHIKV vector competence assays [19,26–29].

(c) Vector competence phenotypes

Owing to the relatively high blood-meal titre of $10^{7.5}$ PFU ml^{-1}, 100% of engorged mosquitoes were considered to have established a midgut infection [26]. Vector competence was assessed based on two conventional phenotypes: viral dissemination from the midgut and transmission potential [27]. A difference in viral dissemination or transmission potential among experimental treatments can result from a difference in the maximum value and/or from a difference in the temporal dynamic. For each experimental condition, viral dissemination and transmission potential were tested in 12–20 mosquitoes 6 days post-infection (pi). CHIKV dissemination and transmission were previously found to reach a maximum at 6 days pi in *Ae. albopictus* maintained at 28°C [26,28]. Transmission potential was measured by forced salivation as previously described [27]. Briefly, legs and wings of each mosquito were removed and the mosquito's proboscis was inserted into a micropipette tip containing 5 µl of fetal bovine serum (FBS). After 45 min, the saliva-containing FBS was expelled into 45 µl of Leibovitz L15 medium (Invitrogen Life Technologies, Carlsbad, CA). Following salivation, the head of each mosquito

was removed and homogenized individually in 135 µl of Leibovitz L15 medium. Homogenates were then supplemented with 10% FBS and stored at −80°C before processing. Transmission efficiency (TE) was calculated as the overall proportion of females that had infectious saliva (i.e. among all tested females with or without a disseminated infection). TE was then broken down into two intermediate indices. Dissemination efficiency (DE) was 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 proportion of females with infectious saliva among those that developed a disseminated infection. Therefore, TE equals the product of DE and TR [27,28].

(d) Virus titration

Viral dissemination and transmission potential were determined by the presence of infectious virus in heads and saliva extracts, respectively, by focus-forming assay in C6/36 cells as previously described [28]. Briefly, 96-well plates were seeded with cells, and each well was inoculated with 50 µl 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 medium supplemented with 10% FBS and 1.5× of an antibiotic–antifungal solution (Dutscher, Brumath, France). After 3 days of incubation, cells were fixed for 20 min at room temperature with formaldehyde 3.7%, washed three times in PBS 1× and incubated 15 min with 0.5% Triton X-100 in PBS 1×. Cells were then incubated for 1 h with a hyper-immune ascetic fluid specific to CHIKV as the primary antibody, washed three times with PBS 1× and incubated for 1 h at room temperature with a goat anti-mouse conjugate as the second antibody (BioRad, Hercules, CA). The number of focus-forming units was determined under a fluorescence microscope. The data were analysed qualitatively (i.e. presence or absence of infectious virus in the sample).

(e) Phylogenetic analysis

Sequence analysis, contig assembly and CHIKV genome alignments were performed using the program BioNumerics v. 6.5 (Applied-Maths, Saint-Martens-Latem, Belgium). For phylogenetic analysis, a maximum-likelihood tree was constructed using MEGA v. 5 (www.megasoftware.net), based on the Tamura-Nei model. Reliability of nodes was assessed by bootstrap resampling with 1000 replicates.

(f) Statistical analysis

The study was run in two separate experiments that involved the same set of virus strains, mosquito populations and temperature conditions, therefore experiment was included as a covariate. Viral dissemination and transmission potential were analysed as a binary response (0 = absence and 1 = presence of virus in heads or saliva) with a full-factorial generalized linear model that included the factors experiment, mosquito population, viral strain, temperature and all their interactions. The model was fitted with a binomial error structure and a logit link function. Statistical significance of the effects was assessed by an analysis of deviance [30]. Effects were considered statistically significant when $p < 0.05$. All analyses were performed in the R statistical environment (http://www.r-project.org/).

3. Results

CHIKV dissemination and transmission were examined in a total of 940 *Ae. albopictus* females from six mosquito populations (France, Italy, Montenegro, Brazil, La Reunion and Vietnam) infected with two strains of CHIKV (2010-1909 and 06-021) following incubation for 6 days pi under two temperature regimes (20°C or 28°C). Phylogenetic analysis of the complete viral genome sequences (11 237 nucleotides) showed that the two strains belonged to the East–Central–South Africa phylogroup (figure 1; electronic supplementary material, tables S1 and S2). The study was run in two separate experiments that consisted of 464 and 476 individual females, respectively. All 24 combinations of mosquito population, viral strain and temperature were represented in both experiments. For each combination, 12–20 individual 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 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 population, viral strain and temperature ($p = 0.00024$; table 1). The effect of the four-way 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 consistent across experiments. The three-way interaction can be represented graphically as differing patterns of two-way interactions between temperature and viral strain among mosquito populations (figure 2). The strongest two-way interaction between temperature and viral strain was observed for *Ae. albopictus* from Brazil: TE were 2.25-fold lower at 20°C than at 28°C for CHIKV 06-021 (37.5 versus 85%) and 2.15-fold higher at 20°C than at 28°C for CHIKV 2010-1909 (75 versus 35%) (figure 2). In other mosquito populations, TE values 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).

TE is a composite phenotype that encapsulates the ability of the virus to disseminate from the midgut, invade the salivary glands and be released in the saliva. Failure to transmit 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 three-way interaction underlying TE could be specifically attributed to one of these intermediate steps of transmission, we analysed DE and TR separately. While TR was strongly influenced by the three-way interaction between mosquito population, viral strain and temperature ($p = 0.0016$; table 3), DE was only slightly influenced ($p = 0.0486$; table 2). The effect of the four-way interaction between experiment, mosquito population, viral strain and temperature did not significantly affect TR ($p = 0.2317$; table 3), indicating that the three-way interaction was consistent across experiments. The four-way interaction could not be included in the analysis of DE because of 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.

4. Discussion

In this study, we provide evidence that CHIKV transmission potential depends on a complex interaction between the

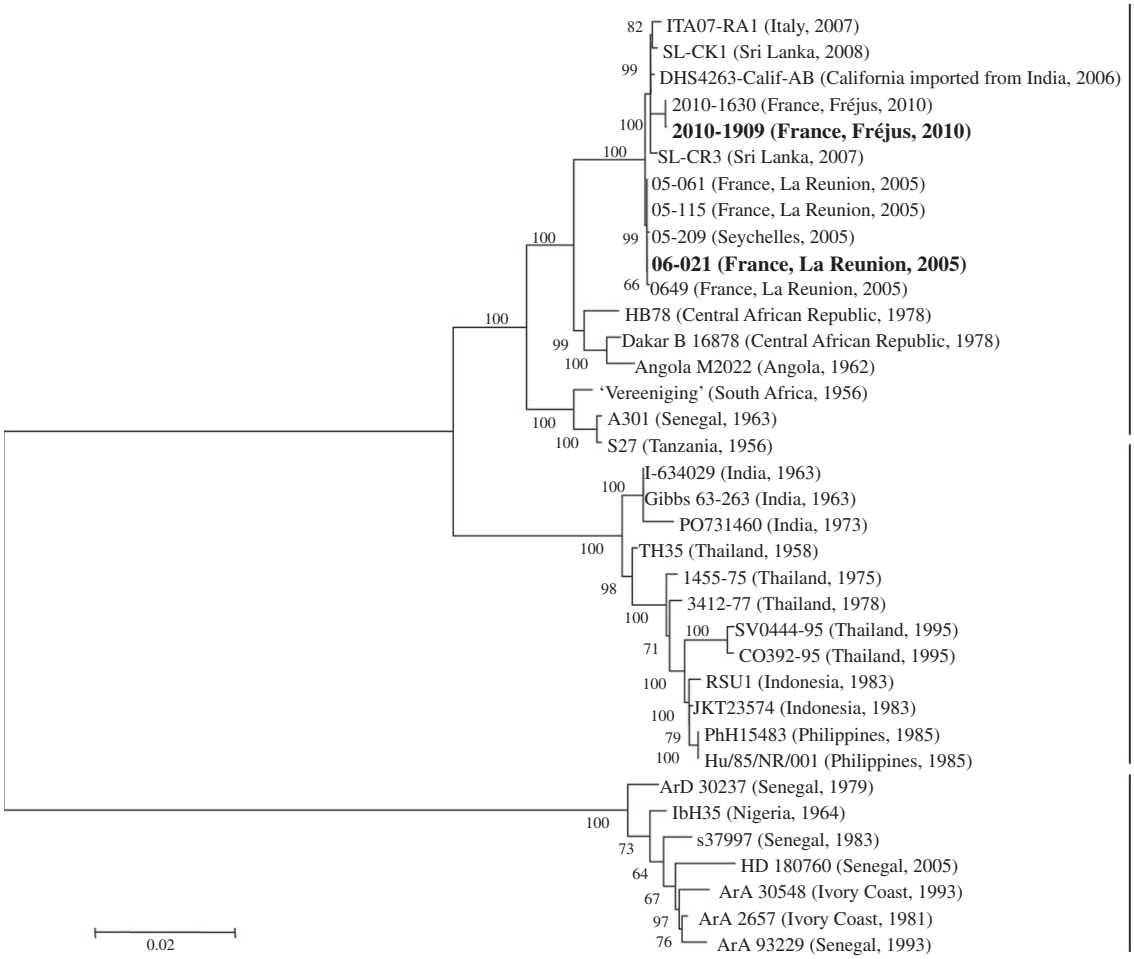

Figure 1. Phylogenetic relationship among CHIKV strains inferred from complete genome sequences (11 237 nucleotides). Bootstrap support values (1000 replicates) are indicated at major nodes. Scale bar indicates the number of base substitutions per site. French strains used in this study are shown in bold.

Table 1. Test statistics of virus TE. TE is calculated as the overall proportion of females that had infectious saliva (i.e. among all tested females with or without a disseminated infection). d.f., degrees of freedom; res. dev., residual deviance.

factor	d.f.	res. dev.	*p*-value
experiment	1	0.1	0.70797
population	5	63.0	2.9×10^{-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	1.6×10^{-5}
experiment × temperature	1	0.2	0.64037
population × temperature	5	15.4	0.00893
viral strain × temperature	1	22.3	2.3×10^{-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

mosquito vector population, the viral strain and the ambient temperature. Prior to and during our experiments, *Ae. albopictus* mosquitoes were maintained in controlled insectary conditions and we interpret phenotypic differences between populations as primarily genetic variation. Likewise, we consider the viral strain effect to reflect the underlying

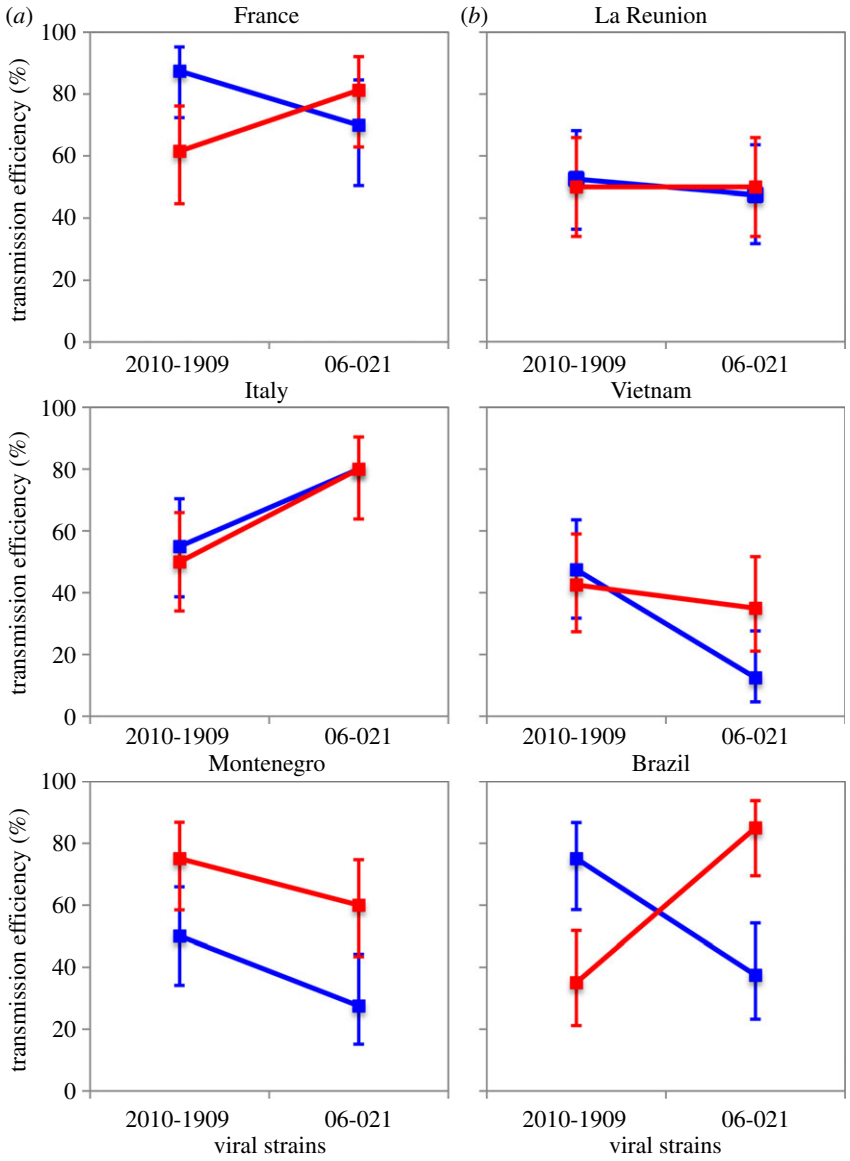

Figure 2. Effect of mosquito population, viral strain and temperature on TE. TE is the overall proportion of females that had infectious virus in their saliva 6 days after exposure to the virus during an infectious blood-meal. Different panels represent different mosquito populations: (*a*) temperate regions; (*b*) 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.

genetic make-up (figure 1; electronic supplementary material, 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 the blood meal. Therefore, we conclude that the three-way interaction between mosquito population, viral strain and temperature that we observed reflects a G × G × E interaction. Our analyses of intermediate phenotypes suggest that the G × G × E interaction influencing CHIKV transmission potential 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 than differences in DE. Although the molecular mechanism(s) underlying this complex interaction remains to be elucidated, our data suggest that it occurs during viral invasion of the salivary glands and/or release in saliva.

The existence of G × G × E interactions has been documented in various mutualistic or parasitic systems [31–35]. However, to the best of our knowledge, this is the first time G × G × E interactions have been demonstrated in the case of a mosquito-borne pathogen of public health 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 example, the adaptive E1-A226 V amino acid change conferring enhanced transmission by *Ae. albopictus* [18,19] might not be favoured to the same extent in different environments. The two CHIKV strains of this study differed mainly by three substitutions in E1 and E2 glycoproteins at positions E1–211, E1–226 and E2–264 (see the electronic supplementary material, table S1). CHIKV 2010-1909 has an alanine, whereas CHIKV 06-021 has a valine at position E1–226. Consistently with the Indian Ocean emergence scenario [16,22,36], CHIKV 06-021 was always better or equally transmitted at 28°C compared with CHIKV 2010-1909 in our experiments (red lines in figure 2). However, it was the opposite pattern at 20°C, with CHIKV 2010-1909 being better or equally transmitted compared with CHIKV 06-021 in all mosquito populations except one (Italy) (blue lines in figure 2). Therefore, the probability of emergence of a viral strain in a particular

Table 2. Test statistics of virus DE. DE 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 (more than 90% of mosquitoes had a disseminated infection). d.f., degrees of freedom; res. dev., residual deviance.

factor	d.f.	res. dev.	*p*-value
experiment	1	4.9	0.0272
population	5	60.4	1×10^{-11}
viral strain	1	33	9.3×10^{-9}
temperature	1	25.4	4.6×10^{-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
population × viral strain × temperature	*5*	*11.1*	*0.0486*

Table 3. Test statistics of virus TR. TR is calculated as the proportion of females with infectious saliva among those that developed a disseminated infection. d.f., degrees of freedom; res. dev., residual deviance.

factor	d.f.	res. dev.	*p*-value
experiment	1	1.5	0.2235
population	5	42.6	4.6×10^{-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	3.8×10^{-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

mosquito species or population 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. albopictus* populations. Accordingly, we have previously demonstrated that TR and TE of *Ae. aegypti* from temperate Argentina experimentally infected with dengue virus were higher when mosquitoes were incubated at 20°C than at 28°C [29]. Exposure to cooler temperatures was recently shown to increase mosquito susceptibility to CHIKV infection through destabilization of the antiviral immune response [37]. This is in contrast with the notion that cooler temperature is less permissive to arbovirus transmission [38].

In this study, all immature stages were reared at the same temperature of 26°C and adults were maintained at 28°C prior to the experimental infection with CHIKV. As we were primarily interested in the effect of the extrinsic incubation temperature, this experimental design allowed us to rule out the confounding effects of life-history traits that are influenced

by the larval rearing temperature. Several life-history traits, such as adult body size, vary with the temperature of immature development [39,40]. Immature stages exposed to cooler temperatures are expected to become larger adults with increased CHIKV vector competence [41], but in this case the respective effects of adult body size and rearing temperature are confounded. A study on *Ae. albopictus* infected with dengue virus suggested that the rearing temperature of immature stages and the holding temperature of adults may have independent effects on life-history traits and susceptibility to virus infection [42]. In this study, a direct effect of the larval rearing temperature can be excluded. On the other hand, we cannot disentangle an effect of temperature *per se* from an effect of the change in temperature experienced by mosquitoes in the 20°C experimental treatment. For example, a cold shock has been shown to increase resistance to fungal infection in *Drosophila melanogaster* [43]. Clearly, temperature may influence vector competence through a complex suite of direct (e.g. impact on virus replication) and indirect effects (e.g. changes in mosquito physiology), and this merits further investigation. In addition, the amplitude of diurnal temperature fluctuations that are typical of natural mosquito habitats can affect pathogen transmission dynamics. Recent studies demonstrated that the range of daily temperature variation, in combination with the mean temperature, could impact mosquito susceptibility to infection and/or virus dissemination [12,13]. Taken together, temperature has complex, multiple and sometimes opposite effects on virus dissemination and 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 × G interactions that have previously been 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 competence [9]. These additional factors can be abiotic or biotic. For example, bacterial communities can modulate mosquito–pathogen interactions. Bacteria diversity, which is mosquito population-specific [44,45], is an essential determinant of vector competence [45]. Pathogens coexist and/or directly interact with bacteria colonizing the midgut or with intracellular symbionts such as *Wolbachia*. Bacterial communities therefore may alter vector competence by acting directly on virus replication or by modulating the host immune system [46]. A change in the composition or density of bacteria in mosquitoes [46–48] triggered by external factors such as temperature may alter mosquito susceptibility to pathogens [49].

In conclusion, we detected strong G × G × E interactions underlying CHIKV transmission by *Ae. albopictus*. This finding underlines the public health significance of questions that are usually addressed in an ecological or evolutionary context. It also highlights the need to consider the sources of variation that may complicate the interpretation of observations from an experimental system. While previous laboratory studies that usually used one mosquito population and/or a single virus strain under constant and/or single environmental conditions definitely improved our understanding of host–pathogen interactions, accurately assessing the potential of vector-borne diseases to emerge will require accounting for the complex interplay between genetic and environmental variabilities.

Laboratory mice were used to blood-feed mosquitoes for egg production. The Institut Pasteur animal facility has received accreditation from the French Ministry of Agriculture to perform experiments on live mice in accordance with the French and European regulations on care and protection of laboratory animals (EC Directive 2010/63, French Law 2013–118, 6 February 2013). Protocols were approved by the veterinary staff of the Institut Pasteur animal facility and were performed in compliance with NIH Animal Welfare Insurance no. A5476-01 issued on 31 July 2012.

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