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

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Running title: Complex interactions influence chikungunya virus transmission
Abstract

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 ignore genetic and 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 additively and may combine in the form of 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 temperatures (20°C and 28°C). Overall, CHIKV transmission by *Ae. albopictus* strongly depended on the three-way combination of mosquito population, virus strain and temperature. Such genotype-by-genotype-by-environment (G x G x 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.

Keywords: mosquito, arbovirus, chikungunya, temperature, transmission, interaction
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, 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 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 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 governed by complex genotype-by-genotype-by-environment (G x G x 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 incriminated was the typical vector *Ae. aegypti* ensuring 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 an alanine to a valine at position 226 of CHIKV E1 glycoprotein (E1-A226V) 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 in 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.
2. Material and methods

(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.

(b) 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. 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$_2$ generation); and three tropical overseas regions: Brazil (Manaus, F$_3$ generation), Vietnam (Bình Phước, F$_8$-F$_9$ generation) and La Réunion island (Providence, F$_8$-F$_9$ generation). After hatching, larvae were split into pans of 150 individuals and supplied every two days with a yeast tablet dissolved in 1 liter of dechlorinated tap water. Emerging adults were maintained at 28°C±1°C with a light: dark cycle of 16h: 8h, 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 Réunion 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 titer estimated by plaque assay on Vero cells [19], was 10⁹ plaque-forming units per mL (PFU/mL) and 10⁸ PFU/mL for 06-021 and 2010-1909 CHIKV strains, respectively. Phylogenetic analysis of the complete viral genome sequences (11,237 nucleotides) showed that the two strains belonged to the East-Central-South Africa phylogroup (ECSA) (Figure 1; Tables S1 and S2).

(c) 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. 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 titer of 10⁷.5 PFU/mL. 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 minutes 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 light: dark cycle of 16h: 8h and 70% relative humidity. The temperature 20°C was chosen as
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].

(d) Vector competence phenotypes

Due to the relatively high blood-meal titer of $10^{7.5}$ PFU/mL, 100% of engorged mosquitoes were considered to have established a midgut infection [25]. Vector competence was assessed based on two conventional phenotypes: viral dissemination from the midgut and transmission potential [26]. For each experimental condition, viral dissemination and transmission was 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 [25,27]. Transmission potential was measured by forced salivation as previously described [26]. Briefly, legs and wings of each mosquito were removed and the mosquito’s proboscis was inserted into a micropipette tip containing 5 μL of foetal bovine serum (FBS). After 45 minutes, the saliva-containing FBS was expelled into 45 μL of Leibovitz L15 medium (Invitrogen Life Technologies, Carlsbad, CA). After 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 in 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].

(e) Virus titration

Viral dissemination and transmission were determined by the presence of infectious virus in 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 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 1.5X of an antibiotic-antifungal solution (Dutscher, 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 0.5% Triton X-100 in PBS 1X. 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 1X, 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 (FFU) was determined under a fluorescence microscope. The data was analyzed qualitatively, that is, presence of absence of infectious virus in the sample.

(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.
(g) Statistical analyses

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 were analyzed 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 [29]. Effects were considered statistically significant when $P<0.05$. All analyses were performed in the statistical environment R (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, 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 study was run in two separate experiments that consisted of 464 and 476 individual females, respectively. All twenty-four 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 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 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 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 for CHIKV 2010-1909 (75% vs. 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 analyzed dissemination efficiency (DE) and transmission rate (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 depends on a complex interaction between the mosquito vector population, the viral strain and the ambient temperature. Prior 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 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 the blood meal. Therefore, we conclude that the three-way interaction between mosquito population, viral strain and temperature that we observed reflects a G x G x E interaction. Our analyses of intermediate phenotypes suggest that the G x G x E interaction influencing 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 than differences in dissemination efficiency. 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 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 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-A226V amino-acid change conferring enhanced transmission by *Ae. albopictus* [18,19] might not be favored 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 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 [16,22,34], CHIKV 06-021 was always better or equally transmitted at 28°C than 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 than CHIKV 06-021 in all mosquito populations but one (Italy) (blue lines in Figure 2). Therefore, the probability of emergence of a viral strain in a particular 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 [28]. This is in contrast with the notion that cooler temperature is less permissive to arboviral transmission [35]. However, exposure to cooler temperatures was recently shown to increase mosquito susceptibility to CHIKV infection through destabilization of the antiviral immune response [36]. Thus, temperature has multiple, and sometimes opposite effects on the efficiency of virus 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
competence [9]. These additional factors can be abiotic or biotic. For example, mosquito-pathogen interactions can be modulated by bacterial communities. Bacteria diversity, which is mosquito population-specific [37], is an essential determinant of vector competence [38]. Pathogens co-exist and/or directly interact with bacteria colonizing the midgut or with intracellular symbionts such as Wolbachia. So, bacterial communities may alter vector 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 external factors such as temperature may alter mosquito susceptibility to pathogens [42].

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

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Figure and Table Legends

Figure 1. Phylogenetic relationship among CHIKV strains inferred from complete genome sequences (11,237 nucleotides)
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.

Figure 2. Effect of mosquito population, viral strain and temperature on transmission efficiency
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.

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

Table 2. Test statistics of virus dissemination efficiency
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).

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 infection.

Table S1. Amino acids differences in the structural protein (nsp) between CHIKV 2010-
1909, CHIKV 06-021 and different chikungunya strains*

*Molecular signatures were based on the analysis of complete amino-acid sequence of nsp1, nsp2 and
nsp3 (2474 amino acids). The numbering of amino-acid positions refers to the African isolate S27.
CHIKV 2010-1909 and CHIKV 06-021 strains are highlighted in red. Colors correspond to amino-
acid changes among ECSA phylogroup (green) or Asian phylogroup (pink). CHIKV, chikungunya
virus. ECSA, East Central South Africa.

Table S2 Amino acids differences in the non structural protein between CHIKV 2010-
1909, CHIKV 06-021 and different chikungunya strains*

*Molecular signatures were based on the analysis of complete amino acid sequence of C, E3,
E2, 6K and E1 glycoprotein (1244 amino acids). The numbering of amino-acid positions
refers to the African isolate S27.
CHIKV 2010-1909 and CHIKV 06-021 strains are highlighted in red. Colors correspond to amino-
acid change among ECSA phylogroup (yellow) or Asian phylogroup (purple). CHIKV, chikungunya
†The amino acid substitution E2-V264A was unique to France 2010-1909 CHIKV isolate.
Table 1. Test statistics of virus transmission efficiency

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d.f.: degrees of freedom; res. dev.: residual deviance. Transmission efficiency is calculated as the overall proportion of females that had infectious saliva (*i.e.*, among all tested females with or without a disseminated infection).
Table 2. Test statistics of virus dissemination efficiency

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</table>

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).
Table 3. Test statistics of virus transmission rate

<table>
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<th>Factor</th>
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<th>res. dev.</th>
<th>P-value</th>
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</table>

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.